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Contents

Original papers

Reviews

IL-4-polarized BV2 microglia cells promote angiogenesis by secreting exosomes

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Conflict of interest
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Abstract

Background. The microglia cell transfer has been shown to play a protective role in ischemic stroke. Microglia cells may play this nerve-protective role via the promotion of angiogenesis. However, the underlying mechanisms are largely unknown and need further investigation.

Objectives. The aim of this study was to investigate the pro-angiogenesis effects of unpolarized, interleukin 4 (IL-4)-polarized or lipopolysaccharide (LPS)-polarized BV2 microglia cells both in vivo and in vitro. We also investigated the potential mechanisms of these pro-angiogenesis effects.

Material and methods. BV2 cells were polarized using phosphate-buffered saline (PBS), LPS or IL-4, respectively. The gene expression pattern was analyzed with reverse transcription polymerase chain reaction (RT-PCR). The transfer of polarized BV2 cells was performed with an intravenous injection into mice 45 min after the middle cerebral artery (MCA) occlusion. Angiogenin expression was assessed with immunofluorescence. We also examined the angiogenesis effect of polarized BV2 cells and their exosomes through 3-dimensional co-cultures in vitro. Finally, the microRNA (miRNA) profiles of exosomes released by BV2 cells under different polarization conditions were examined using miRNA microarray.

Results. The IL-4-polarized BV2 transplantation promoted angiogenin expression in the ischemic brain. Interleukin 4-polarized microglia increased the tube formation of endothelial cells by secreting exosomes. The miRNA profiles of exosomes released by BV2 cells under different polarization conditions were different. Exosomes from IL-4-polarized BV2 cells contained higher amounts of miRNA-26a compared to those from the LPS-polarized and unpolarized BV2 cells.

Conclusions. Interleukin 4-polarized microglia cells might ameliorate the damage caused by ischemic stroke by promoting angiogenesis through the secretion of exosomes containing miRNA-26a.

Key words: angiogenesis, exosomes, microglia, interleukin 4

Cite as
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Introduction

Stroke is the second leading cause of death worldwide. It is most often caused by a thrombus or an embolus in the middle (MCA) or anterior cerebral artery (ACA), where an infarct develops after a few minutes of ischemia. Microglia are a type of neuroglia (glial cells) located throughout the central nervous system; they account for 10–15% of all cells within the brain and spinal cord. Microglia are also a type of resident macrophage cells, and are the first responding cells during the development of a stroke.[1,2] There have been reports indicating that transplanting unstimulated HMO6 human microglial cells could protect animals from neural damage in a stroke by reducing neuronal cell death.[3]

Microglia/macrophages are known to have distinct phenotypes with various and even opposing functions. It has recently been reported that microglia with the M2-like phenotype (interleukin 4 (IL-4)-polarized microglia) are initially recruited at the injury site after the middle cerebral artery occlusion (MCAO), but they polarize to the M1-like phenotype (lipopolysaccharide (LPS)-polarized microglia) at a later stage.[4,5] The M2-like microglia which are induced by type II cytokines like IL-4 and IL-13 could secret cytokines that promote regenerative processes and neurogenesis while suppressing type I immunity; thus, the M2-like microglia could be beneficial in spinal cord injury and in stroke.[6] Moreover, IL-4 has been proven to be essential for the treatment of ischemic brain damage, as well as for polarizing microglia/macrophages to a M2-like subset.[7] Therefore, manipulating the polarization of microglia/macrophages might be a better treatment method not only for experimental stroke but in clinical treatment as well.[8,9] A direct injection of M2-type bone marrow-derived macrophages (BMDM) after ischemia did not lead to a significant improvement,[10] but this may be due to different causes of stroke, and different micro-environments may drive BMDM to different phenotypes with opposing functions under hypoxia conditions.[11] In short, the therapeutic effect of a direct M2 microglia/macrophages transplantation has not yet been proven. Polarizing resident microglia/macrophages in vivo may be a more suitable approach to the treatment of stroke.

Exosomes are small vesicles originating from the fusion of multivesicular bodies with a plasma membrane. The contents of exosomes, including proteins, mRNAs and microRNAs (miRNAs), play a crucial role in the activation, polarization or inhibition of immune cells. Recent findings indicate that exosomes derived from mesenchymal stromal cells significantly improved functional recovery by promoting angiogenesis in stroke rats.[12] Angiogenesis stimulates other downstream events, including neurogenesis, synaptogenesis, and neuronal and synaptic plasticity, which are all involved in the long-term repair and restoration process of the brain after an ischemic event. Particularly, recent studies have shown that the conditioned medium from metformin-treated BV2 cells can promote angiogenesis in vitro.[13] Whether IL-4-polarized microglia may promote angiogenesis by secreting exosomes needs further investigation.

In this work, we transferred polarized microglia to the brain in a mouse stroke model to determine the protective effect of microglia in vivo; we also investigated the underlying mechanisms in vitro by co-culturing endothelial cells with polarized microglia cells or exosomes secreted by those cells. Our results showed that M2-like microglia could protect the central nervous system, probably through the secretion of pro-angiogenesis exosomes.

Material and methods

BV2 cell culture and polarization

Mouse microglial cell line BV2 cells were cultured in a complete medium (Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin) (North China Pharmaceutical Group Corp., Shijiazhuang, China). In order to eliminate exosomes, FBS was ultracentrifuged at 120,000 g for 90 min using a Ti70 rotor (Optima™ LE-80K Ultracentrifuge; Beckman Coulter Life Sciences, Indianapolis, USA) before being added to the medium. The cells were maintained at 37°C in a 5% CO_2 incubator. BV2 cells were seeded at 1×10^6 per well in a 25 cm^2 plate for 24 h before being stimulated with LPS (1 µg/mL) or murine IL-4 (25 ng/mL) for 48 h to generate classically activated macrophages (M1) or alternatively activated macrophages (M2), respectively.

Gene expression of polarized BV2 cells by reverse transcription polymerase chain reaction

For the reverse transcription polymerase chain reaction (RT-PCR) assay, BV2 cells were seeded in a 25 cm^2 flask and polarized with LPS (1 µg/mL) and IL-4 (25 ng/mL) to generate M1 and M2, respectively. After polarization, total RNA was extracted with TRIzol™ Reagent (Invitrogen, Carlsbad, USA), following the manufacturer's protocol, and then reverse-transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen). The gene primers are shown in Table 1. The parameters for RT-PCR were as follows: denaturing at 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 51°C for 1 min and 72°C for 1 min, and then extension at 72°C for 2 min.

Middle cerebral artery occlusion model and transplantation of microglia

The animal procedures were approved by the Stanford Institutional Animal Care and Use Committee (Stanford University, USA) and were in accordance with the National

Table 1. Forward and reverse gene primers sequences

Gene	Primers
iNOS (forward) *iNOS* (reverse)	ATGGCAACATCAGGTCGG GCACAACTGGGTGAACTCC
TNF-α (forward) *TNF-α* (reverse)	ACTGAACTTCGGGGTGATCG CCACTTGGTGGTTTGCTACG
Arg1 (forward) *Arg1* (reverse)	CAGTCTGGCAGTTGGAAGC GGTTGTCAGGGGAGTGTTG
TGF-β (forward) *TGF-β* (reverse)	GGACTACTACGCCAAAGAAG TCAAAAGACAGCCACTCAGG
GAPDH (forward) *GAPDH* (reverse)	GACTTCAACAGCAACTCCCACTC TAGCCGTATTCATTGTCATACCAG
VEGF (forward) *VEGF* (reverse)	CTGCTGTAACGATGAAGCCCTG GCTGTAGGAAGCTCATCTCTCC
HGF (forward) *HGF* (reverse)	CTCCTGAAGGCTCAGACTTGGT CCAGAAGTAAATACTGCAAGTGG
FGF (forward) *FGF* (reverse)	AAGCGGCYCYACYGCAAGAACG CCTTGATAGACACAACTCCTCTC
EGF (forward) *EGF* (reverse)	ACTGGTGTGACACCAAGAGGTC CCACAGGTGATCCTCAAACACG
PGF (forward) *PGF* (reverse)	TGCTGTGGTGATGAAGGTCTGC GCATTCACAGAGCACATCCTGAG
PDGF (forward) *PDGF* (reverse)	GTGGTCCTTACCGTCATCTCTC GTGGAGTCGTAAGGCAACTGCA
MMP2 (forward) *MMP2* (reverse)	CAAGGATGGACTCCTGGCACAT TACTCGCCATCAAGCGTTCCCAT
MMP9 (forward) *MMP9* (reverse)	GCTGACTACGATAAGGACGGCA TAGTGGTGCAGGCAGAGTAGGA

Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Male C57BL/J (20 ±2 g) mice were purchased from The Jackson Laboratory (Bar Harbor, USA). Transient MCAO was induced using the method of intraluminal vascular occlusion described in our previous studies.[14] Briefly, the mice were initially anesthetized with 5% isoflurane, and maintained at 1–2% during the surgery in a 70% N_2O and 30% O_2 mixture using a face mask. In order to maintain the body core temperature of the mice at 37 ±0.5°C, a surface heating pad was used and the temperature was monitored by a rectal probe during the entire procedure. Then, the left common carotid artery, external carotid artery and internal carotid artery were surgically exposed by a ventral midline neck incision. The mice were subjected to MCAO with a 6-0 nylon monofilament suture (Doccol Corp., Sharon, USA), coated with silicone. At 45 min after MCAO, the occluded animals were re-anesthetized, the nylon monofilament suture was removed and the end of the external carotid artery was tied. The mice were allowed to wake up from anesthesia and returned to the cages.

The mice were randomly divided into 4 groups as follows (n = 5–7 mice per group): 1. vehicle treatment; 2. treatment with unpolarized microglia (the control group); 3. treatment with LPS-polarized microglia; and 4. treatment with IL-4-polarized microglia. One million microglia cells in 0.1 mL of phosphate-buffered saline (PBS) were administered to each mouse with vein infusion immediately after the transient MCAO; the vehicle-treated mice were injected with an equal volume of PBS.

Immunofluorescence staining

At 2 days after MCAO, 3 groups of 5 experimental animals each were sacrificed and their organs were fixed by transcardial perfusion with PBS. The brain tissues were immersed in 4% paraformaldehyde (PFA) in 0.1 mol/L PBS (pH 7.4) for 48 h. The brain tissues were cut into equal samples (thickness: 50 μm).

The slides were fixed with methyl alcohol and acetone (1:1) for 10 min, and then washed 3 times with PBS. After washing, the slides were blocked with 5% bovine serum albumin (BSA) and incubated overnight with anti-angiogenin Ab (diluted 1:200; Abcam, Cambridge, USA). The slides were washed 3 times in a wash buffer (PBS with 0.05% Tween® 20 (Institute of Chemical Technology, Beijing, China) for 15 min each time. After washing, the secondary antibody coupled with fluorescence was added and incubated for 2 h at room temperature. Then, 4′,6-diamidino-2-phenylindole (DAPI) was added for staining for 5 min, followed by washing. The slides were sealed with Fluoromount™ (Sigma-Aldrich, St. Louis, USA) and observed with confocal laser scanning microscopy using an Axio Vert inverted scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

Endothelial cell co-culture with polarized BV2 cells and tube formation assay

In order to analyze the mechanism of the therapeutic effect of IL-4-polarized microglia and to investigate whether these cells can promote angiogenesis, we introduced a co-culture of endothelial cells and microglia. For all the in vitro experiments, the human umbilical vein endothelial cells (HUVEC) were used. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin. Then, 48-well plates were filled with 150 μL Matrigel® (BD Biosciences, Franklin Lakes, USA) and allowed to solidify at 37°C for 30 min. Subsequently, BV2 microglia subsets (4×10^3 cells/well) were co-incubated with the HUVEC (4×10^4 cells/well). After 24 h, network structures were analyzed at ×40 magnification using the AxioVision Microscopy software (Carl Zeiss AG) and photographed with a digital camera (ECLIPSE TS 100-F; Nikon, Tokyo, Japan).[15,16] The number of tubes per picture was counted using the ImageJ program (National Intitutes of Health, Bethesda, USA).

Assessment of vascular endothelial growth factor secreted by polarized BV2 cells

The supernatants of the polarized BV2 cells were collected after 48 h of LPS and IL-4 stimulation. Then, the concentration of vascular endothelial growth factor (VEGF) secreted by the polarized BV2 cells was detected using the SMMV00 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, USA). The detection of the concentration of VEGF in the control, M1 and M2 supernatants was repeated 3 times.

Isolation and identification of microglia exosomes

The microglia exosome isolation procedures were performed at 4°C as described in the literature, using an exosome extraction kit (System Biosciences, Palo Alto, USA). Briefly, the cell supernatants were centrifuged at 3000 g for 15 min to remove cells and cell debris; the supernatants were added to an exosome extraction reagent and mixed gently. After 12 h at 4°C, the mixture was centrifuged at 10,000 g for 30 min; the pellets were collected and resuspended in 50–100 µL of PBS, and used for the analysis of the exosome-enriched fraction. For the transmission electron microscopy (TEM) morphology investigation, the pellets obtained by this process were subjected to uranyl acetate negative staining on the formvar/carbon-coated 400-mesh copper electron microscopy grids (FCF400-Cu; Electron Microscopy Sciences, Hatfield, USA). Twenty microliters of the sample were applied to the grid and incubated for 1 min at room temperature, and then the excess solution on the grid was wicked off and dried for 30 min with filter papers. An equal part of 10% uranyl acetate was added to the grid for 1 min for negative staining. The preparations obtained were examined at 70 kV with a Philips 208 electron microscope (Philips Healthcare, Bothell, USA) with the DigitalMicrograph™ (Gatan, Inc., Pleasanton, USA). Western blot was used to identify TSG101, CD81 and CD63 (primary antibody, 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, USA), the specific exosomal protein markers. The protein concentrations of the exosome preparations were determined using the micro bicinchoninic acid protein assay (Thermo Fisher Scientific, Lafayette, USA).

Tube formation by microglia exosomes

Exosomes from other cells, such as multiple myeloma cells and mesenchymal stem cells, can promote angiogenesis. The effect of exosomes from IL-4-polarized microglia on angiogenesis has not been clear. The HUVEC were cultured as described above, then 48-well plates were filled with 150 µL Matrigel (BD Biosciences) and allowed to solidify at 37°C for 30 min. The exosomes were separated by the method described above. Briefly, the exosomes from 5×10^6 polarized cells were co-incubated with the HUVEC. After 12 h, network structures were analyzed at ×40 magnification using the AxioVision Microscopy software (Carl Zeiss AG) and photographed with a digital camera (Nikon). The number of tubes per image was counted using ImageJ (NIH).

RNA extraction and microRNA array

RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol, and the Nanodrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used to assess the RNA present. The samples extracted from M0, M1 and M2 type microglia exosomes were then delivered to GMINIX Co. (Shanghai, China). Commercial mouse miRNA microarrays, containing 1,908 mouse mature microRNAs from the Sanger mirBase database v. 20.0 (2 probes for each miRNA on each chip) were used to analyze the expression of miRNA in diferent types of BV2 cells. The tagged miRNAs were purified and hybridized with the GMINIX microRNA Microarray-Single according to the manufacturer's instructions. After the hybridization, the chips were subjected to a stringent wash and fluorescence data were collected using the GeneChip Scanner 3000 (Thermo Fisher Scientific, Waltham, USA); the chips were scanned at a pixel size of 10 µM with Cyanine 3 (Cy3) Gain at 460 nm and Cyanine 5 (Cy5) Gain at 470 nm scanning. Equal RNA from 6 individual cell samples with the same treatment was mixed and each mixture sample was repeated twice.

The data was shown as mean ± standard deviation (SD). The p-values were calculated using Student's t-test and post hoc test for multiple comparisons.

Results

Interleukin 4-polarized BV2 cells upregulated the expression of angiogenin in the ischemic brain

Angiogenin is a potent angiogenic growth factor that degrades the basement membrane, thereby facilitating cell invasion and migration. To check whether BV2 cells could induce angiogenesis by producing angiogenin, we polarized BV2 cells with either LPS or IL-4. The gene expression pattern was analyzed by RT-PCR (Fig. 1). We also detected the expression of angiogenin in the ischemic brain after IL-4-polarized BV2 treatment using immunofluorescence staining. The results showed that IL-4-polarized microglia significantly increased the expression of angiogenin and vascular density after 14 days compared with the control mice (Fig. 2).

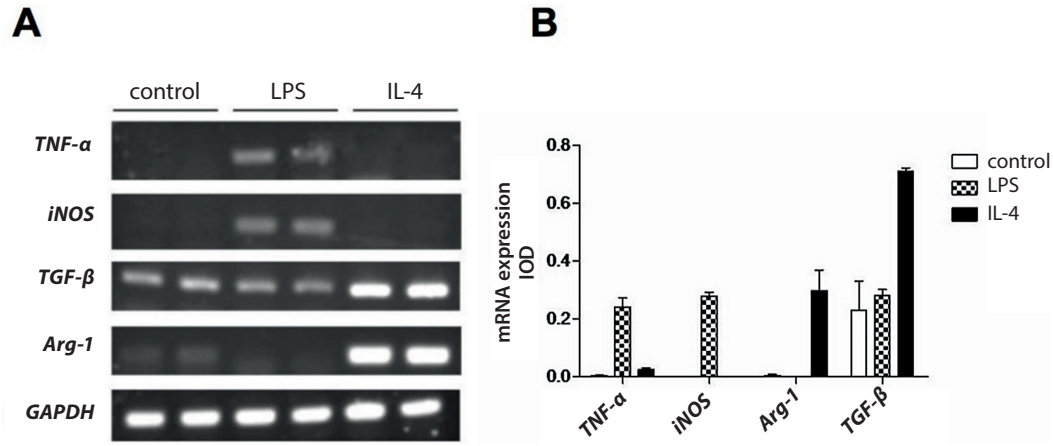

Fig. 1. Gene expression of polarized BV2 cells

A. Reverse transcription polymerase chain reaction (RT-PCR) results showing that BV2 express M1 markers (*TNF-α* and *iNOS*) or M2 markers (*Arg-1* and *TGF-β*)
B. Quantitative data for the expression of *TNF-α*, *iNOS*, *Arg-1*, and *TGF-β*

Data is presented as mean ± standard deviation (SD) from triplicates; *Arg-1* – arginase 1; *GAPDH* – glyceraldehyde 3-phosphate dehydrogenase; *IL-4* – interleukin 4; *iNOS* – inducible nitric oxide synthase; IOD – integrated optical density; LPS – lipopolysaccharide; *TGF-β* – transforming growth factor β; *TNF-α* – tumor necrosis factor α; M1 – classically activated macrophages; M2 – alternatively activated macrophages.

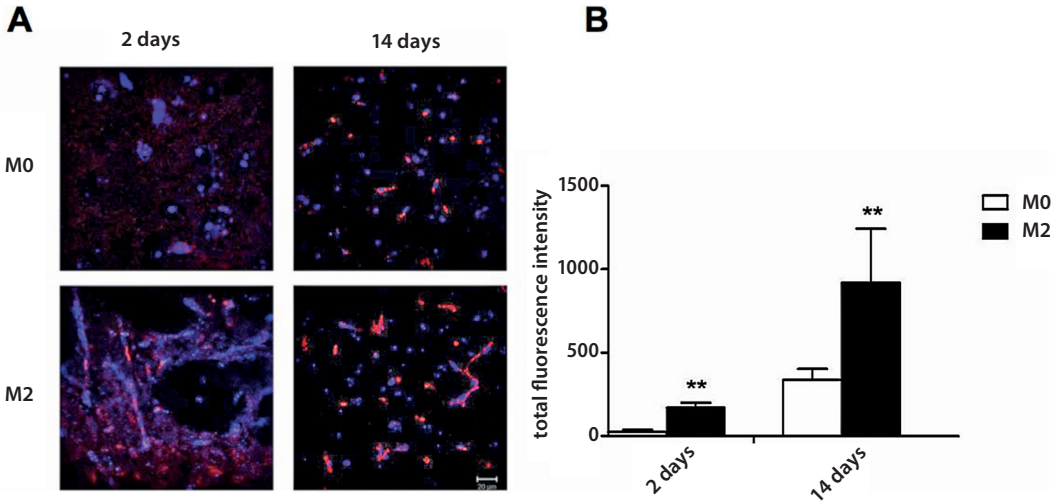

Fig. 2. Expression of angiogenin in ischemic mice increased 14 days after the M2 microglia transplantation

A. Immunostaining of angiogenin in the ischemic brain on day 2 and day 14 after control and interleukin 4 (IL-4)-polarized BV2 treatment (4′,6-diamidino-2-phenylindole (DAPI): blue; angiogenin: red; n = 3 per group; the scale bar represents 20 μm)
B. Total fluorescence of angiogenin in the ischemic brain on day 2 and day 14

M0 – control; M2 – IL-4--polarized BV2 treatment; ** $p < 0.01$ compared to the M0 group.

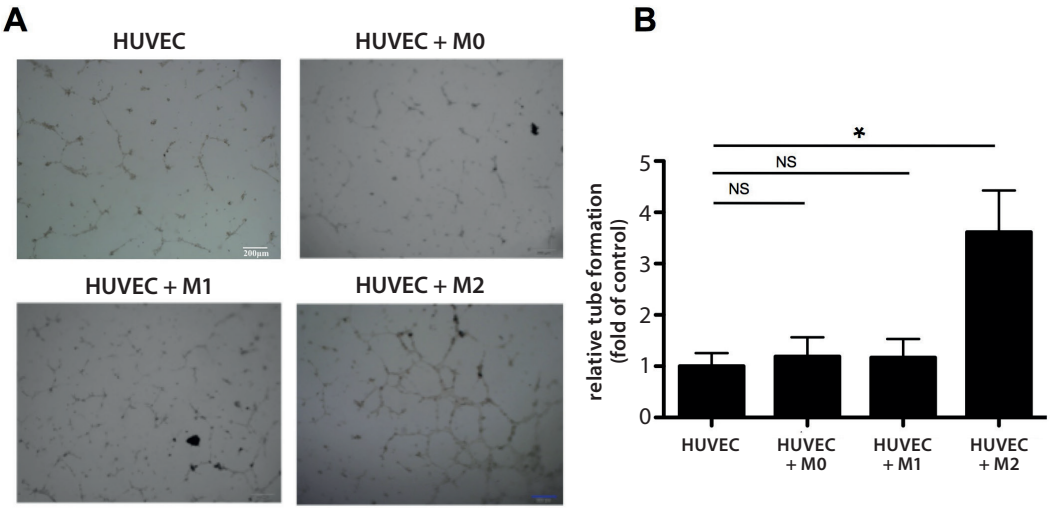

Fig. 3. Interleukin 4-polarized microglia promoted angiogenesis in vitro. BV2 microglia subsets (4×10^3 cells/well) were co-incubated with human umbilical vein endothelial cells (HUVEC) (4×10^4 cells/well) for 24 h

A. Network structures were analyzed at ×40 magnification and photographed with a digital camera (n = 3 per group; the scale bar represents 200 μm)
B. The number of tubes per picture was counted using ImageJ software

M1 – LPS-polarized BV2 treatment; * $p < 0.05$ compared to the HUVEC group; NS – statistically nonsignificant.

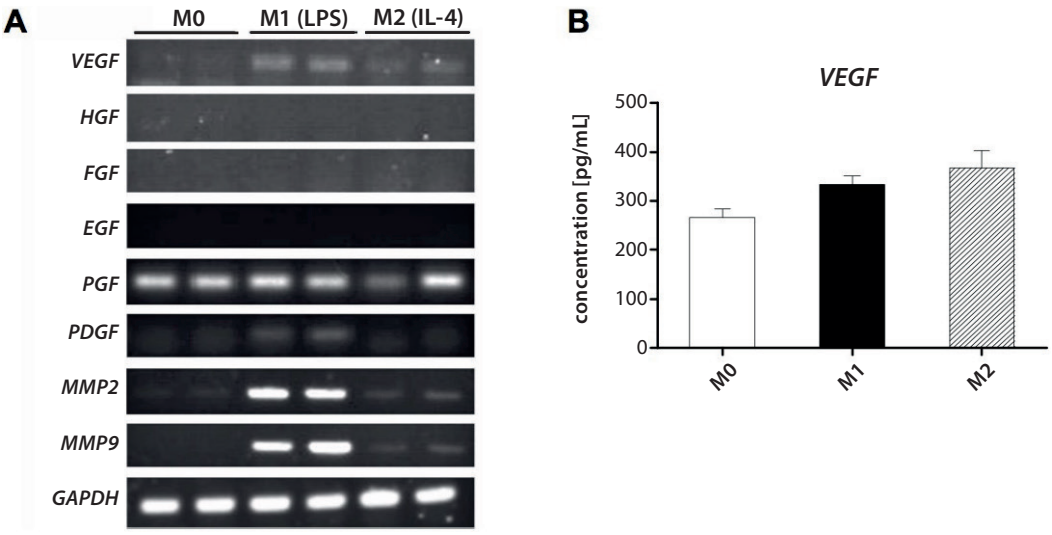

Fig. 4. Angiogenesis-related gene expression of polarized BV2 cells. The expression of angiogenesis-related genes was detected with RT-PCR and the release of VEGF was detected in the culture supernatants of polarized BV2 cells using enzyme-linked immunosorbent assay (ELISA)

A. Expression of genes related to angiogenesis in M0, M1 and M2 BV2 cells
B. Concentrations of VEGF in the culture supernatants of M0, M1 and M2 BV2 cells; there were no significant differences between these cells ($p > 0.05$)

EGF – epidermal growth factor; *FGF* – fibroblast growth factor; *HGF* – hepatocyte growth factor; *MMP2* – matrix metalloproteinase 2; *MMP9* – matrix metalloproteinase 9; *PDGF* – platelet-derived growth factor; *PGF* – placental growth factor; *VEGF* – vascular endothelial growth factor.

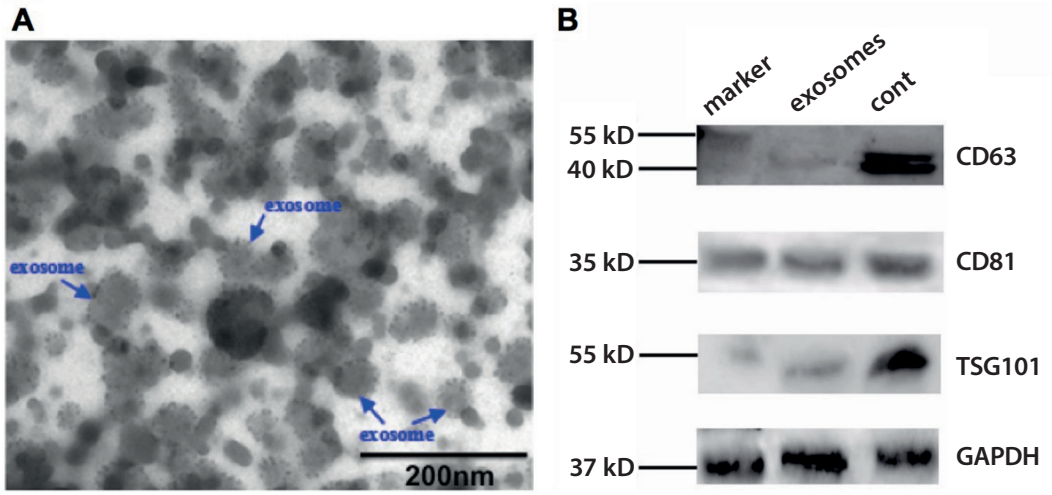

Fig. 5. Characteristics of exosomes

A. Transmission electron micrographs of the exosomes derived from BV2 cells stimulated by IL-4 (the scale bar represents 200 nm)
B. Western blotting analysis for exosome markers CD63, CD81 and TSG101

cont – positive control.

Interleukin 4-polarized BV2 cells increased the tube formation in vitro

To study the role of macrophage subsets in angiogenesis, we performed in vitro tube formation assays using a co-culture of endothelial cells and macrophages on a Matrigel base. Culturing endothelial cells in these settings led to the formation of tubular structures after 24 h. Adding IL-4-polarized BV2 cells to endothelial cells increased the number of tubes as compared to the control situation (Fig. 3).

Interleukin 4-polarized BV2 cells promote the tube formation by secreting exosomes

In order to ascertain the mechanism by which IL-4-polarized BV2 cells promote angiogenesis, we first detected the gene expression of secreted growth factors related to angiogenesis, such as *VEGF*, hepatocyte growth factor (*HGF*), fibroblast growth factor (*FGF*), epidermal growth factor (*EGF*), placental growth factor (*PGF*), platelet-derived growth factor (*PDGF*), matrix metalloproteinase 2 (*MMP2*), and matrix metalloproteinase 9 (*MMP9*). Although the *VEGF* gene expression increased in the IL-4-polarized BV2 group compared with the control group, its level was lower than in the LPS-polarized group (Fig. 4A). There were no significant differences in the ELISA results for VEGF between the control culture, LPS-polarized BV2 cells and IL-4-polarized BV2 cells (Fig. 4B). The expression of other genes showed no significant increase in the IL-4-polarized BV2 group compared with the LPS-polarized group (Fig. 4A). These results showed that the tube formation of endothelial cells

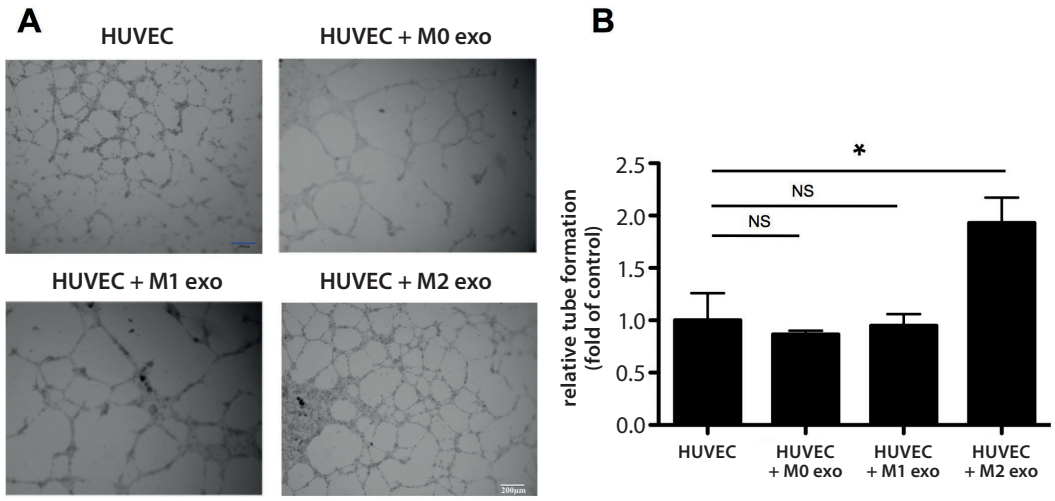

Fig. 6. IL-4-polarized microglia exosomes promoted angiogenesis in vitro. The exosomes from 5×10^6 polarized cells were co-incubated with HUVEC cells for 12 h

A. Network structures were analyzed at ×40 magnification and photographed with a digital camera (n = 3 per group; the scale bar represents 200 μm)
B. The number of tubes per picture was counted using ImageJ software

exo – exosomes; * $p < 0.05$ compared to the HUVEC group; NS – statistically nonsignificant.

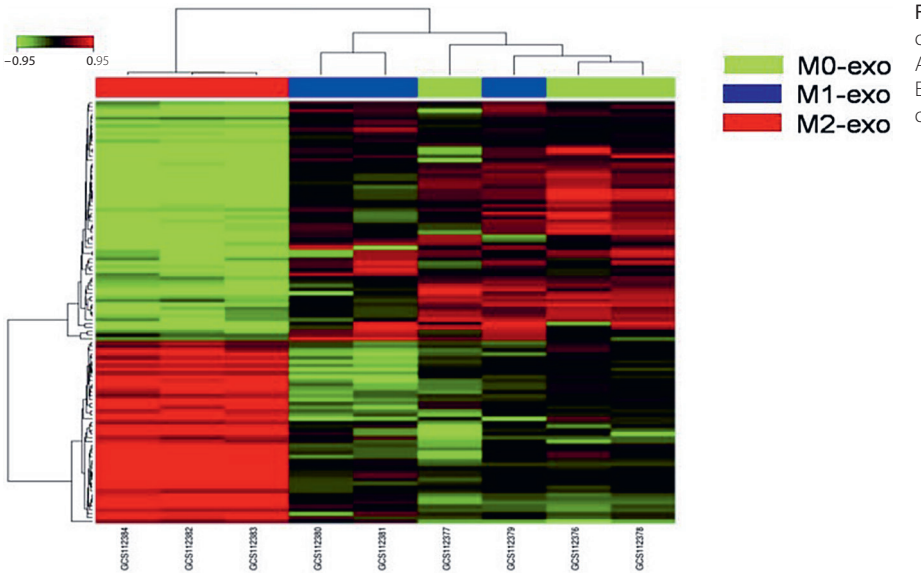

Fig. 7. Heat map of microRNA expression array of M0, M1 and M2 microglia-derived exosomes. An intensity key is given next to the heat map. Each sample is represented as an average of duplicates

promoted by IL-4-polarized macrophages was not related to the secretion of VEGF, HGF, EGF, PGF, PDGF, MMP2, and MMP9.

We next investigated the exosomes secreted by polarized BV2 cells in terms of size, ultrastructures and quantity, and then analyzed their activities promoting angiogenesis. Transmission electron microscopy revealed that the size of the exosomes was about 50–100 nm and each vesicle showed the classic cup-shaped appearance (Fig. 5A). Western blotting results showed the expression of common exosome markers like CD63, CD81 and TSG101 (Fig. 5B). Then, a tube formation assay was performed with exosomes from different microglia subsets. In comparison with the controls, the exosomes from IL-4-polarized BV2 cells stimulated the tube formation: the quantities of tubes increased significantly (Fig. 6). There was no difference between the LPS-polarized group and the controls. This showed that the exosomes from IL-4-polarized BV2 microglia had pro-angiogenic properties.

The promotion of angiogenesis by the M2-type BV2 cells may be due to the secretion of miRNA-26a. Exosomes have been shown to play an important role in the regulation of cell activities. Recent studies have also shown that the content of exosomes, like miRNA, could enter a recipient cell once the exosome membrane fuses with the cell membrane. In this work, we analyzed the different miRNA profiles of unpolarized, LPS-polarized and IL-4-polarized BV2 cells (Fig. 7). We found that miRNA-26a, which has been shown to have angiogenic properties, was selectively upregulated by IL-4 polarization (Table 2).[17]

Table 2. MicroRNAs of exosomes released by M0-, M1- and M2-type microglia

Transcript ID	Mean signal of group M0	Mean signal of group M1	Mean signal of group M2
miR-466b-3p	5.521937	4.806909	2.125578
miR-466c-3p	5.521937	4.806909	2.125578
miR-466p-3p	5.521937	4.806909	2.125578
miR-8117	9.017336	8.827255	7.226154
miR-151-5p	8.958132	8.5831889	6.680118
miR-3620-5p	11.610691	11.805563	13.094678
miR-7221-3p	12.137256	12.323571	13.408188
miR-677-3p	9.540374	9.610824	11.439959
miR-1956	7.252635	7.637256	7.637256
miR-6360	4.987463	4.509745	2.048182
miR-6970-5p	11.378213	11.735456	12.896675
miR-346-3p	12.529241	12.37427	13.65795
miR-26a-5p	9.300976	8.937378	7.642079
miR-7033-5p	10.858716	10.955668	12.375583
miR-702-5p	9.151745	9.149582	8.055301
miR-15b-5p	9.812329	9.292808	7.689586
miR-7235-5p	11.251865	11.324551	12.264028
miR-1940	7.170623	7.549589	5.388706
miR-7049-5p	7.799654	7.473127	6.029101
miR-466a-5p	5.572361	5.641108	2.280584

Discussion

Our study offers a few points on the therapeutic effect of IL-4-polarized microglia in the acute and chronic phases of ischemic stroke. We showed that IL-4-polarized BV2 cells may promote the tube formation in vitro and angiogenesis in vivo through the secretion of exosomes containing miRNA-26a. Moreover, exosomes released by IL-4-polarized microglia may have a potential therapeutic value in the treatment of stroke.

Current stroke therapies include regulatory T cells,[18] BMDM,[19] human mesenchymal stem cells,[20] and neural stem cells,[21] all of which might have the potential to shift the inflammatory environment and restore the neurological function after a stroke. However, as a kind of resident macrophages, microglia could be polarized or stimulated directly by the changes in the microenvironment in the brain during all stages of a stroke. Angiogenesis is essential in physiological processes, such as embryonic development and wound-healing tissue repair.[22] New capillaries are formed from pre-existing blood vessels, allowing the recovery of the supply of anti-inflammatory factors and neuron growth factors. Microglia/macrophages are not only key players in inflammatory diseases, but also in promoting angiogenesis.[23] In this work, our results showed that microglia could promote angiogenesis not by conventional pathways (i.e., direct cell–cell contact or VEGF signaling), but by secreting exosomes, which concurs with previous reports.[24–26]

The proteomic analysis of exosomes has revealed the presence of specific proteins involved in cell motility, angiogenesis, inflammatory regulation, or neuromodulation, suggesting that glial cells use unconventional pathways for protein secretion.[27] Some reports have shown that the overexpression of CXCR4 in exosomes secreted from mesenchymal stem cells (ExoCR4) also promotes the recovery of cardiac functions after myocardial infarction (MI).[25,28] Other reports have also shown that the exosome miRNAs from breast cancer (let-7a, miR-23b, miR-27a/b, miR-21, let-7, and miR-320b) are known to present anti-angiogenic activity.[29] In our study, the expression of miRNA-26a in M0, M1 and M2 microglia was quite different, and there have been numerous reports showing that miRNA-26a is closely related to angiogenesis. Qian et al. and other authors found that miRNA-26a promoted tumor growth and angiogenesis in glioma by directly targeting prohibitin.[17] On the other hand, miRNA-26a suppresses the epithelial mesenchymal transition in human hepatocellular carcinoma; miRNA-26a is also a key factor in angiogenesis in diabetic wound healing.[30,31] These results indicate that different exosomes from of various cell types have different effects on angiogenesis under special circumstances.[32]

The hypoxic state is a situation in which exosome-mediated signaling promotes angiogenesis in some solid tumors and ischemic infarction.[32–34] In the neural system, the hypoxia-inducible factor (HIF) pathway is involved in angiogenesis in an exosome-dependent manner.[35] The study of exosomes provided a platform for the diagnosis and monitoring of neurodegenerative progression.[36] In our experiment, the M2-like microglia could secrete specific exosomes to promote neovascularization, and then carry more Th2/M2-type cells into the ischemic region, which would be beneficial in the recovery from ischemic stroke.

We injected the M1-type microglia into mice along with the M2-type microglia, but the mice died rapidly, perhaps due to strong inflammatory responses. The HIF pathway may be involved in the pro-angiogenic activity of the exosomes secreted from the M2 microglia.

Taken together, our data demonstrates that IL-4-polarized microglia could ameliorate the damage caused by ischemic stroke by promoting angiogenesis through the secretion of exosomes. Still, the exact mechanisms of how IL-4-polarized microglia secrete these exosomes or how these exosomes help in recovery still need further investigation.[37] The exact contents of these exosomes and the potential effects of this substance need to be elucidated as well. Nevertheless, our current data suggests that using exosomes derived from IL-4-polarized microglia may be considered a novel therapeutic method for the treatment of ischemic stroke.

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Impact of fabrics from transgenic flax on cultures of skin cells

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Abstract

Background. The development of a new type of wound dressing material that can support skin regeneration is an important challenge to improve treatment of chronic, non-healing wounds.

Objectives. The objective of this study was to compare the impact of flax fabrics from transgenic plants overexpressing phenolic acids and flavonoids (W92) and polyhydroxybutyrate (M48), as well as fabric from non-transgenic plant (Nike) on cultures of human skin cells.

Material and methods. Flax fabric pieces as well as water extracts from the fabrics were co-cultured with human skin cells: keratinocytes, fibroblasts, dermal microvascular endothelial cells, and with monocytoid cell line (THP1) for 48 h. Cell viability and proliferation were assessed with the sulforhodamine B colorimetric assay. Intracellular reactive oxygen species (ROS) was estimated with the 2'7 dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation method. Endothelial cell migration was measured with the scratch assay. The results were compared with the multi-criteria analysis (MCA) procedure.

Results. Tested flax fabrics released flavonoids and polyhydroxybutyrate to cell culture media, as it was determined by means of the high performance liquid chromatography (HPLC) method. Fabrics from transgenic plants W92 and M48 promoted proliferation of keratinocytes and fibroblasts. Water extracts from flax fabric diminished the proliferation of monocytoid cells, decreased oxidative burst in activated THP1 cells and accelerated the velocity of dermal microvascular cell migration. The MCA proved that the sum of beneficial effects estimated in human skin cell cultures was higher (by 47% and by 34% with W92 and M48, respectively) than that of non-transgenic flax fabric (Nike).

Conclusions. The W92 and M48 fabrics should be further studied as candidates for elaboration of new types of bandages, able to improve skin wound healing.

Key words: flax fabric, skin cell cultures, flax biotechnology, transgenic flax fabrics

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Introduction

Flax (*Linum usitatissimum*), an annual plant primarily cultivated for industrial purposes, was recently genetically modified in order to enhance wound healing properties of the fibers.[1–3]

These new types of flax have been genetically modified in order to provide stable overexpression of enzymes for polyhydroxybutyrate (PHB) synthesis,[1] and enzymes of phenylpropanoid pathway.[3] Three-gene construct with cDNA encoding β-ketothiolase (*phb A*), acetoacetyl-CoA reductase (*phb B*) and PHB synthase (*phb C*) generated a new flax type called M type composite fiber. It contains a new kind of cellulose-polyhydroxybutyrate polymer with components chemically bound via hydrogen and ester bonds. Those fibers were previously found to promote the proliferation rate of cultured fibroblasts.[4] The W type flax plants were generated by the introduction of 3 genes from the phenylpropanoid pathway (chalcone synthase, chalcone isomerase and dihydroflavonol reductase).[3] New flax fibers overexpressing these genes accumulate components like phenolic acids and flavonoids with antioxidant activity.[2]

Previously, we reported the impact of wound dressings made of fibers from W92 plants on healing chronic skin ulcers.[5,6] A 4-week application of bandages composed of the W92 flax fabric led to a marked reduction of wound exudates and significantly decreased wound size in 55% of patients. Beneficial effect of flax fabrics impact on a healing wound needs further studies in cultures of cells engaged in skin regeneration and repair. In this paper, we describe the impact of fabrics from fibers of M48 and W92 transgenic flax plants and from non-transgenic Nike flax on proliferation in vitro of human primary skin cells: keratinocytes and fibroblasts. Flax fabrics were cut into small pieces of 0.25 cm^2, 0.5 cm^2 and 1 cm^2, sterilized and added to cell cultures for 48 h. Also, the proliferation of non-adherent human monocytoid cells (THP1 cell line) and a content of intracellular reactive oxygen species (ROS) in those cells as well as a velocity of endothelial cell migration in vitro were established in cultures with the water extracts from fabrics. Results obtained with the tests were compared by means of the multi-criteria analysis (MCA) procedure.[7,8] The MCA calculations allowed us to compare the effects of tested linen fabrics in order to choose the most favorable material as a candidate for a new type of bandage that can improve wound healing.

Material and methods

Plant material

Genetically modified flax were obtained according to the procedures described previously[3,9]; the M48 flax expressed genes for PHB synthesis: β-ketothiolase (*phb A*), acetoacetyl-CoA reductase (*phb B*) and PHB synthase (*phb C*), and the W92 overexpressed genes from the phenylpropanoid pathway: chalcone synthase, chalcone isomerase and dihydroflavonol reductase. Plants were grown in a field in the 2010 vegetative season. After 4 months of field growth, the plants were harvested and the flax fiber was prepared via the standard dew retting process, which has previously been described.[10] The fabrics were sterilized by autoclaving at 120°C for 20 min.

Reagents and cell culture media

KBM-Gold medium (Keratinocyte Cell Basal Medium), medium supplements, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Lonza (Verviers, Belgium). Endothelial Cell Growth Medium MV and its supplements were obtained from PromoCell (Heidelberg, Germany). The solution of antibiotics (10,000 U/mL penicillin and 10,000 µg/mL streptomycin) containing 29.2 mg/mL L-glutamine was purchased from HyClone Laboratories Inc. (Logan, USA) and the Trypsin EDTA solution from Lonza. Sulforhodamine B (SRB), phorbol 12-myristate 13-acetate (PMA) and Trizma-base were purchased from Sigma-Aldrich (St. Louis, USA). Phosphate buffered saline (PBS) and 0.4% of trypan blue solution were obtained from POCH SA (Gliwice, Poland).

Estimation of phenolic components released from the flax fabrics to cell culture medium

In order to determine the amount of phenolic components released from the flax fabrics to cell culture medium, 10 cm^2 fragments of the tested fabrics were incubated in 10 mL of medium (DMEM, without FBS) for 48 h at 37°C. After incubation, the solution was adjusted to pH 3 and extracted 3 times with ethyl acetate. The ethyl acetate fraction was dried under a vacuum, resuspended in 1 mL of methanol and analyzed using liquid chromatography in Ultra Performance Liquid Chromatography (UPLC; Waters Corp., Milford, USA).

Cells and cell growth conditions

Three types of primary skin cells from adult donors: normal human epidermal keratinocytes (NHEK), normal human dermal fibroblasts (NHDF) and human dermal microvascular endothelial cells (HDMEC) were purchased from PromoCell (Heidelberg, Germany). Established monocytoid cell line (human acute monocyte leukemia, THP1) was obtained from the Laboratory for Therapeutic Innovation, Faculty of Pharmacy, University of Strasbourg, France.

Cell pellets were suspended with PBS, counted and inspected under a microscope for cell viability with the standard trypan blue exclusion test. Then, the cells were plated on 24-well plastic culture plates and sterile pieces (0.25 cm^2, 0.5 cm^2 and 1.0 cm^2) of flax fabrics were placed in each culture-well of adherent cells, or respective

volume of aqueous extract from the flax fabric was added to the cultures of non-adherent cells. Cultures were incubated in a CO_2-incubator at 37°C for 48 h.

Determination of the cell density/cell proliferation

Cell density/cell proliferation was determined with the sulforhodamine B (SRB)-colorimetric assay.[11] Sulforhodamine absorbance was estimated at 490 nm with the Victor 2 microplate reader (PerkinElmer, USA).

Evaluation of the intracellular free radical level

Free radical content in THP-1 cells was estimated with the dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation method.[12] The standard activator of free radical generation (PMA, 200 nM) was added for 2 h to selected cultures. Fluorescence of intracellular, oxidized DCFH-DA was inspected at 525 nm (FL1) with the CyFlow Space cytometer (Partec, Germany), equipped with a 488 nm argon laser lamp.

Estimation of endothelial cell migration

The impact of the aqueous extract from the flax fabric on migration of human dermal microvascular endothelial cells was analyzed on plastic Petri dishes using the scratch assay in vitro.[13] An analysis of cell migration distances and calculations of the velocity of cell migration (μm/h) were carried out with AxioVision software (Carl Zeiss, Germany).

Statistical analysis

The statistical significance of the results (mean ± standard deviation – SD; n = 5) was assessed with the paired t-test with Microsoft Excel 2010 software (Microsoft Corp., Redmond, USA) and significant results were marked on histogram columns with asterisks (*$p < 0.05$, **$p < 0.001$, ***$p < 10^{-4}$).

The multi-criterial analysis (MCA) procedure was calculated according to literature data[7,8] with own modifications. In brief, the results obtained with 6 in vitro tests with 1.0 cm^2 area of the flax fabrics pieces or equivalent dose of water extract from fabrics were compared to the relative control cultures (E/E_0) and the statistical distances between the expected (exp.) and observed (obs.) results were calculated with the standard formula: $b = (exp. - obs.)^2/exp.$ The expected values in each test were assumed as the most favorable results estimated for 3 tested flax fabrics. Results of the calculation were expressed as 1/b ratios and then multiplied by the indices of importance. The indices of importance were assumed for the results of each test, given their potentially beneficial role in skin wound healing. Finally, the results of the MCA procedure of compared fabrics were obtained according to the following equation: $M = \Sigma\ 1/b$.

Results

Tested flax fabric released detectable amount of phenolic compounds to aqueous media, as revealed by HPLC analysis.

Table 1 shows that, despite differences in the content and release of individual identified phenolic compounds, the total amount of phenolic compounds released into aqueous solutions after 48 h of incubation of the tested flax fabrics in the PBS solution was more than twice as high for W92 as compared to the Nike flax. In the case of M48 fabric, during the 48-hour incubation, 30% less phenolic compounds were released into the aqueous solution than from the Nike fabric, although the total antioxidant activity of this extract was comparatively strong as the Nike extract.

Additionally, the M48 fabric released polyhydroxybutyrate, which degrades to D,L-β-hydroxybutyrate in contact with body fluids (data not shown).[1] Similar results were obtained with the flax fabrics incubated for 48 h in cell culture media (without FBS) – all identified phenolic compounds were released to the cell culture media.

Table 1. Content of phenolic compounds [ng] released from 1 cm^2 of fabric after 48 h incubation in phosphate-buffered saline solution (PBS) and antioxidant activity of the water extracts

Estimated phenolic compound	Nike		W92		M48	
	ng	% of total	ng	% of total	ng	% of total
3,4 dihydroxybenzoic acid	18.34	25.06	23.19	27.27	10.68	11.23
4-hydroxybenozoic acid	10.31	6.73	22.71	10.97	9.06	3.67
4-hydroxy-3-methoxybenzoic acid (vanilic acid)	21.82	7.76	10.37	4.82	12.57	4.29
Vanilin	14.50	1.90	16.08	1.73	11.13	0.59
Ferulic acid	4.40	5.40	5.88	4.28	4.50	3.22
Vitexin	8.35	7.69	6.97	4.77	4.15	2.86
Total	77.82	5.30	163.02	10.20	56.29	3.10
Antioxidative properties (% of oxidation inhibition)	42		48		43	

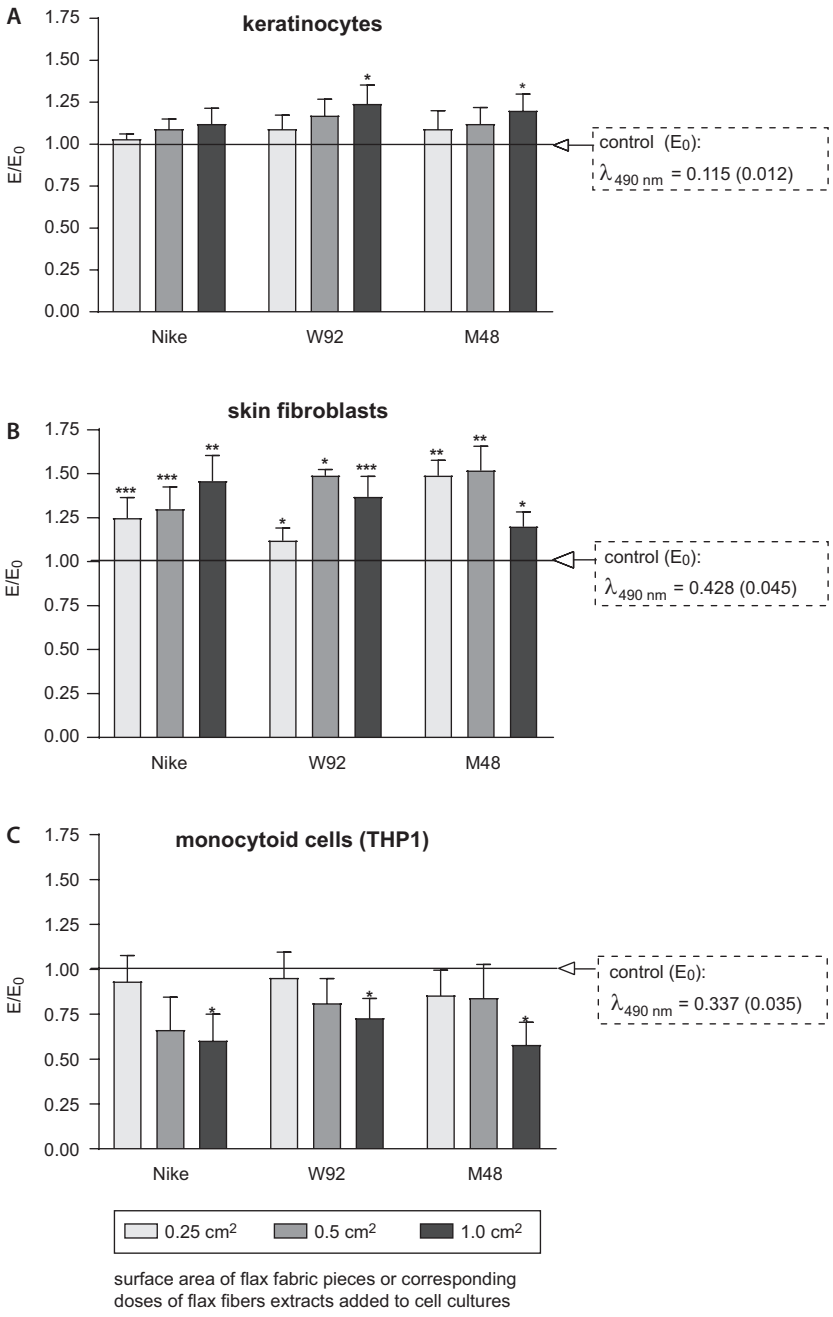

Fig. 1. Proliferation of keratinocytes, skin fibroblasts and monocytoid cells (THP1) estimated with the sulforhodamine B (SRB) test after culture of cells for 48 h in the presence of tested flax fabrics pieces (A and B) or in the presence of water extract from equivalent area of fabric pieces (C). The results (E) were compared to those obtained in the relative control cultures (without flax fabrics; $E_0 = 1.0$) and shown as E/E_0 ratios. Control E_0 values (λ 490 nm) were given as mean (standard deviation – SD; n = 5). Statistical significance of the results obtained in tested and in control cultures was calculated with t-test (* $p < 0.05$; ** $p < 0.001$; *** $p < 10^{-4}$)

Incubation of keratinocytes and skin fibroblasts in the presence of the tested flax fabrics pieces lead to marked increase of cell proliferation, measured with the SRB test. On the other hand, monocytoid cells (THP1) cultured in the presence of fabrics water extract exhibited significant decrease of cell proliferation.

Impact of flax fabric pieces (keratinocytes and fibroblasts) and water extract from fabrics (THP1) on skin cell proliferation was shown in Fig. 1. The results were compared to relative control cultures (without flax fabric, $E_0 = 1$) and presented as E/E_0 ratio in histograms in Fig. 1A–C.

Figure 1A shows a moderate increase of cell number of human keratinocyte cultures after 48-hour incubation with the tested flax fabrics. The effect was directly proportional to the area of fabric pieces present in keratinocyte cultures, and for 1 cm^2 pieces of flax fabrics was by 24% higher in the case of W92 than in the control culture (without flax fabric) and by 20% higher in the case of M48 flax fabric.

Figure 1B demonstrates the impact of flax fabrics on skin fibroblast proliferation. The effect is the strongest in the presence of 0.5 cm^2 of flax pieces when compared to the control cultures (without flax fabric) both in the case of M48 (increase of 52%) and W92 (increase of 49%). The results obtained with the greater (1 cm^2) and smaller (0.25 cm^2) area of flax fabric fragments were not so pronounced, although statistically significant. The impact of non-transgenic Nike flax fabric on fibroblast proliferation was directly proportional to the area of fabric

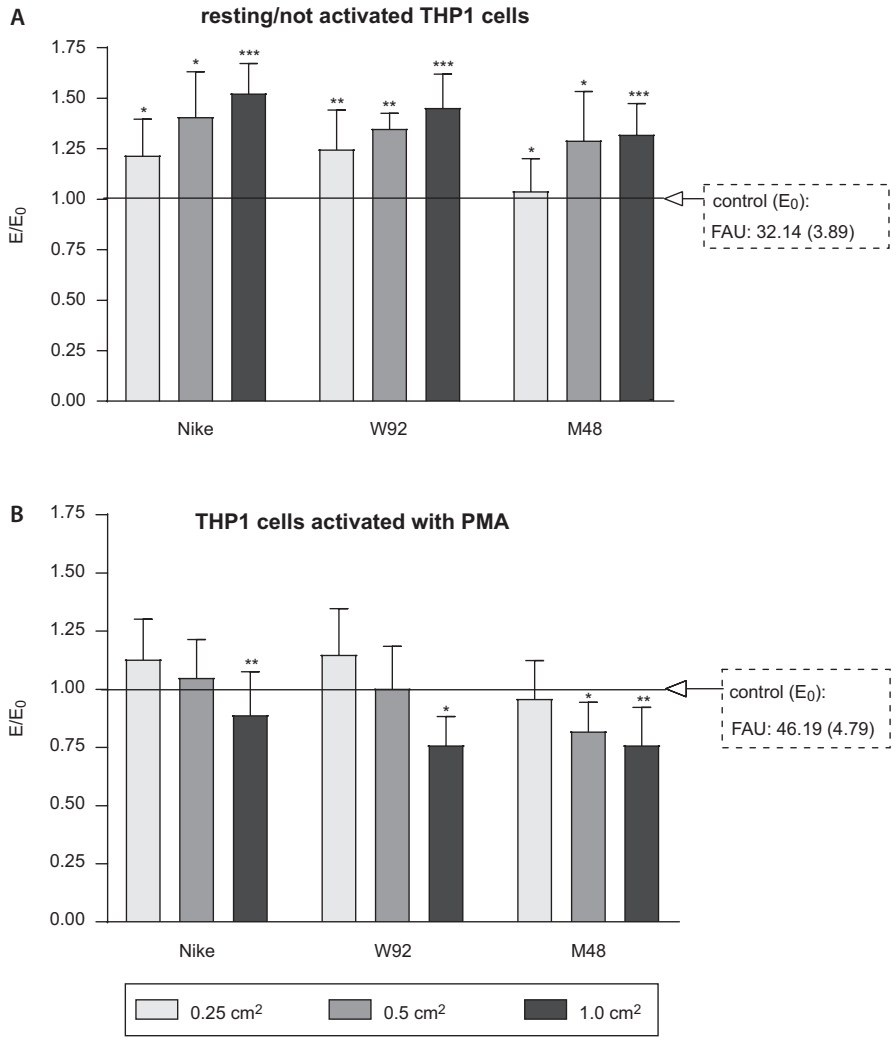

Fig. 2. Intracellular ROS contents in monocytoid cells (THP1) estimated with the dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation test after 48-hour incubation of THP1 cells in the presence of water extracts from the tested flax fabric pieces. THP1 cells were not activated/resting cells (A) or activated with phorbol 12-myristate 13-acetate (PMA; 200 nM, 2 h) (B). The results (E) were compared to those obtained in the relative control cultures (without flax fabrics extracts; $E_0 = 1.0$) and shown as E/E_0 ratios. Control E_0 values (fluorescence arbitrary units – FAU) were shown as mean (standard deviation – SD; n = 5). Statistical significance of the results obtained in tested and in control cultures was calculated with t-test (* $p < 0.05$; ** $p < 0.001$; *** $p < 10^{-4}$)

present in cell culture, and in the presence of 1 cm^2 pieces of the fabric the cell proliferation was even by 46% higher than in control culture.

Figure 1C illustrates the influence of flax fabric water extracts on proliferation of human monocytoid cell line (THP1). Tested flax fabrics markedly decreased the proliferation of THP1; decrease by 42% (M48), by 40% (Nike) and by 28% (W92) were estimated for 1 cm^2 – area of the pieces of the fabric, compared to the control culture.

The intracellular contents of ROS in the THP1 cells cultured for 48 h with the extracts from tested flax fabrics (resting/not activated cells) and in THP1 cells stimulated to oxidative burst by the treatment with phorbol ester (PMA, 200 nM for 2 h) were estimated with the standard DCFH-DA oxidation test. The results were compared to those obtained in relative control cultures (E_0; cells cultured without the presence of flax fabric extracts) and shown in Fig. 2 as E/E_0 ratios.

In resting THP1 cells, not activated to oxidative burst, the aqueous extracts from the 3 types of flax fabric enhanced ROS production in THP1 cells, as was shown in Fig. 2A. The increase was the highest at the dose of 1.0 cm^2 of extracted flax fabric, by more than 50% in the case of the Nike, by 45% for the W92, and by 32% for the M48 fabric when compared to the level of ROS in the control culture (without fabric extract; $E_0 = 1.0$).

In THP1 cells activated to oxidative burst with PMA, lower doses of water extracts from the tested flax fabrics (0.25 cm^2 of fabric extracted) caused a small increase (by about 10% in the case of the Nike and W92 extracts) of intracellular ROS, whereas higher doses of extracts (1.0 cm^2 fabric extracted) led to a marked decrease of the ROS contents in THP1 cells by about 10% (Nike) and by almost 25% (W92 and M48), as is presented in Fig. 2B.

Mean velocity of HDMEC migration (μm/h) in the presence of aqueous extracts from 1.0 cm^2 pieces of the flax fabrics and in the control cell culture (without tested extracts) is presented in Fig. 3. Flax fabric extracts differed markedly in their impact on endothelial cell migration. The cell migration velocity was increased by 33% in the presence of the extract from M48 fabric and by 20% with the extract from W92 fabric, whereas the extract from non-transgenic Nike fabric caused a decrease by 50% of cell migration velocity when compared to the control culture (without tested extracts).

The results obtained with 6 tests were evaluated in order to choose the most advantageous flax fabric type

Table 2. Multi-criterial analysis (MCA) of 3 flax fabric in the aspect of their potential enhancement of skin wound healing

Rating and ranking criteria			Tested flax fabric		
Tests	expected result	index of importance	Nike	W92	M48
Proliferation of keratinocytes (NHEK)	increase	0.25	6.280	26.880	14.620
Proliferation of fibroblasts (NHDF)	increase	0.15	9.145	3.212	1.362
Proliferation of monocytoid cells (THP1)	decrease	0.20	4.194	1.220	5.830
ROS in resting THP1 cells	increase	0.05	3.820	1.730	0.711
ROS in activated THP1 cells	decrease	0.20	1.390	4.540	4.540
Migration of endothelial cells (HDMEC)	increase	0.15	0.199	2.112	6.522
Total MCA (sum of 1–6)		1.00	25.028	36.694	33.585

NHEK – normal human epidermal keratinocytes; NHDF – normal human dermal fibroblasts; ROS – reactive oxygen species; HDMEC – human dermal microvascular endothelial cells.

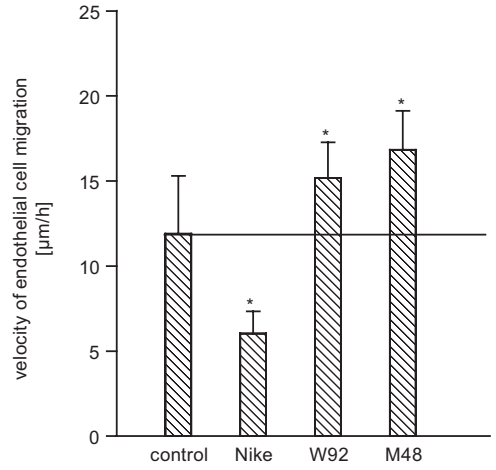

Fig. 3. Velocity of human endothelial cell migration in vitro in the presence of water extracts from the tested flax fabrics (1.0 cm^2 of flax fabric pieces extracted with water). Significance of the results obtained with flax fabric extracts and those obtained in control cultures (without the extracts) were calculated with t-test (* $p < 0.05$)

for elaboration of new skin wound healing bandage. The results of the MCA are given in Table 2.

The overall beneficial effect on skin cell cultures (the MCA index) was higher by about 47% in the case of W92 fabric and by 34% in the case of M48 than that of non-transgenic Nike flax fabric. The analysis proved that W92 flax fabric exhibited the most favorable overall effect on skin cell cultures, which could be important in the promotion of wound healing.

Discussion

An increasing number of patients suffering from chronic, non-healing skin wounds are a serious challenge for modern medicine. Thus, the search for new type of dressing materials able to promote skin wound healing becomes an urgent need. The aim of our study was to find a flax fabric for a new type wound dressings, whose effects on the skin cells cultures could suggest possible improvement in skin wound healing. A favorable dressing material should release active compounds to the moist environment of a wound, which could promote healing the skin injuries. The tested flax fabrics released a detectable amount of polyphenolics and polyhydroxybutyrate to water solutions and to cell culture media; also, they differed in the capacity of the release – the compounds from the W92 fabric were the most easily released. The reason for this is unknown yet, but earlier experiments suggested that the cellulose polymers in W92 fiber were less tightly bound than those in M48 and this might affect extraction efficiency.[2] Probably the more dense fiber structure of the M48 fabric[2] results in a slower release of phenolic compounds from this fabric to the aqueous solution in a 48-hour incubation procedure.

We compared the impact of 2 types of transgenic flax fabrics (W92 and M48) and non-transgenic fabric (Nike) on the proliferation, intracellular ROS and migration of human skin cells. The tested primary cell cultures of keratinocytes, fibroblasts and HDMEC were taken from adult human donors, since chronic skin wounds afflict primarily elderly people.[14] In chronic wounds, the ability of keratinocytes and fibroblasts to proliferate and to release cytokines and growth factors is markedly reduced.[15,16] Therefore, new medicinal materials able to promote wound healing should stimulate the proliferation of these cells as a crucial element of connective tissue repair and epithelial wound closure. The tested flax fabrics enhanced the proliferation of keratinocytes and fibroblasts in cell culture, and the effect of the W92 fabric was the strongest.

It was documented that cells at the non-healing edges of chronic wounds reveal a decreased ability to migrate and differentiate,[17] whereas endothelial cell migration is an indispensable condition of angiogenesis and vasculogenesis, prerequisite for effective wound healing.[18] Therefore, we checked the impact of the flax fabrics on the in vitro endothelial cell migration, In our study, the extracts from the engineered flax fabrics (W92 and M48) enhanced cell migration, while the extract from non-transgenic flax fabric (Nike) lowered migration of endothelial cells as compared to the control culture (without fabric extract).

Chronic inflammatory reactions are considered a regular feature of non-healing skin wounds, being induced and sustained by monocytes/macrophages, which proliferate and release ROS inside a wound.[19] Thus, it was suggested that treatment focusing on the reduction of monocytoid cell proliferation could decrease local chronic inflammation within a wound and enhance the healing processes in chronic wounds. We estimated the proliferation potential and free radical release from established human monocytoid cell line THP1 – the cell line which is often used in studying the mechanisms of monocyte/macrophage participation in inflammatory reactions.[20]

Incubation of the THP1 cells for 48 h in the presence of the aqueous extract from flax fabric decreased proliferation of monocytoid cells. We expected that also in vivo these extracts could decrease monocyte cell burden in human skin wounds, attenuating local inflammatory reactions. The influence of the tested extracts from flax fabrics on ROS generation in resting (not activated) THP1 cells leads to significant increase of the ROS levels, especially in the case of the non-transgenic fabric, when compared to the control cell culture (without fabric extract). However, the intracellular level of ROS was markedly decreased in THP1 cells cultured with the tested extracts and subsequently, stimulated to ROS production with PMA, a strong activator of free radical generating enzymes.[21] The decreasing effect depended on the dose of extracts and was the most significant at the highest extract dose, especially with W92 and M48 extracts.

Although an excess of free radicals is deleterious and apparently should be attenuated to prevent cellular damage and death, a normal, physiological level of free radicals is a necessary component of many intracellular signal transduction pathways, thereby playing a vital role in the cellular stress response and in tissue repair.[22] For instance, free radicals are essential regulators of endothelial cell functions during new vessel formation in wound healing[18,23] and, apparently, are crucial for the host defense against microbial infections.[22] Indeed, a fine-tuning modulator of ROS is required in chronic wound treatment, able to decrease free radical excess without affecting the basic pool of ROS needed for normal physiological processes and intracellular signaling and necessary for resistance against bacterial infection. According to our results, we can conclude that the M48 and W92 flax fabrics are promising modulators of free radical level, able to moderately increase the basic pool of ROS, and to decrease the free radical excess in cells activated to ROS generation.

The results of our in vitro study revealed that the tested flax fabrics from transgenic plants as well as the extracts obtained from the fabrics improved cell activities which could be important for wound healing. A comparison of the results obtained with 6 tests using the MCA shows that the potentially beneficial influence on skin wound healing could be markedly stronger with fabrics from transgenic flax – by 47% (W92) and by 34% (M48) when compared to non-transgenic flax fabric (Nike). The W92 and M48 flax fabrics seem to be a promising material for the development of a new type of dressings, able to improve skin wounds healing.

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Decreased serum level of nitric oxide in children with excessive body weight

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Abstract

Background. Nitric oxide (NO) is an important mediator involved in vascular homeostasis. Changes in NO level are considered to be associated with obesity and its clinical consequences. Previous studies on NO levels in obese children provided inconclusive results, so this issue requires clarification.

Objectives. One of the main goals of this study was to assess whether childhood excessive body weight (EBW) is associated with changes in serum NO level and whether features like age and gender are linked to NO levels in selected groups.

Material and methods. In the present study, the serum NO levels were compared in 43 children with EBW and 37 age- and gender-matched children with normal weight. Moreover, in both groups, body measurements and various clinical parameters, including the serum concentrations of arginine (Arg), a precursor of NO, were determined.

Results. The mean serum NO level in EBW group (8.7 ±3.1 μmol/L) was significantly lower than in normal weight group (22.2 ±11.5 μmol/L). However, the serum Arg concentrations were higher in EBW children than in controls. Serum asymmetric dimethylarginine (ADMA) levels were higher in EBW group and inversely correlated with serum NO. The EBW female subgroup was characterized by slightly lower level of NO than the EBW male subgroup. There were no significant changes in serum NO level among different age subgroups in both groups.

Conclusions. Our results revealed that EBW in children is associated with significantly decreased level of serum NO. The decreased serum NO level in EBW children is not a result of depleted Arg in the blood. Asymmetric dimethylarginine may at least partially contribute to decreased NO levels in children with EBW. A decreased level of NO could be a potential early marker of the risk of cardiovascular disorders developing in children with EBW.

Key words: children, overweight, cardiovascular risk, asymmetric dimethylarginine, nitric oxide

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Introduction

Obesity is a clinical condition defined as an abnormal and excessive accumulation of body fat and it is connected with a wide range of health consequences.[1] For example, obesity (especially central body fatness) is a cardiovascular disease (CVD), diabetes and hypertension risk factor.[2,3] Nowadays, obesity among children and juveniles is recognized as a worldwide epidemic.[4,5] Excessive body weight (EBW) in young age can promote the premature development of CVD. Given the increasing concern for this fact in pediatrics, data on factors related to childhood obesity and early markers of pathological consequences of this complicated disorder is especially needed.

The nitric oxide (NO) is a free radical and a gaseous molecule with a short half-life time. In humans, NO is involved in a variety of physiological and pathological mechanisms. Nitric oxide is critically important in vascular homeostasis and vasomotor tone regulation. This molecule regulates endothelium-dependent vasodilation by the relaxation of smooth muscle cells. Due to this mechanism, NO level plays a role in the regulation of blood flow and blood pressure. Moreover, studies confirmed that NO can modulate cell interaction by decreasing platelet adhesion and aggregation. It is also associated with the suppression of smooth muscle cell proliferation.[1,6] Nitric oxide is produced by NO synthase (NOS) during the transformation of L-arginine into citrulline. Isoforms of this enzyme include: endothelial synthase (eNOS), neuronal synthase (nNOS) and inducible synthase (iNOS).[1] Two of them (eNOS and nNOS) are constitutively expressed and calcium-dependent, and produce stable, low level of NO. In contrast, the 3rd one (iNOS) is calcium-independent and is able to produce huge amounts of NO after induction. The eNOS is expressed primarily in endothelial cells whereas nNOS is expressed primarily in neurons.[7] However, it has been confirmed that all 3 isoforms of NOS are also present in cardiomyocytes.[8] NOS are competitively inhibited by natural methylated metabolite of arginine (Arg) – asymmetric dimethylarginine (ADMA).[9] Changes in NO level have been considered to be associated with juvenile obesity and its clinical consequences like CVD, diabetes and hypertension.[10] However, previous studies on NO levels in obese children provided inconclusive and contradictory results. Codoner-Franch et al. reported increased NO production in obese children, Gruber et al. described the reduction in bioavailability of NO in obese juveniles, whereas Belo et al. found no differences in NO level between obese and eutrophic children.[11–13] Thus, this issue requires clarification.

In the present study, serum NO levels were compared in children with normal body weight and EBW in order to assess whether EBW in children influences NO level. It was also investigated whether features like age and gender are associated with NO levels in selected groups. In addition, we ascertained several other anthropometric and biochemical characteristics of patients in EBW and the normal weight group. Since Arg is a precursor of NO, its concentration in serum was also measured to check if it influences serum NO levels.

Material and methods

Subjects

Forty-three EBW and 37 normal weight age- and gender-matched children were included in this study. The mean age of the subjects was similar in EBW and normal weight group (11.64 ±3.46 and 11.20 ±3.98 years, respectively). The patients were divided into subgroups according to their age (for EBW group: aged 5–9 n = 15, aged 10–14 n = 13 and aged 15–18 n = 15; for normal weight group: aged 5–9 n = 15, aged 10–14 n = 10 and aged 15–18 n = 12) and gender (EBW group: female/male (F/M) = 19/24; normal weight group: F/M = 17/20). The participants of the study were recruited from the patients admitted to the Emergency Department, Orthopedic Ward and Department of Pediatrics, Pediatric Gastroenterology, Hepatology and Nutrition (Medical University of Gdańsk, Poland), with no clinical evidence of chronic diseases and acute infections in 2 weeks preceding blood collection. Children were admitted to the Emergency Department with the suspicion of foreign bodies present in the gastrointestinal tract. Patients from Orthopedic Ward were qualified to corrective surgery of bone alterations. The subjects were also recruited from children participating in a health program funded by Gdańsk City Council (6-10-14 Dla Zdrowia program). None of the children were receiving anti-inflammatory or hypotensive drugs. Basic information about the patients, such as age, gender, body weight, body height, and blood pressure, were recorded. Weight was measured to the nearest 100 g (patients were without shoes and minimally clothed). Body mass index (BMI) was calculated as weight in kilograms divided by height in square meters. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured after 10–15 min resting. The subjects were qualified to EBW or normal weight group based on the BMI percentile and in accordance with International Obesity Task Force (IOTF) criteria.[14] The EBW was defined as a BMI greater than the 85th percentile. All protocols of this study were carried out according to tenets of the Declaration of Helsinki of the World Medical Association.

Blood collection

Blood was collected after informed written consent was given by the patient's parent or legal guardian. Blood samples were obtained from all patients in the morning, after an overnight fast. Blood samples were obtained by venous puncture and centrifuged within 30–45 min. Serum samples were stored at –80°C until analysis.

Analytical procedures

For the purpose of this study, we have assessed NO level in serum samples by an indirect method. Nitric oxide concentration was determined through its stable end products, nitrate and nitrite, by colorimetric detection of the Griess reaction product.[15] Commercial assay Parameter™ Total Nitric Oxide and Nitrate/Nitrite from R&D Systems was used (R&D Systems Inc., Minneapolis, USA). The analysis was performed according to the manufacturer's instruction. Each sample was measured in duplicate.

Serum concentrations of Arg and other amino acids as well as ornithine and ADMA were determined using ion-pair high-performance liquid chromatography and mass spectrometry with electrospray ion source Thermo-Finnigan LCQ Advantage (Thermo Scientific, Waltham, USA), as described previously.[16]

Selected biochemical parameters, i.e., glucose, insulin, total cholesterol, triglycerides (TG), creatinine, C-reactive protein (CPR), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), were measured by the Central Clinic Laboratory of the Medical University of Gdańsk, Poland. The homeostasis model assessment (HOMA) index was calculated as follows: (fasting glucose [mmol/L] × fasting insulin [UE/mL])/22.5.[17]

Statistical analysis

Results are presented as a mean ± standard deviation (SD) or are shown as box plots and the median. The adjustment to normality was verified by using the Kolmogorov-Smirnov test with Lilliefors correction. The significance of the differences between variables was tested by unpaired two-tailed t-test in case of normally distributed variables. The Mann-Whitney U test was used to compare non-normally distributed variables. For the comparison of multiple variables, analysis of variance (ANOVA) test was performed. The p-values less than 0.05 were considered statistically significant. It should be added that the power of unpaired two-tailed t-test was above 0.8. Correlations between selected parameters were determined by linear regression analysis (Pearson's method). All statistical analyses were performed with STATISTICA v. 10 (StatSoft Inc., Tulsa, USA).

Results

General anthropometric and biochemical data of normal weight and EBW children are presented in Table 1. As expected, significant differences were found between groups in terms of anthropometric measurements. Body mass index, centile BMI and weight were higher in EBW group than in normal weight group. Furthermore, the EBW children showed a significant increase in SBP and DBP in comparison to normal weight juveniles. The significant elevations in the levels of ALAT, creatinine, CPR, and lysine (Lys) were also shown. The lipid status of EBW children revealed an insignificant trend to increase in total

Table 1. Anthropometric and biochemical characteristics of studied subjects

Characteristic	Normal weight	Excessive body weight	p-value
Age [years]	11.2 ±3.98	11.6 ±3.46	0.817
Gender (F/M)	17/20	19/24	–
BMI [kg/m^2]	17.6 ±2.83	25.1 ±3.16	0.000*
Centile BMI	42.1 ±24.7	95.8 ±3.05	0.000*
Weight [kg]	41.0 ±18.2	59.5 ±19.3	0.000*
CRP [mg/L]	0.38 ±0.49	1.37 ±1.03	0.000*
Glucose [mg/dL]	88.8 ±17.3	91.0 ±7.48	0.458
Insulin [µIU/mL]	9.86 ±7.32	11.3 ±8.14	0.432
HOMA index	2.24 ±1.73	2.60 ±2.01	0.424
Cholesterol [mg/dL]	154 ±29.6	163 ±24.4	0.169
TG [mg/dL]	75.9 ±28.2	88.4 ±44.1	0.152
Creatinine [mg/dL]	0.58 ±0.16	0.65 ±0.09	0.005*
ASAT [U/L]	21.7 ±7.79	21.1 ±6.79	0.706
ALAT [U/L]	8.85 ±2.89	15.9 ±6.08	0.000*
SBP [mm Hg]	98.9 ±16.2	117 ±15.4	0.000*
DBP [mm Hg]	66.0 ±11.2	73.3 ±11.5	0.008*
Lys [µmol/L]	87.5 ±26.7	106 ±24.7	0.007*
Total aa [µmol/L]	2,148 ±454	2,360 ±375	0.282
Ornithine [µmol/L]	71.5 ±23.5	61.2 ±19.8	0.065
Arg/ADMA ratio	67.6 ±25.1	73.9 ±30.6	0.406

BMI – body mass index; CRP – C-reactive protein; HOMA – homeostasis model assessment; TG – triglycerides; ASAT – aspartate aminotransferase; ALAT – alanine aminotransferase; SBP – systolic blood pressure; DBP – diastolic blood pressure; Lys – lysine; Arg – arginine; ADMA – asymmetric dimethylarginine; * significant difference between groups.

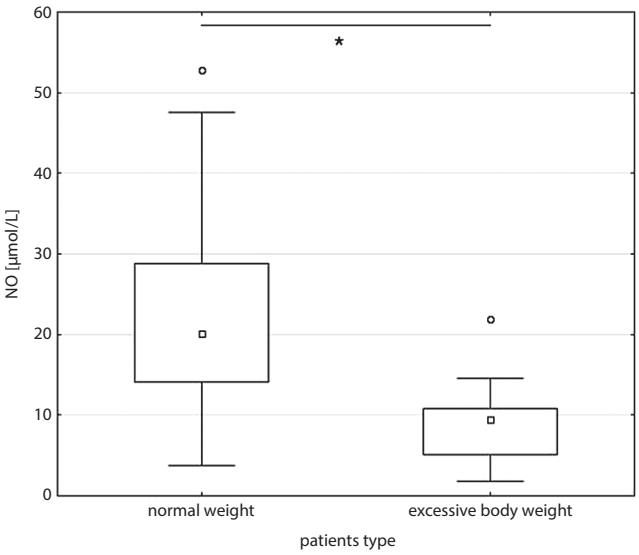

Fig. 1. Box and whiskers plot of serum nitric oxide (NO) levels in normal weight and excessive body weight (EBW) children. The boxes extend from the 25th percentile to the 75th percentile

□ – median, ○ – outliners, * $p < 0.05$.

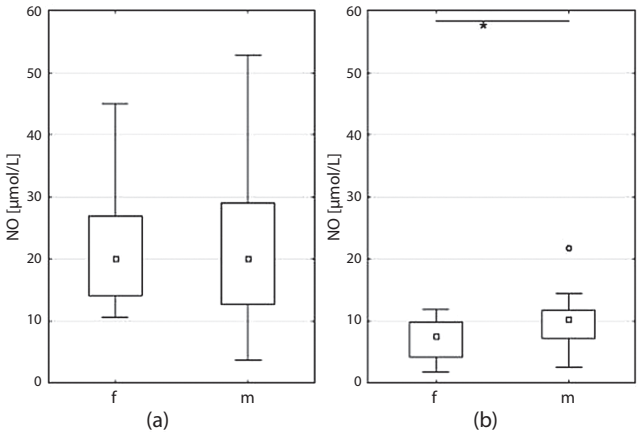

Fig. 2. Box and whiskers plot of serum nitric oxide (NO) levels in normal weight (A) and excessive body weight (EBW) (B) children divided by gender (males – m, and females – f). The boxes extend from the 25th percentile to the 75th percentile

□ – median, ○ – outliners, * $p < 0.05$.

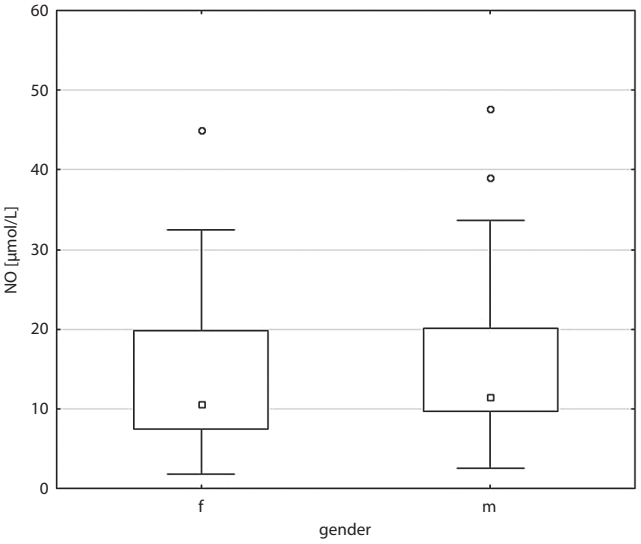

Fig. 3. Box and whiskers plot of serum NO levels in total population of the study divided by gender (males – m, and females – f). The boxes extend from the 25th percentile to the 75th percentile

□ – median, ○ – outliners, * – $p < 0.05$.

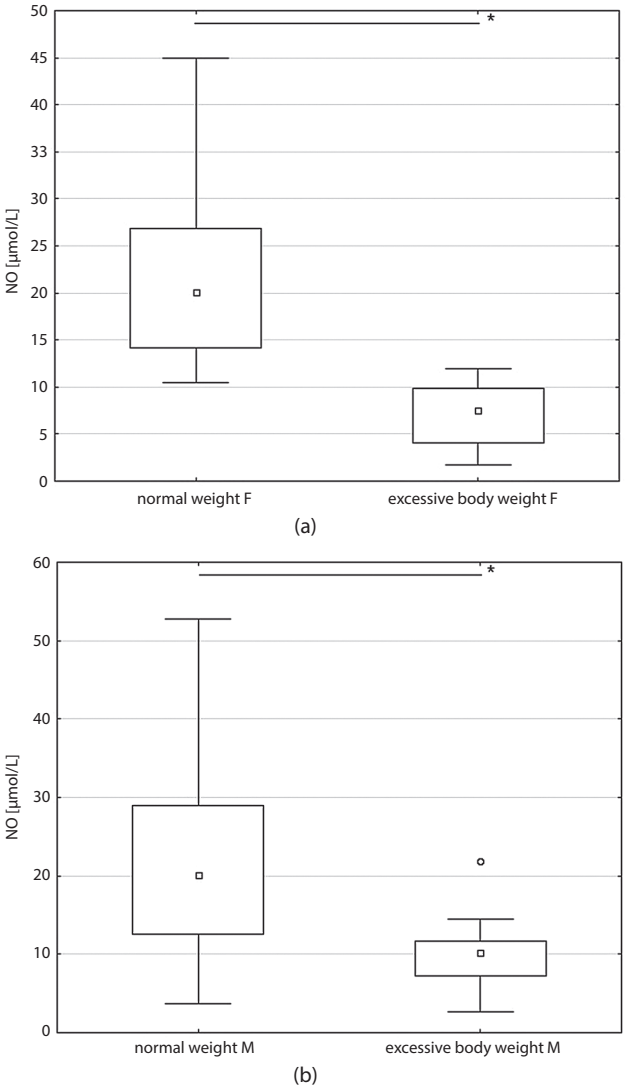

Fig. 4. Box and whiskers plot of serum nitric oxide (NO) levels in females (A) and males (B) subgroup divided by weight category (excessive body weight (EBW), normal weight)

cholesterol and TG. Moreover, no differences in the levels of glucose and insulin, HOMA index, ASAT, ornithine, Arg/ADMA ratio and total amino acids were observed between 2 analyzed groups.

The mean NO level in EBW group was equal to 8.7 ±3.1 µmol/L, whereas in normal weight group the NO level was 22.2 ±11.5 µmol/L. The observed difference was statistically significant (Fig. 1).

It was also investigated whether gender is associated with NO levels. The EBW male subgroup was characterized by higher level of NO than EBW female subgroup (9.9 ±4.2 and 7.2 ±3.0, respectively) (Fig. 2B). The observed difference was statistically significant. However, there was no significant difference in NO levels between normal weight male and female subgroups (Fig. 2A). Moreover, there was no significant difference in NO levels between genders in the total population included in the study (all 80 patients) (Fig. 3). Subsequently, NO levels were analyzed with gender as a main grouping factor (Fig. 4). The normal weight female subgroup was characterized by a higher level of NO than EBW female subgroup (22.4 ±13.2 and 9.8 ±4.2, respectively). The observed difference was statistically significant. Similar statistically significant difference was observed between normal weight male subgroup and EBW male subgroup (22.0 ±9.1 and 7.2 ±3.0, respectively).

Levels of NO in serum among age subgroups were also investigated. There were no significant differences in NO level among age subcategories in both groups, as well as in the total population of the study (Fig. 5).

The Arg (a precursor of NO) and other amino acids levels in EBW and normal weight group were also measured. The Arg level in serum was significantly higher

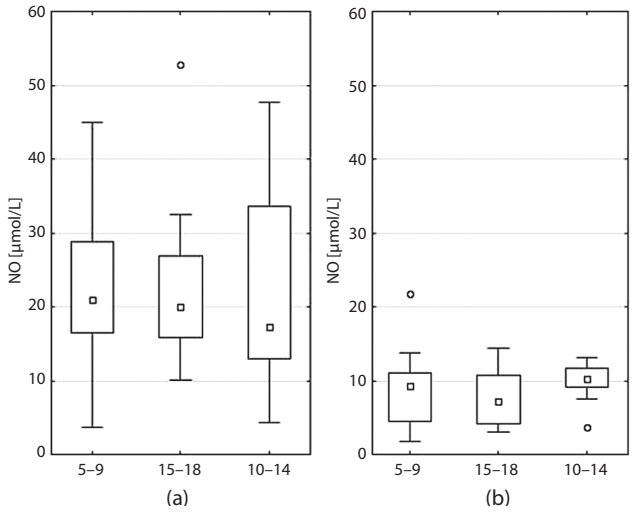

Fig. 5. Box and whiskers plot of serum nitric oxide (NO) levels in normal weight (a) and excessive body weight (EBW) (b) children divided by age (5–9, 10–14 and 15–18). The boxes extend from the 25th percentile to the 75th percentile

□ – median, o – outliners.

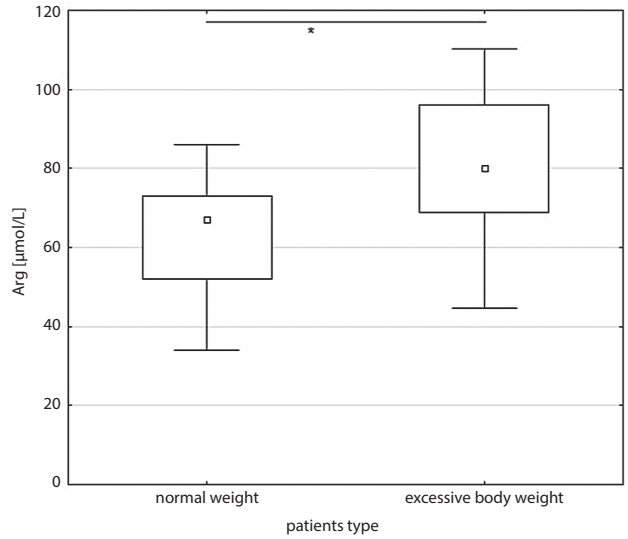

Fig. 6. Box and whiskers plot of serum arginine (Arg) levels in normal weight and excessive body weight (EBW) children. The boxes extend from the 25th percentile to the 75th percentile

□ – median, o – outliners, * $p < 0.05$.

in EBW group that in normal weight group (82.4 ±17.9 and 62.8 ±14.8, respectively) (Fig. 6). Moreover, levels of aspartic acid, glutamine, alanine, valine, tyrosine, Lys, and leucine were significantly higher in EBW children than in normal weight group (data not shown).

We also measured the serum level of ADMA – a competitive inhibitor of NOS. We found significantly higher levels of ADMA in the EBW group than in controls (Fig. 7). However, the Arg/ADMA ratio did not differ between groups (Table 1).

Correlations between NO level or Arg and other biochemical characteristics in the total population of the

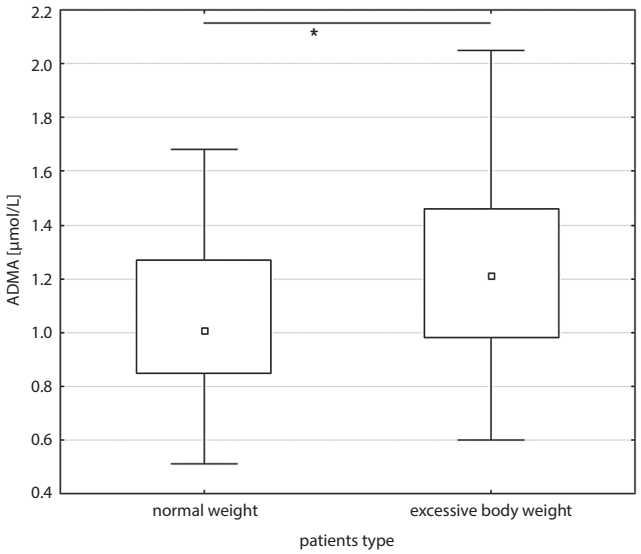

Fig. 7. Box and whiskers plot of serum asymmetric dimethylarginine (ADMA) levels in normal weight and excessive body weight (EBW) children. The boxes extend from the 25th percentile to the 75th percentile

□ – median, o – outliners, * $p < 0.05$.

Table 2. Pearson's analysis of correlation between nitric oxide (NO) or arginine (Arg) and selected parameters in studied subjects

Characteristic	NO [µmol/L]	Arg [µmol/L]
BMI [kg/m^2]	–0.501*	0.441*
Centile BMI	–0.319*	0.393*
Weight [kg]	–0.263*	0.298*
CRP [mg/L]	–0.312*	0.339*
Glucose [mg/dL]	–0.134	0.230
Insulin [µIU/mL]	–0.068	0.202
HOMA index	–0.072	0.188
Cholesterol [mg/dL]	–0.221	–0.049
TG [mg/dL]	–0.001	0.029
Creatinine [mg/dL]	–0.059	0.268*
ASAT [U/L]	0.032	–0.151
ALAT [U/L]	–0.264*	0.144
SBP [mm Hg]	–0.343*	0.439*
DBP [mm Hg]	–0.193	0.457*
Lys [µmol/L]	–0.197	0.631*
ADMA [µmol/L]	–0.269*	0.100
NO [µmol/L]	–	–0.434*

BMI – body mass index; CRP – C-reactive protein; HOMA – homeostasis model assessment; TG – triglycerides; ASAT – aspartate aminotransferase; ALAT – alanine aminotransferase; SBP – systolic blood pressure; DBP – diastolic blood pressure; Lys – lysine, Arg – arginine; ADMA – asymmetric dimethylarginine; * statistically significant correlation ($p < 0.05$).

study were tested (Table 2). The NO level was significantly and negatively correlated with BMI, CRP and SBP. The same biochemical characteristics were positively correlated with Arg level. Furthermore, the significant negative correlation was found between NO level and ADMA as well as Arg.

Discussion

In this study, serum NO level was assessed in normal weight and EBW children. The main findings are as follows: 1. compared with normal weight children, the EBW children had significantly lower serum NO concentration; 2. in EBW children, females had slightly lower serum NO level than males; 3. serum Arg and ADMA levels were higher in EBW group. A significantly lower serum level of NO in EBW children found in our study is in line with studies which suggest a correlation between decreased bioavailability of NO and obesity, both in children and adults.[12,18] However, in some publications, no changes or increase the bioavailability of NO in obese children were reported.[11,19,20] According to Codoner-Franch et al., differences in NO level were caused by dissimilarities in subjects among studies, mainly regarding age.[11] However, we examined children in the age comparable with the subjects studied by Codoner-Franch et al. and we obtained different results. In addition, the subjects included in our study were younger than children studied by Gruber et al., but in both studies, higher levels of NO were observed in normal weight children.[12] Furthermore, we observed no differences between NO levels among age subgroups in our study (no differences within normal weight group, EBW group and total population). In our opinion, these discrepancies may be derived from analyzing obesity on different levels of progression (based on characteristics like BMI, TG, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and CRP). Changes in serum NO level can be a result of complex interactions between factors that regulate NOS activity, NOS mRNA stability and NO degradation.[7] All those factors could differ throughout the development of obesity. It is conceivable that in the initial state of obesity there is a decline of NO level, and, after that, NO level could increase in severe obesity. In obesity increased oxidative stress is observed. This systemic stress can increase along with fat accumulation.[21] Reactive oxygen species (ROS) can diminish NO bioactivity. For instance, ROS can decline the effect of NO by inactivating it directly. It has been proven that superoxide reacts with NO, and peroxynitrite is produced. This reaction both reduces the level of NO and produces more damaging ROS. Moreover, ROS can also disturb NO actions by direct competition (oxidation of the sites of protein which NO reacts with) or allosteric modulation (influence on NO binding sites).[8] The decrease in the NO concentration beneath critical value would facilitate the activation of the compensatory mechanisms and increase NO level. Enhanced NO production may occur through induction of iNOS expression. First of all, it has been shown that NO inhibits the transcription of the gene-encoding iNOS in several cell types, which is why a decrease in NO concentration removes the inhibitory effect.[22–24] Secondly, obesity is considered an inflammatory condition and overproduction of NO is a consequence of inflammatory condition, since iNOS requires INF-γ, IL-1β and TNF-α for induction.[7,25,26]

In the current study, a significant difference was found in NO concentration between genders in EBW group. In female EBW subgroup, NO level was lower than in the male EBW subgroup. However, EBW girls were slightly older compared to EBW boys (12.9 ±3.0 vs 10.7 ±3.5). What is surprising, despite the same age difference in the normal weight group, no differences in NO level between genders were observed in this group. There were also no differences in NO level among age subgroups in the general population of the study. Other authors also reported a lack of such difference between NO level and gender in the general population of their studies.[11,20] It has been proven that obesity may perturb pubertal development by advancing puberty in girls and delaying puberty in boys.[27] Therefore, it is reasonable to assume that puberty is somehow involved in the regulation of NO level. Since in the estimated group, the girls were slightly older than boys, puberty among girls may be more advanced and this may result in some gender-related differences between both groups, including higher NO level in male EBW group than in female EBW group (Fig. 2B).

In our study, the serum level of Arg was significantly higher in the EBW group than in children with normal weight. This observation is in line with findings of other authors (e.g., Gruber et al.).[12] Therefore, it seems that the lower level of NO cannot be explained by the lack of substrate. Moreover, we have found a negative correlation between NO level and Arg concentration in serum. This relationship between Arg (substrate) and NO (product) may be explained by the phenomenon described in the literature as "arginine paradox".[28] Plasma concentration of Arg is a result of protein catabolism and synthesis from other amino acids. The majority of synthesized Arg is derived from citrulline (by argininosuccinate synthase and argininosuccinate lyase). However, it seems that an extracellular/exogenous Arg concentration (Arg transported into cells) is the major determinant of NO production.[29–31] Furthermore, it has been proven that the transport of extracellular Arg by the cationic amino acid transporter I (CAT-1) can be suppressed by L-lysine.[32] In our EBW group, the concentration of serum Lys was increased in comparison with normal weight children. This fact may also explain why higher concentration of serum Arg does not translate directly into higher NO production; however, only weak, nonsignificant negative correlation between serum NO and Lys was found in our subjects. Children with EBW had slightly, but significantly increased serum levels of natural NOS inhibitor – ADMA. Moreover, we found a significant inverse correlation between serum ADMA concentration and NO levels. Thus, ADMA may at least partially contribute to decreased NO levels in children with EBW. Also, other authors found a correlation between obesity and increased ADMA.[33] Moreover, they found that ADMA may be implicated in the increase of blood

pressure. It should be noted that our EBW children had also increased both ADMA and SBP. Another factor that contributes to increased NO synthesis is serum adiponectin concentration.[13,34] Since decreased serum adiponectin level in children obesity is well-documented, it could be another reason for decreased serum NO levels in EBW children.[35] The nutritional differences may also influence the serum levels of NO and Arg in different individuals. Unfortunately, the subjects from the control group did not fill a nutritional questionnaire, so we were unable to verify this, and this is a limitation of the study. However, all participants of the study were recruited from the same population (residents of Gdańsk and its surroundings), so we suppose that diets in both groups were generally similar. Our findings may also be limited by a relatively small number of subjects enrolled in the present study.

We found significantly increased SBP and DBP in EBW children, and a statistically significant correlation between NO level and SBP. A similar observation was made by Codoner-Franch et al., and Ghasemi and Zahediasl.[11,19] This may suggest that obesity-related NO reduction is involved in the development of hypertension in children and may contribute to CVD development in later age. Because of the complex nature of the interactions between serum NO level and obesity, at this point, NO concentration has no prognostic implications as an individual marker. Further studies on the molecular mechanism of changes in serum NO level during development of obesity may increase the clinical value of this marker. However, monitoring of serum NO level in EBW and obese children together with well established CVD biomarkers (e.g., lipid profile, inflammation, arterial structural vulnerability) may be beneficial in risk assessment process.[36]

Conclusions

In conclusion, we showed that EBW in children is associated with decreased serum level of NO. The decreased level of NO in this group is not a result of deficient accessibility of serum Arg, but may be associated with increased serum concentration of ADMA. Our results suggest that decreased level of NO could be involved in the development of cardiovascular disorders among children with EBW. Future studies should apply analyses in a larger group of patients to evaluate the generality of the obtained results.

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Analysis of adnexal mass managed during cesarean section

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D – writing the article; E – critical revision of the article; F – final approval of the article

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Conflict of interest
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Abstract

Background. Pregnancy with an adnexal mass is one of the most common complications during pregnancy and clinicians are sometimes caught in a dilemma concerning the decision to be made regarding clinical management.

Objectives. The objective of this study was to outline and discuss the clinical features, management and outcomes of adnexal masses that were encountered during a cesarean section (CS) at a university-affiliated hospital in China.

Material and methods. The medical records of the patients with an adnexal mass observed during a CS were retrospectively collected at Women's Hospital, Zhejiang University School of Medicine, Hangzhou, China, from January 1991 to December 2011.

Results. The incidence of adnexal masses was 16.40 per 1000 CSs. The most common pathologic diagnosis was benign ovarian tumor, the 2nd was ovarian endometrioma and the 3rd was theca lutein cyst. Thirteen cases of ovarian malignancies were diagnosed during a CS. Only 388 cases (29.78%) were detected using an ultrasonography (USG) examination before a CS. Eight cases required emergency CS due to abdominal pain; all other patients were clinically asymptomatic. The reasons for abdominal pain included torsion (n = 5), rupture (n = 2) and ovarian enlargement (n = 1). In 13 cases with ovarian endometrioma, cysts ruptured during a CS without any clinical manifestation. No maternal and fetal complications related to surgery were observed.

Conclusions. Preconception care and routine prenatal care, including USG examination, may optimize the detection and management of an adnexal mass. The presumptive ovarian endometrioma detected before pregnancy could be the indication for surgery due to the possibility of spontaneous hemoperitoneum. Theca lutein cysts might be huge and exist throughout the whole pregnancy period. Expectant management is reasonable for an adnexal mass that emerged during pregnancy without suspicion of malignancy. Abdominal pain might be a clue for cyst torsion or rupture.

Key words: pregnancy, cesarean delivery, adnexal mass

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Introduction

Pregnancy with an adnexal mass is one of the most common complications during pregnancy, with an incidence of 1–5.3%, according to previous reports.[1–5] Improper management of an adnexal mass during pregnancy can endanger the maternal and neonatal safety, owing to the particularity of the pregnancy. Both the fetus and mother should be taken into account when the doctors try to manage the adnexal mass. Due to the development of ultrasonography (USG) technology and the awareness regarding prenatal health care, an increased number of adnexal masses are detected during pregnancy.[6,7] As we know, the risk of malignancy of adnexal masses during pregnancy cannot be determined by an USG examination alone, and the true pathologic type can be determined only by surgical examination of the masses. Thus, the clinician sometimes would be caught in a dilemma concerning whether to choose surgery or expectant management. Moreover, despite the widespread use of USG technology, the incidence of adnexal masses incidentally discovered during cesarean section (CS) remains high. This would potentially carry risk for clinical management. The aim of the present study was to compare and report the clinical characteristics of adnexal masses with different pathologic types that were encountered during CS at Women's Hospital, Zhejiang University School of Medicine, Hangzhou, China. It is also the intention of this study to provide a reference for diagnosis and treatment of adnexal masses during pregnancy.

Material and methods

This is a clinical retrospective study of gravid women with an adnexal mass diagnosed during CS at our hospital in the 21-year period between January 1991 and December 2011. Approval from the Ethics Committee of the hospital was obtained before the study was conducted. Owing to the retrospective character of the study, informed consent was not needed. The following data was collected: patient's age, gestational age, gravidity and parity history, symptoms, USG records before CS, indication for CS, the size and site of the adnexal mass at CS, surgical procedure, pathologic diagnosis, perioperative complications, and neonatal outcome. Final diagnosis was confirmed by a routine paraffin section after surgery. The pathology specialists at our hospital reviewed all pathologic sections. The present conditions of the patients with malignancy were followed up and the information was retrospectively recorded up to the date of the last contact. For theca lutein cysts, the manifestations were followed up until the masses dissolved. Data was analyzed using the χ^2 test. Statistical analysis was performed with SPSS v. 20.0 software (IBM Corp., Armonk, USA). The p-values below 0.05 were considered statistically significant.

Results

Basic information

There were 79,548 CSs at our hospital during the studied 21 years. Briefly, 1,303 cases of adnexal masses were diagnosed during CS. The incidence was 1.64%. Among patients undergoing CS, the median age was 29 years (20–42 years). The median pregnancy duration was 37 weeks (28–42 weeks), including 1,263 (96.93%) women with a duration ≥34 weeks and 40 (3.07%) women with a duration <34 weeks; 1,215 (93.25%) women were primipara and 88 women were multipara (6.75%). All women underwent CS for different indications (Table 1). Of the

Table 1. The indications for cesarean section (CS)

Indication	n [%]	Indication	n [%]
Social factor	238 (18.27)	cord entanglement	44 (3.38)
Fetal distress	150 (11.51)	placenta previa/abruption	39 (2.99)
Adnexal mass	148 (11.36)	multiple pregnancy	32 (2.46)
Abnormal fetal position	147 (11.28)	IVF-ET	27 (2.07)
Cephalopelvic disproportion/arrested labor[1]	124 (9.52)	medical disease[2]	26 (2.00)
Oligohydramnion	88 (6.75)	surgical diseases[3]	14 (1.07)
Intrahepatic cholestasis of pregnancy	60 (4.60)	symphysis separation	12 (0.92)
High myopia	54 (4.14)	postterm pregnancy	7 (0.53)
Scar uterus[4]	45 (3.45)	induced labor failure	2 (0.16)
Preeclampsia	45 (3.46)	verruca acuminatapubic	1 (0.08)

IVF-ET – in vitro fertilization and embryo transfer; [1] cephalopelvic disproportion/arrested labor: macrosomia, uterine inertia, cervix edema, pendulous abdomen, protracted active phase, contracted pelvic outlet, prolonged latent phase; [2] medical diseases: cardiac disease, kidney disease, hematological system disease, gestational hypertension, gestational diabetes mellitus, pregnancy-induced eczema; [3] surgical diseases: uterine malformation, tail bone fracture, varicosity, pendulous abdomen, lumbar disc herniation; [4] scar uterus: 41 cases were previous cesarean deliveries and 4 cases had history of uterine surgery; categorical variables were expressed as number and percentage.

1,303 patients, 1,290 (99.00%) cases received a prenatal visit. Only 388 (29.78%) masses were detected by a USG examination before surgery; 132 (34.02%) masses were found before pregnancy, 120 masses (30.93%) in the 1st trimester, 63 masses (16.24%) in the 2nd trimester, and 73 masses (18.81%) in the 3rd trimester. No sign of malignancy was observed for these masses during pregnancy. The remaining 915 (70.22%) cases were incidentally discovered during CS.

Clinical manifestations

Most masses were clinically asymptomatic, except for 8 (0.61%) cases which required emergency CS due to acute abdominal pain. The reasons for abdominal pain included torsion (n = 5, 3 for benign ovarian tumor, 1 for ovarian endometrioma and 1 for theca lutein cyst), rupture (n = 2; 1 for benign ovarian tumor and 1 for ovarian endometrioma) and ovarian enlargement (n = 1; theca lutein cyst) (Table 2). In 13 patients with ovarian endometrioma, the cyst ruptured during CS without any obvious abdominal pain and discomfort during pregnancy. Among them, 4 masses were detected before pregnancy, 3 cases in the 1st trimester and 6 cases at CS. Among 179 cases with theca lutein cyst, only 19 cases were secondary to assisted reproductive technologies.

Diagnosis and management

A frozen section was applied for suspected malignant mass. The final diagnosis was made according to the pathologic report by the paraffin section. The clinical characterstics and management are presented in Table 3 and the pathologic types of adnexal masses are shown

Table 2. Cases that received emergency cesarean section (CS) due to acute abdominal pain

Pathology	GA [weeks]	Complication	Management	Lateral	Size [cm]
Mature teratoma	39	torsion	cystectomy	R	9
Serous cystadenoma	37	torsion	cystectomy	R	7
Mucinous cystadenoma	37	torsion	SO	R	15
Mucinous cystadenoma	38	rupture	SO	L	30
Ovarian endometrioma	28	torsion	cystectomy	Bi	4, 4
Ovarian endometrioma	35	rupture	cystectomy	R	6
Theca lutein cyst	38	torsion	SO	L	5
Theca lutein cyst	38	NA	SO	Bi	9, 10

GA – gestational age; SO – salpingo-oophorectomy; R – right; L – left; Bi – bilateral; NA – not applicable.

Table 3. Clinical characteristics and management of 1,303 adnexal masses

Pathology	n [%]	Cystectomy	SO	Uni	Bi	<5 cm	5 cm	>5 cm
Benign ovarian tumor								
Mature teratoma	390 (29.93)	380	10	353	37	140	84	166
Serous cystadenoma	155 (11.90)	153	2	149	6	109	19	27
Mucinous cystadenoma	76 (5.83)	62	14	75	1	18	9	49
Fibroma	26 (2.00)	25	1	26	0	23	1	2
Mixed cystadenoma	8 (0.61)	8	0	7	1	4	3	1
Ovarian leiomyoma	1 (0.08)	1	0	1	0	1	0	0
Brenner tumor	1 (0.08)	1	0	1	0	0	1	0
Theca cell tumor	1 (0.08)	1	0	1	0	0	0	1
Ovarian endometrioma	251 (19.26)	243	8	219	32	168	32	51
Ovarian tumor-like condition								
Theca lutein cyst*	179 (13.74)	74	2	73	106	54	27	98
Simple cyst	103 (7.90)	103	0	101	2	73	12	18
Stromal hyperplasia	34 (2.61)	34	0	34	0	34	0	0
Paraovarian-paratubal cyst	65 (4.99)	65	0	64	1	50	6	9
Ovarian malignancy**	13 (1.00)	3	9	13	0	6	2	5

SO – salpingo-oophorectomy; Uni – unilateral; Bi – bilateral; * 103 theca lutein cysts were not listed in this table, they received biopsy (40), paracentesis (2) or were untreated (61); ** 1 patient with ovarian carcinoma, who received radical surgery, was not listed here (the detailed data of ovarian malignancy was published in Reference 8); categorical variables were expressed as number and percentage.

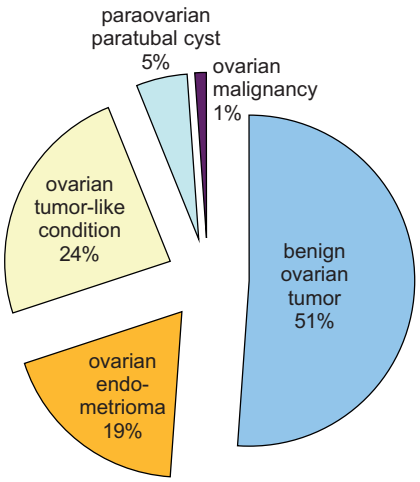

Fig. 1. The pathological types distribution of 1,303 adnexal masses

in Fig 1. In brief, the most common histological type was mature teratoma (390, 29.93%), the 2nd was ovarian endometrioma (251, 19.26%), the 3rd was theca lutein cyst (179, 13.74%), and the 4th was serous cystadenoma (155, 11.90%); 13 (1.00%) were ovarian malignancies.[8] The median adnexal mass size for benign ovarian tumor was 5 cm (range from 0.1 to 30 cm), 6 cm for theca lutein cyst (from 0.3 to 16 cm), 4 cm for ovarian endometrioma (from 0.5 to 13 cm), 3 cm for paraovarian-paratubal cyst (from 0.5 to 10 cm), and 5 cm for ovarian malignancy (from 2.5 to 30 cm). The frequencies of unilateral and bilateral of the adnexal mass were 1,117 (85.73%) and 186 (14.27%), respectively. After excluding the rare pathologic types, we found that most masses were unilateral (84.80%), except for theca lutein cysts (40.80%); the differences were significant ($\chi^2 = 330.799$, $p = 0.000$). Most masses had the size of 5 cm or less (65.70%), except for theca lutein cysts (45.30%); the differences were significant ($\chi^2 = 142.134$, $p = 0.000$). A cystectomy was performed in 1,153 (88.49%) cases. Unilateral salpingo-oophorectomy (SO) was performed in 45 (3.53%) cases. Bilateral SO was performed in 1 case with theca lutein cyst due to misdiagnosis as malignancy. One ovarian carcinoma received radical surgery, including total abdominal hysterectomy, SO and pelvic lymphadenectomy during CS. Briefly, 103 cases with theca lutein cysts were treated with biopsy (40), paracentesis (2) or were untreated (61). All the 103 theca lutein cysts resolved spontaneously within 1–12 months after CS, which was confirmed by an USG examination, and no complications, such as cyst torsion or rupture, presented during the follow-up period. A total of 1,338 healthy live newborns were recorded, including 178 premature newborns (28–37 weeks). No fetal mortality and congenital malformations were detected in the newborns. No complications, morbidity and mortality related to surgical removal of the masses during CS were observed. No tumor metastasis to placenta and fetus was recorded for ovarian malignancies at the mean follow-up time of 108.7 months. All patients with malignancies are currently in complete remission and tumor-free.

Discussion

Depending on the method one uses and how one defines a clinically significant adnexal mass, the prevalence of pregnancies complicated by an adnexal mass varies.[1–3] In accordance with previous reports, we found that the incidence of adnexal masses diagnosed during CS was about 1.64%. As we know, a USG examination is the most helpful method in the detection and evaluation of an adnexal mass in women, regardless of whether the woman is pregnant or not. Moreover, it can detect masses smaller than those usually detected during a physical examination, especially during pregnancy.[3] Aggarwal and Kehoe reviewed the studies conducted between 1984 and 2009 and found that a USG examination increased the detection of adnexal masses in pregnancies from 0.04–1.32% (1/76–1/2,328) to 1.14–5.23% (1/19–1/88).[6] The majority of masses found during pregnancies are small (<5 cm) and represent functional cysts that typically resolve spontaneously by the 2nd trimester.[1] Our data reflects the actual rate of adnexal masses in CS patients, which can explain why the rate is situated in the low limitation of the previous reports. Nevertheless, the incidence of masses discovered during CS was much higher than that (0.49%) detected before and during pregnancy in the present study. Only 29.78% of the adnexal masses were detected before CS, although about 99.00% gravida women received a prenatal examination. This means that more than half of the masses were diagnosed incidentally during CS. This is similar to the results of a study by Baser et al., which revealed that 83 (55.0%) adnexal masses were incidentally discovered during CS.[7] The probable reasons for this low diagnostic rate during pregnancy were addressed here. Most adnexal masses were asymptomatic and small (≤5 cm); the pregnant patients refused a pelvic examination and a transvaginal USG examination for the fear of abortion during early pregnancy; some adnexal masses might emerge after pregnancy and gravid uterus may obscure the correct visualization and detection. Thus, it is important to promote the use of USG and improve the USG technique during preconception and prenatal visits in China.

Once the adnexal mass is detected during pregnancy, the obstetrician would have to make the clinical decision of how to manage the mass, since the masses can affect the pregnancy outcome due to the risk for torsion, rupture, bleeding, obstruction, or malignancy. Even today, the management of adnexal masses during pregnancy is controversial.[9–10] While some obstetricians prefer elective removal in the 2nd trimester, others state that a conservative approach results in the resolution of most masses and avoid unnecessary surgery.[6] Surgery during pregnancy carries some inherent intraoperative and perioperative risks, including the added risks of fetal loss, preterm contractions and an increased risk of embolic events.[1,11] However, observing a mass during pregnancy might delay the treatment if the adnexal mass is malignant or develops an acute

event, such as ovarian torsion, cyst rupture or obstruction of labor, which often necessitates emergency intervention.[1] The pathologic types in the present study are concordant with those in previous reports.[7,12–14] Owing to their characteristic USG appearance, the differential diagnosis for most masses is relatively easy with the improvement of the USG technique, especially for an experienced sonographer. However, some malignancies were still misdiagnosed before CSs in our study and in the reports from other researchers. The rate of malignancy in the present study was 1.00% (13/1303), including 8 low-malignant potential tumors, 3 invasive epithelial carcinomas and 2 malignant germ cell tumors.[8] All malignancies were clinically asymptomatic and were diagnosed in stage I with good prognosis. Thus, we agree that delaying surgery may be feasible to avoid any unnecessary risks to the pregnancy, depending on the clinical suspicion of malignancy.[1] For asymptomatic adnexal masses, surgery should only be considered in a pregnancy for suspicious or obvious malignant tumors.[15] No new malignances were diagnosed after 2008 in the present study. This might be due to the improved technique of USG and most suspicious adnexal masses receiving surgery before CS. Nevertheless, routine puerperium USG is strongly recommended for vaginal delivery, since it raises the possibility for delaying the diagnosis of an adnexal mass.

In the present study, we found that 20 (1.61%) cases experienced torsion or rupture. A previous study reported that ovarian torsion was most commonly (60% of the time) seen in pregnant women with an adnexal mass.[1] Interestingly, 75% of complications were connected to ruptures and most of them in the present study were ovarian endometriomas. However, the rate of torsion would be increased to 71.43% (5/7) if the asymptomatic ruptures of ovarian endometrioma were excluded. The complications managed during early or mid-pregnancy were excluded, because we only collected the information of the patients with an adnexal mass observed during CS; as a result, the actual rate of torsion or rupture here was lower than in previous reports. During the last decade, the increased use of assisted reproductive technologies has led to higher fertility rates in patients with ovarian endometrioma. Therefore, the number of pregnant women with ovarian endometrioma and associated complications may rise, despite most investigators reporting regression or cessation of growth of the endometriomas during pregnancy.[4,16,17] Theoretically, ovarian endometrioma torsion and rupture may be less common during pregnancy due to its adhesion to the neighboring tissues, and pregnancy may have a beneficial effect on endometriosis by promoting involution of decidualizing ectopic endometrium.[18–20] However, we found that 71.43% (15/21) of the masses with complications were ovarian endometriomas and most of them (14/15) were ruptures. This is concordant with a previous study, which reports that ovarian endometrioma is a major risk factor for spontaneous hemoperitoneum in pregnancy.[18] Fortunately, no massive hemoperitoneum presented in this study. Of the 15 cases, only 2 received emergency CS due to acute abdomen pain. Other 13 cases with ruptures were asymptomatic and diagnosed at the time of the CS for other surgical indications. Although ovarian endometrioma is widely studied, little is known about the incidence of ovarian endometrioma complications during pregnancy. This study is the first detailed report on the rupture and torsion in ovarian endometrioma observed during a relatively large number of CSs. The possibility of spontaneous hemoperitoneum should be always kept in mind, since 7.97% of ovarian endometriomas experienced a rupture in the present study.[17]

Functional cysts usually resolve spontaneously by the 2nd trimester, while we found that 179 (13.74%) were theca lutein cysts, which persisted until CS. Theca lutein cysts, also known as hyperreactio luteinalis (HL), are a type of functional ovarian cyst.[21,22] They are typically multiple and bilateral, and are usually associated with gestational trophoblastic disease, ovulation induction and very rarely with a normal pregnancy. Interestingly, only 19 cases received ovulation induction in the present study. Production of high concentrations of human chorionic gonadotropin (HCG) and increased ovarian sensitivity to prolonged exposure to HCG may be manifested as an exaggerated ovarian response, leading to theca lutein cyst formation.[23,24] In the present study, 1 case with HL was misdiagnosed as a malignancy during CS and received bilateral SO (Table 2). The bilateral masses were 10 cm in size and the surgeon did not recognize them. After that, all obstetricians in our hospital were alerted to the possibility that huge theca lutein cysts can exist through the whole trimester and mimic malignancy. That is why doctors should be aware that theca lutein cysts may mimic malignancies and lead to an unnecessary ovarian resection.[21,25] Furthermore, most theca lutein cysts are bilateral (54.7%), which might be helpful for a differential diagnosis. In the present study, most theca lutein cysts were asymptomatic and were found incidentally with USG or during CS; 34.08% of cases were managed expectantly by experienced obstetricians and showed to resolve spontaneously postpartum. Thus, the management should be carefully chosen depending on the manifestation of the masses. It is important to exclude malignancies with a biopsy (or wedge resection) and then freeze the section in order to avoid unnecessary surgical excision.[21] Paracentesis is acceptable for huge masses to minimize their volume and avoid complications if no malignancy signs are present, while a cystectomy and SO are not recommended unless there is a definite indication.

Conclusions

Based on our results and previous reports, an USG examination is still the preferred auxiliary examination to rule out the adnexal mass, although most masses in the present study were incidentally discovered during CS. Preconception care, routine prenatal care and puerperal checks,

including USG examinations, may be used to optimize the detection and management of adnexal masses, since most masses are unilateral and ≤5 cm. Active treatment is advised for the persistent adnexal masses or presumptive ovarian endometriomas found before pregnancy, to avoid acute abdominal disease or other complications related to pregnancy that could threaten maternal-fetal safety. For the masses detected during pregnancy, expectant management is recommended if no malignancy is suspected and closer observation is recommended.[6,26] The presumed risk of torsion or rupture should not be considered as an indication for surgery.[27] Moreover, the patients should also be aware that antenatal surgery might become necessary once the mass becomes symptomatic or its features change.[6] For the masses discovered during CS, surgical removal is preferred to prevent subsequent complications (torsion or rupture) or a future requirement for surgery, except for functional cysts such as theca lutein cysts.[6,28,29]

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Evaluation of antioxidant activity of extracts from the roots and shoots of *Scutellaria alpina* L. and *S. altissima* L. in selected blood cells

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D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. It is widely known that reactive oxygen species (ROS) can cause oxidative damage in cells and have been linked to the pathogenesis of oxidative diseases, such as atherosclerosis, ischemia, neurodegenerative disease, diabetes, or cancer. Recently, much attention has been focused on preventive strategies for oxidative stress and related diseases. Plants represent a source of bioactive compounds whose antioxidant activity may be useful in protecting against pro-oxidative reactions.

Objectives. The study determines the in vitro biological activity of the ethanolic extracts from the shoots and roots of *Scutellaria* species (*S. altissima* and *S. alpina*) in selected blood cells (blood platelets and lymphocytes).

Material and methods. Platelet activity, both resting and after thrombin stimulation, was used to indicate the ability of the plant extracts to inhibit the production of superoxide anion radicals ($O_2^{\bullet -}$) and platelet lipid peroxidation. The generation of superoxide anion radicals was measured with cytochrome c reduction. Lipid peroxidation in blood platelets was measured by the level of thiobarbituric acid reactive substances (TBARS). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to determine the protective effect of *Scutellaria* extracts on lymphocyte cells against oxidative damage induced by hydroxyl radicals.

Results. Extracts (5–50 µg/mL) containing phenolic compounds from both *Scutellaria* species distinctly reduced nonenzymatic lipid peroxidation and arachidonic acid metabolism by blood platelets in vitro. When given at the tested concentration, the extracts reduced the generation of $O_2^{\bullet -}$ in resting blood platelets and platelets activated by thrombin in vitro. All *Scutellaria* extracts (10 µg/mL) containing phenolic compounds also protected human lymphocytes against oxidative stress induced by hydrogen peroxide (H_2O_2).

Conclusions. The present study suggests that the natural extracts from *S. altissima* and *S. alpina* have antioxidant properties and, therefore, may be beneficial in the prevention of diseases in which blood platelets and lymphocytes are involved, i.e., cancer or inflammatory and infective diseases.

Key words: oxidative stress, lymphocytes, blood platelets, polyphenols, *Scutellaria*

Introduction

Reactive oxygen radicals are released under conditions of stress and cause a number of pathological changes in all cells of the human organism. In recent years, increased attention has been given to the antioxidant activity of plants. Many studies suggest that several medicinal plants containing polyphenolic compounds can protect cells against destructive oxidative damage and limit the risk of various diseases associated with oxidative stress. Natural antioxidants are known to be radical scavengers or radical-chain breakers, which can inhibit or delay the oxidation process.

Extracts from *Scutellaria* plants have been used in traditional Chinese medicine for their hepatoprotective, anti-inflammatory, antihistaminic, hyperlipidemic, antibacterial, antiviral, antitumor, and other pharmacological properties for centuries.[1] Today it is known that the dominant role in these therapeutic effects may be attributed to their antiradical properties. Flavones present in *Scutellaria* extracts, which can bind and eliminate heavy metals and scavenge free radicals, are strong antioxidants. Experiments have shown that the flavonoids present in skullcap plants protect hepatocytes against necrosis and mutagenesis induced by toxins, high doses of paracetamol or hydrogen peroxide (H_2O_2).[2–4] *Scutellaria* metabolites have demonstrated protective activity towards nervous cells and have effectively limited the development of neurodegenerative diseases. For example, baicalin is known to have a neuroprotective effect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a factor causing changes in dopaminergic neurons, which can translate into the occurrence of the Parkinson's disease.[5] *Scutellaria* extracts have also exhibited a protective effect on erythrocytes and prevented their membranes from free radical damage.[6] However, no studies have examined the effects of skullcap extracts on the viability and function of the other cellular components of blood under conditions of oxidative stress.

Blood platelet activation results in the production of reactive oxygen species (ROS), which play a crucial role in hemostasis and thrombosis. Reactive oxygen species can behave as 2nd messengers during platelet activation and participate in signaling pathways.[7] Several sources of ROS in blood platelets are postulated, one of which being the metabolites of arachidonic acid.[8] Under pathological conditions, oxidants generated by activated blood platelets or inflammatory cells may promote oxidative stress and influence platelet functions, which can damage platelet structure.[9] In fact, one of the therapeutic strategies used in the treatment and prophylaxis of cardiovascular disease is the prevention of platelet activation during exposure to oxidative stress. Another important component of the blood are lymphocytes, which act as the basis of the specific resistance of the organism by initiating the immune response. Lowering their amounts and activity leads to the inhibition of the immune system.[10]

Previously, we have demonstrated the antioxidant properties of *Scutellaria* extract in human blood plasma.[11] The aim of the paper is to determine the in vitro protective effect of ethanolic extracts, derived from the shoots and roots of *Scutellaria altissima* and *S. alpina*, on blood platelets and lymphocytes. The aim of the study was to investigate the antioxidant activity of skullcap extracts against the effect of a strong biological oxidant, H_2O_2, a known hydroxyl radical donor, on human lymphocytes. The study also examines the effect of *Scutellaria* extract on lipid peroxidation, and on superoxide anion ($O_2^{\cdot -}$) production in resting blood platelets and platelets activated by thrombin, a strong physiological agonist. Experimental models used in this study are similar to reactions which take place in human cells under conditions of oxidative stress or during blood platelet activation.

Material and methods

Plant material

The roots and aerial parts of *S. altissima* L. and *S. alpina* L. were used for the study. The plants had previously been growing for 2 years under field conditions in the Medical Plant Garden of the Department of Pharmacognosy, Medical University of Lodz, Poland. The plants were identified on the basis of the Flora Europaea by I. Grzegorczyk-Karolak and voucher specimens were deposited in Department of Biology and Pharmaceutical Botany, Medical University of Lodz, Poland.[12]

Preparation of extracts

The lyophilized plant material (1 g) was pre-extracted with chloroform overnight. After filtration, the plant material was extracted 3 times in a 30 mL ethanol–water (7:3) mixture for 15 min in an ultrasonic bath. The extracts were combined and evaporated under reduced pressure.

Total phenolic determination

Total phenolic content was measured using the Folin-Ciocalteu method as described by Singleton and Rossi.[13] Briefly, 400 µL of each extract was mixed with 2 mL of Folin-Ciocalteu reagent (diluted 10-fold) and 1.6 mL of 7.5% sodium carbonate (Na_2CO_3). The absorbance was determined by spectrophotometry at 765 nm (Beijing Rayleigh Corp., Beijing, China) after 30 min of incubation at room temperature. The results were expressed as mg gallic acid equivalents (GAE) per gram of dry extract. The calibration curve was obtained by preparing a gallic acid solution in the concentration range 1–400 mg/mL. The results are mean values of 9 independent experiments.

Blood platelet isolation

Peripheral blood was collected from 5 non-smoking volunteers into ACD solution (citric acid/citrate/dextrose; 5:1; v/v; blood/ACD). Donors were healthy men and women aged 35–45 years, with normal body mass (body mass index [BMI] between 22 and 25). They did not take any medications or addictive substances, including tobacco, alcohol, antioxidant supplementation, aspirin, or any other anti-platelet drugs. They did not take treatment for any kind of systemic disease in their histories. The protocol was approved by the Committee for Research on Human Subjects, University of Lodz, Poland (reference: 2/KBBN-UŁ/III/2014). Platelet-rich plasma (PRP) was prepared by centrifugation of fresh human blood at 250 × g for 10 min at room temperature. Platelets were then sedimented by centrifugation at 500 × g for 10 min at room temperature. The platelet pellet was washed twice with Tyrode's buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 140 mM sodium chloride (NaCl), 3 mM potassium chloride (KCl), 0.5 mM magnesium chloride ($MgCl_2$), 5 mM sodium bicarbonate ($NaHCO_3$), and 10 mM glucose (pH 7.4), and the platelets were suspended in Tyrode's buffer. Spectrophotometric evaluation found the concentration of platelets in platelet suspensions to be about 5×10^8/mL.[14] The suspensions of blood platelets were incubated with plant extracts at final concentrations of 0.5–50 µg/mL (15 min, at 37°C), with or without the addition of thrombin (5 U/mL, 5 min, 37°C).

Superoxide anion radical measurement

The generation of $O_2^{\cdot-}$ in the control platelets and in platelets incubated with tested extracts was measured by an inhibitable reduction of cytochrome c using superoxide dismutase (1 µg/mL), as described earlier.[15] Briefly, an equal volume of modified Tyrode's buffer containing cytochrome c (160 µM) (Sigma-Aldrich, St. Louis, USA) was added to a platelet suspension. After incubation, the platelets were sedimented by centrifugation at 2,000 × g for 5 min and the supernatants were transferred to cuvettes. Any reduction in cytochrome c was measured spectrophotometrically at 550 nm. To calculate the molar concentration of $O_2^{\cdot-}$, the molar extinction coefficient for cytochrome c was taken as 18,700/M × cm.

Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring the concentration of thiobarbituric acid reactive substances (TBARS) (Sigma-Aldrich). Incubation of platelets was stopped by cooling the samples in an ice bath. Samples of platelets were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 M HCl and centrifuged at 1,200 × g for 15 min. The clear supernatant was mixed with 0.12 M thiobarbituric acid in 0.26 M Tris at pH 7.0 in a ratio of 5:1 by volume and immersed in a boiling water bath for 15 min. Absorbance was measured at 532 nm (Spectrophotometer UV/Vis Helios alpha; Unicam, Cambridge, UK).[16] The TBARS concentration was calculated using the molar extinction coefficient (ε = 15,600/M × cm).

Lymphocyte cultures

Lymphocytes were isolated from peripheral human blood obtained from medication-free, regular non-smoking donors at the blood bank (Łódź, Poland) by centrifugation in a density gradient with Histopaque 1077 (Sigma-Aldrich) at 300 × g for 15 min. The protocol was approved by the Committee for Research on Human Subjects, University of Lodz, Poland (reference No.: 2/KBBN-UŁ/III/2014). The cells were suspended in Roswell Park Memorial Institute (RPMI) 1640 culture medium with Glutamax, 15% inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin, and 1% mitogen PHA (added 24 h before application of the tested compound). The lymphocytes were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

3-(4,5-dimethylthiazol-2-yl)-2,5--diphenyltetrazolium bromide assay

Lymphocyte viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[17] The MTT test is based on the reduction of tetrazolium to colored formazan by dehydrogenase inside living cells. The assay only detects living cells and the signal generated is dependent on their degree of activation. A 24-hour culture of lymphocytes seeded on to 96-well microplates at 10^3 cells/well was treated with extracts of shoot and root *S. altissima* and *S. alpina* at a concentration of 10 µg/mL. Fifteen min before the end of the incubation period, H_2O_2 solutions at concentrations of 0.1, 0.5, 1 or 1.5 mM were added to the wells. After this time, 20 µL of fresh MTT solution, i.e., 5 mg/mL MTT in sterile phosphate-buffered saline (PBS), was added to each well and the plates were incubated for the next 3 h. Following this, a 100 µL mixture of 20% sodium dodecyl sulfate (SDS) and 50% dimethylformamide (DMF) was added to each well and left for 24 h. The absorbance of purple formazan solution was measured spectrophotometrically using a microplate reader (BioTek Instruments Inc., Winooski, USA) at 595 nm. A lymphocyte culture treated with H_2O_2, but without any plant extracts, was used as a positive control, while a culture of cells not exposed to any of the tested compounds was used as a negative control. Any observed reduction of MTT after treatment with the tested compounds was compared to the negative control, which represented a 100% MTT reduction.[18] All results were presented as the means of the replicates from 6 independent experiments.

Data analysis

Several tests were used in the statistical analysis. In order to eliminate uncertain data, Dixon's Q test was performed. Significant differences were assessed with the Kruskal-Wallis test. Differences were considered significant at $p < 0.05$. All the values in this study were expressed as mean ± standard error (SE). The statistical analysis was performed with STATISTICA v. 10.0 software (StatSoft Inc., Tulsa, USA).

Results

We have observed that *Scutellaria* extracts may decrease the oxidative alteration of lipids and the level of ROS in both resting blood platelets and those activated by thrombin. However, the exposure of blood platelets to thrombin (5 U/mL) resulted in stronger oxidative changes than observed in the resting blood platelets (controls). Thrombin, which is a known effective inducer of platelet activation, increased both $O_2^{\cdot-}$ production (2.26 nM of $O_2^{\cdot-}/10^8$ of platelets vs 1.6 nM of $O_2^{\cdot-}/10^8$ of platelet) and lipid peroxidation (0.7 nM/mL of platelets vs 0.49 nM/mL of platelets).

Cytochrome c reduction was used to test the ability of analyzed extracts to influence ROS generation in platelets. Extracts from the shoots and roots of *S. altissima* and *S. alpina* at concentrations of 0.5–50 µg/mL decreased the production of $O_2^{\cdot-}$ in resting blood platelets and those activated by thrombin in vitro in a dose-dependent manner (Fig. 1). Root and shoot extracts from both species had similar inhibitory effects at the same concentrations. The highest dose of skullcap extracts reduced $O_2^{\cdot-}$ production in platelets by about 30% (Table 1). The *Scutellaria* extracts were also observed to bestow a protective effect at lower concentrations (5 µg/mL); however, only slight inhibition of $O_2^{\cdot-}$ generation was observed at the lowest concentration (0.5 µg/mL).

The TBARS level was measured as nonenzymatic lipid peroxidation in resting blood platelets and as enzymatic lipid peroxidation of arachidonic acid in blood platelets stimulated by thrombin. The tested extracts decreased

Table 1. Inhibitory effects of extracts from shoots and roots of *Scutellaria altissima* and *S. alpina* (50 µg/mL; 15 min) on the production of $O_2^{\cdot-}$ in blood platelets

Plant material	Inhibition of the production of $O_2^{\cdot-}$ in resting blood platelets [%]	Inhibition of the production of $O_2^{\cdot-}$ in blood platelets activated by thrombin [%]
S. altissima shoots	35.4 ±6.07[a]	32.9 ±2.93[a]
S. altissima roots	35.3 ±3.96[a]	34.6 ±2.29[a]
S. alpina shoots	33.3 ±4.01[a]	33.3 ±2.34[a]
S. alpina roots	33.0 ±4.26[a]	32.7 ±2.74[a]

The results are mean values ± standard error (SE) of 3 replicates for each of the 5 donors. Means with the same letters for the same column are not significant according to the Kruskal-Wallis test at $p < 0.05$.

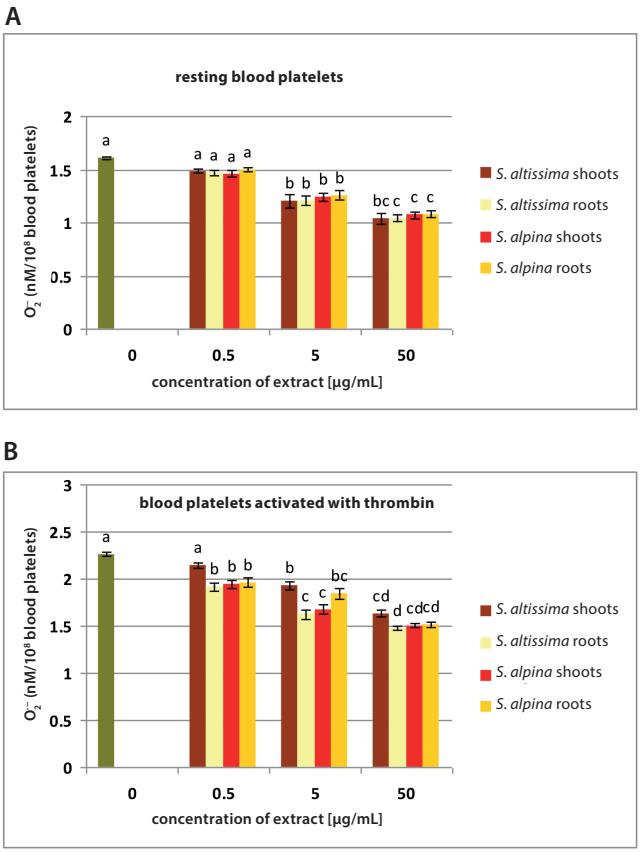

Fig. 1. The effects of shoot and root extracts of *Scutellaria altissima* and *S. alpina* (0.5–50 µg/mL; 15 min, 37°C) on $O_2^{\cdot-}$ generation in resting blood platelets (A) and blood platelets activated by thrombin (5 U/mL, 5 min, 37°C) (B). The data represents means of 3 replicates for each of the 5 donors ± standard error (SE). Means with the same letters for treatment (resting blood platelets or blood platelets activated by thrombin) are not significant according to the Kruskal-Wallis test at $p < 0.05$

TBARS level. After a 15-minute pre-incubation of blood platelets with both shoot and root extracts of *S. altissima* and *S. alpina* at tested concentrations (0.5–50 µg/mL), the amount of TBARS in resting platelets and thrombin-activated blood platelets was seen to diminish. The activity of the 4 tested extracts was concentration-dependent (Fig. 2). Differences were found between resting platelets and induced platelets regarding lipid peroxidation. In the presence of the highest extract concentrations (50 µg/mL), TBARS production in activated platelets was reduced by about 43% in the shoot extract of *S. altissima* and about 40% in the root extract of *S. altissima* (Table 2). The tested extracts demonstrated less effective antioxidant action regarding the protection of the resting blood platelet lipids; extracts at a concentration of 50 µg/mL inhibited the peroxidation of 22–27%. Even the lowest concentration of the tested extracts (5 µg/mL) was able to reduce TBARS production by about 20% in activated platelets. In control experiments, dimethyl sulfoxide (DMSO) (the solvent) added to platelet suspensions at a final concentration below 0.05% did not influence platelet activation in the studied assays.

Table 2. Inhibitory effects of extracts from shoots and roots of *Scutellaria altissima* and *S. alpina* (50 µg/mL; 15 min) on blood platelet lipid peroxidation

Plant material	Inhibition of lipid peroxidation in resting blood platelets [%]	Inhibition of lipid peroxidation in blood platelets activated by thrombin [%]
S. altissima shoots	21.5 ±2.03[a]	43.2 ±4.39[a]
S. altissima roots	21.3 ±2.96[a]	40.6 ±3.44[a]
S. alpina shoots	23.6 ±2.87[a]	40.3 ±3.44[a]
S. alpina roots	27.4 ±3.25[a]	40.5 ±3.38[a]

The results are mean values ± standard error (SE) of 3 replicates for each of the 5 donors. Means with the same letters are not significant according to the Kruskal-Wallis test at $p < 0.05$.

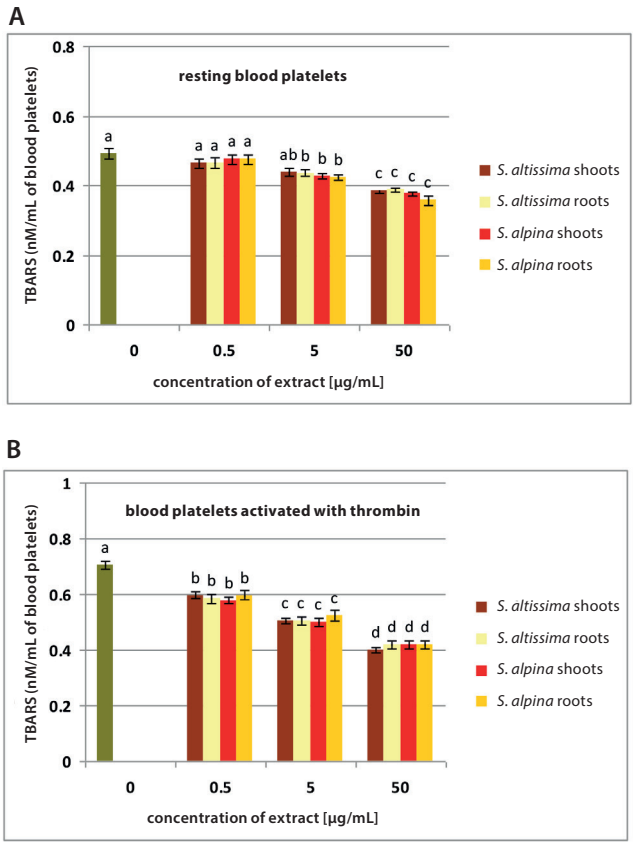

Fig. 2. The effects of shoot and root extracts of *Scutellaria altissima* and *S. alpina* (0.5–50 µg/mL; 15 min, 37°C) on the level of thiobarbituric acid reactive substances (TBARS) in resting blood platelets (A) and blood platelets activated by thrombin (5 U/mL, 5 min, 37°C) (B). The data represents the means of 3 replicates for each of the 5 donors ± standard error (SE). Means with the same letters for treatment (resting blood platelets or blood platelets activated by thrombin) are not significant according to the Kruskal-Wallis test at $p < 0.05$

The results of lymphocyte survival assessment after the treatment with tested plant extracts in the absence and in the presence of H_2O_2 are presented in Table 3. Only the lowest concentration of H_2O_2 (0.1 mM) had no significant effect on lymphocyte survival rate. Higher concentrations (0.5–1.5 mM) induced oxidative damage in the lymphocytes resulting in a 40% decrease in cell viability (Table 3). The results of the MTT assay found that all *Scutellaria* extracts at concentration of 10 µg/mL increased the viability of the human lymphocyte cells and protected them against damage induced by H_2O_2. No significant difference was found in this regard between the plant species or organ used for preparing the extract. In the presence of plant extracts, lymphocyte survival ranged from 90.2 to 98.7%, which was close to that demonstrated by cells grown without stress conditions. Dimethyl sulfoxide added to lymphocyte culture at the concentration used in the study (0.05%) did not result in any decrease in the survival rate of lymphocytes.

The main group of bioactive compounds found in *Scutellaria* extracts includes polyphenols such as flavonoids and phenylethanoids.[1] Both analyzed species have been found to contain flavonoids such as baicalin, wogonoside, luteolin, and cynaroside, as well as the phenylethanoid verbascoside.[11] Total polyphenol levels in *S. altissima* roots and shoots (30.51 ±0.32 and 30.52 ±0.09 mg GAE/g dry weight of extract, respectively) were found to be 30% lower than those identified in the *S. alpina* extracts (49.76 ±0.52 and 44.55 ±0.16 mg GAE/g dry weight of extract, respectively).

Discussion

Recently, much attention has been focused on plants containing various bioactive compounds which may have pharmacological properties, such as antioxidant activity. The present study uses lymphocytes, cells important for the immune system, and blood platelets, which are not only important components of hemostasis or the pathomechanisms of several arterial diseases, but also participate in tumor progression and allergic inflammation.[9]

Blood platelet activation can be strongly induced by thrombin, a proteolytic enzyme and platelet physiological agonist. Thrombin activates platelets by several independent and interactive signal transduction pathways.[9] Our findings confirm that thrombin stimulates $O_2^{\cdot-}$ formation and TBARS production in blood platelets (Fig. 1,2). A signal is transmitted into the cell that invokes various biochemical events, including ROS production. This signal also exerts an influence on arachidonic acid metabolism. The ensuing multistage reactions in the cyclooxygenase pathway involve the activation of phospholipase A_2, which catalyzes the cleavage of arachidonic acid from membrane phospholipids. Free arachidonic acid in platelets is converted mainly to thromboxane A_2 (TXA_2) and malonyldialdehyde (MDA).[19] Thromboxane A_2 acts as a signaling molecule (2^{nd} messengers) in the regulation of blood platelet activation and thrombus formation.

A significant finding of this study is that extracts from shoots and roots of *S. altissima* and *S. alpina* inhibit the peroxidation of arachidonic acid and ROS generation in blood platelets by enzymatic and nonenzymatic means,

Table 3. The effect of extracts from shoots and roots of *Scutellaria altissima* and *S. alpina* (10 µg/mL) in the presence of different concentration of hydrogen peroxide (H_2O_2) on lymphocyte growth after 24-hour incubation evaluated with MTT assay

Concentration of H_2O_2 [mM]	% lymphocytes survival				
	H_2O_2	*S. altissima* shoots	*S. altissima* roots	*S. alpina* shoots	*S. alpina* roots
0 (control)	100 ±5.16[a]	100 ±2.00[a]	100 ±3.02[a]	100 ±2.35[a]	100 ±1.62[a]
0.1	97.08 ±4.62[a]	97.54 ±3.18[a]	97.88 ±1.02[a]	97.57 ±2.25[a]	93.91 ±2.43[a]
0.5	63.66 ±1.71[b]	94.53 ±2.42[a]	94.08 ±2.87[a]	98.51 ±2.05[a]	91.46 ±1.97[a]
1	59.92 ±2.23[b]	94.41 ±3.45[a]	93.33 ±2.54[a]	97.79 ±2.01[a]	91.46 ±1.97[a]
1.5	59.08 ±1.96[b]	93.15 ±3.95[a]	92.33 ±3.92[a]	96.25 ±2.41[a]	90.27 ±2.23[a]

The results are mean values ± standard error (SE) of 3 replicates for each of the 6 samples. Means with the same letters are not significant according to the Kruskal-Wallis test at $p < 0.05$.

which implies that the tested extracts may inhibit cyclooxygenase activity in platelets. However, $O_2^{\cdot-}$ generation was also observed in resting platelets. Wachowicz et al. suggested that radical production can be partially associated with glutathione metabolism, or that platelet isolation and resuspension may also stimulate their activation.[20]

The antiplatelet properties of *Scutellaria* species have only been partly recognized so far. Lee et al. report that a herbal extract named Soshiho-tang containing *Scutellaria baicalensis* Georgi root demonstrated an anti-thrombotic effect via antiplatelet activity.[21] *Scutellaria baicalensis* flavonoids are also known to inhibit platelet aggregation, demonstrating an inhibition rate of 45.5% in 1 study, which was close to the value of 55% noted for the control group based on aspirin.[22] The present study found that *S. alpina* extracts were able to reduce $O_2^{\cdot-}$ production in platelets activated by thrombin by 33%, and reduce TBARS production in activated platelets by about 40%. Inhibition of lipid peroxidation, measured by the level of TBARS in blood platelets treated with $ONOO^-$, by *Aronia melanocarpa* (Michx.) Elliott, a species known for its strong antioxidant properties, was about 30%.[23] Vitamin C at a concentration of 1 mM was found to have comparable activity, inhibiting TBARS production in pig resting blood platelets by about 30%.[24]

The pharmacological properties of the *Scutellaria* plants are mainly due to the presence of polyphenolic compounds, among them flavonoids, in the plant extracts. Some studies have shown that flavonoids such as baicalin, baicalein, wogonin, or luteolin exhibited generally antioxidant activity, but little research has addressed the antiplatelet properties of the flavonoids.[1] Kubo et al. report that baicalein, baicalin, wogonin, or wogonoside inhibits the platelet aggregation induced by arachidonic acid by 48%, 31%, 46%, and 20%, respectively, at a concentration of 1 mM.[25] In comparison, the positive control, aspirin, inhibited aggregation by 30%. At a concentration of 0.5 mM, baicalein and baicalin also inhibited thrombin-induced conversion of fibrinogen to fibrin, slowing it from 193 s for controls to 413 s for baicalein and 478 s for baicalin. Incubation of fibrinogen with heparin (10 U/mL) prolonged the clotting time only to 281 s.

The 2nd blood cell type used in the present study, the lymphocytes, are an important part of the human immune defense against infection and cancer. When their functioning is disturbed, their activity can be wrongly directed against healthy human tissue, resulting in autoimmune disease.[10] The results of the MTT assay in the present study found *Scutellaria* extracts to have a protective effect against oxidative damage induced by a strong biological oxidant, H_2O_2, in lymphocyte cells. In previous studies, Zhang et al. found the aqueous extract of *S. baicalensis* to have protective effects at concentrations of 50 and 100 µg/mL against acrolein-induced oxidative stress in cultured human umbilical vein endothelial cells.[26] Shojaee et al. described the protective effects of *Scutellaria litwinowii* Bornm & Sint. ex Bornm. root extract against H_2O_2-induced DNA changes in normal fibroblasts (NIH/3T3 cell line); however, their MTT and comet assay results suggest that the methanolic *S. litwinowii* extract demonstrated a protective effect against DNA damage caused by H_2O_2 only at high extract concentrations (1,000 µg/mL).[27]

Although this is the first report to describe the effect of *S. altissima* and *S. alpina* extracts on lymphocyte oxidative damage, some other authors have reported that other plant species and their metabolites have a protective effect against toxic and mutagenic factors. Porrini and Riso used H_2O_2 at a concentration of 0.5 mM to stimulate oxidative stress damage in lymphocytes.[28] According the authors, the blood cells are excellent markers of the body state and could be a reliable model for studying the effect of the dietary supplementation with antioxidants on the responses of the body to factors causing oxidative stress. In the study, tomato products offered significant protection to lymphocytes against oxidative stress. Lycopene, the main carotenoid isolated from tomatoes, characterized by a great ability to quench singlet oxygen, effectively protected blood lymphocytes from NO_2 radical damage.[29] Elsewhere, Lin et al. examined the protective effects of several plant species extracts against DNA damage in lymphocytes induced by means of H_2O_2.[30] They report that the inhibition of DNA changes in the presence of tested plants ranged from 74.51% for *Bidens alba* (L.) DC. to 91.45% for *Mentha arvensis* L. extract at a concentration of 25 µg/mL.

Pool-Zobel et al., using the comet assay, found a significant decrease in endogenous levels of strand breaks in lymphocyte DNA after the intake of tomato juice containing 40 mg lycopene, carrot with 38 mg carotene, or spinach containing 11.3 mg lutein.[31] Meanwhile, American ginseng extract was effective in protecting human peripheral lymphocytes against radiation-induced oxidative stress.[32]

In the present study, a spectrophotometric method was used to determine the total phenol content in the tested plant extracts.[13] It has been previously demonstrated that the polyphenol content of *Scutellaria* extracts was significantly correlated with the antioxidant properties estimated by ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and lipid hydroperoxide (LPO) assays.[33] The findings suggest that the polyphenol compounds were the main contributors to the antioxidant properties of the extract, which is consistent with the findings of many other authors.[34,35] Our present findings indicate that all tested extracts exhibited similar activity in blood platelets and lymphocytes, although higher levels of polyphenolic compounds were found in *S. alpina* than *S. altissima* extracts. Also, *M. arvensis* extract was more effective against H_2O_2-induced DNA damage in lymphocyte cells than *Centella asiatica* L. extract, despite the lower content of bioactive compounds (respectively, 21.2 and 32.0 mg gallic acid/g dry weight).[30] The authors suggested that this could be connected with the synergism present among antioxidant compounds, indicating that the properties of the mixture are dependent not only on the concentrations of the antioxidants, but also on their type and the interaction between them.

The unclear relationship between the protective activity of *S. altissima* and *S. alpina* extracts on blood cells and the flavonoid content could also be connected with other factors. The scientific interest in pharmacological activity of *Scutellaria* plants has been focused mainly on the effects of their flavonoid compounds, but other groups of compounds, for example diterpenoids, could also act as antioxidant and antiplatelet compounds. Several compounds of this group have been described as having antioxidant effects, for example royleanonic acid, tanshinones or carnosol.[36–38] Diterpenoids have been identified in both *S. altissima* and *S. alpina*; however, the plants contain different diterpenoids, whose levels have not yet been evaluated in detail.[39,40] These differences could account for the results observed in the present study.

Conclusions

A significant finding of this paper is that it describes the antioxidant properties of polyphenol-rich *Scutellaria* extracts. When added at a concentration of 10 μg/mL, the extracts reduced H_2O_2-induced oxidative stress in human lymphocytes and increased cell viability to more than 90% in MTT assay. The analyzed plants may also be valuable in the protection of platelets against oxidative stress and its consequent pathological platelet activation and aggregation. Therefore, *S. altissima* and *S. alpina* may represent promising new sources of compounds offering significant benefits in diseases typified by an imbalance between oxidative reactions and the antioxidant process. However, future studies should be extended to include other groups of compounds besides flavonoids, which can play a role in the antioxidant activity of *Scutellaria* extracts.

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Effectiveness comparison of various atrial fibrillation ablation methods in patients with common venous trunk

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Abstract

Background. Atrial fibrillation (AF) is a common clinical problem. The left atrium anatomy makes up a factor that may significantly affect the effectiveness of the AF ablation.

Objectives. The aim of the study was to evaluate a long-term effectiveness ablation in patients with common pulmonary vein trunk (CPVT) and AF.

Material and methods. The outcomes of 129 procedures in 95 patients with CPVT out of 1,475 procedures carried out in 1,150 patients with AF treated with ablation, were analyzed. Ablation with CARTO 3 system (Johnson & Johnson, New Brunswick, USA), cryoballoon, and the circular multipolar duty-cycled radiofrequency-based pulmonary vein ablation producer with catheter (PVAC) were considered as advanced methods. The following data was recorded for every patient: age, gender, AF duration and type, previous antiarrhythmic drugs, weight, height, any prior cardioversion, and comorbidities, including hypertension, diabetes, hypothyreosis, thyrotoxicosis, heart failure, and stroke/transient ischemic attack. The following anatomical factors were assessed: the presence of patent foramen ovale (PFO) and localization of the CPVT on the basis of venography or computed tomography (CT). In the 1st year after ablation, 24-hour Holter monitoring was performed 3–5 times, and the patients were encouraged to visit their doctor or an emergency department if a cardiac arrhythmia occurred. Long-term ablation effectiveness was assessed based on a telephone interview and patients' answers to the questionnaires including 12-lead electrocardiography (ECG).

Results. Sinus rhythm was maintained in 44 patients (43.6%) after a median of 42 months (range: 12–120). A lower number of clinical factors (odds ratio (OR) = 0.09; 95% confidence interval (95% CI) = 0.02–0.56; $p < 0.01$), and advanced ablation methods (OR = 3.1; 95% CI = 1.4–7.1; $p < 0.01$) were related to a better long-term effectiveness.

Conclusions. The long-term effectiveness of pulmonary vein (PV) isolation in patients with AF and CPVT is higher when advanced ablation techniques are used. Accumulation of clinical factors was found to be the most tremendous predictor of AF recurrence.

Key words: long-term outcome, atrial fibrillation, ablation, common trunk

Introduction

Atrial fibrillation (AF) is a common clinical problem associated with the occurrence of sudden and non-sudden cardiac death, heart failure, stroke, an increased risk of hospitalizations, and decreased quality of life.[1–3]

In the treatment of patients with symptomatic AF, isolation of pulmonary vein (PV) is recommended by the creation of circumferential lesions around the right and the left PV ostia.[2–4] However, invasive procedures may lead to complications.[4–7] Looking for predictors of atrial fibrillation relapse facilitates the process of qualifying the patient for the procedure, as well as the selection of the most effective methods to perform ablation.[8–11]

The left atrium anatomy makes up a factor that may significantly affect the effectiveness of the procedure. An unusual anatomy of PV, usually in the form of a common pulmonary vein trunk (CPVT), occurs in 13–29% of patients.[12,13] The vascular anomaly, which usually refers to single-sided PVs, is more common on the left side.[4,13] The presence of the CPVT hampers PV isolation, the main reason for that possibly being a fact that systems for isolating the PVs have been developed for a typical PV anatomy, and their effectiveness might alter in the case of an unusual anatomy. The aim of the study was to evaluate the long-term effectiveness of different methods used to ablate/isolate PV in patients to treat AF.

Material and methods

The study was designed as the retrospective analysis of the long-term results of ablation procedures performed in patients with AF and CPVT between 2003 and 2012.

Out of the group of 1,150 patients (1,475 procedures) with AF, 107 (9.3%) had the CPVT recognized. Twelve patients did not appear at the follow-up after the 1st procedure. Finally, the effectiveness of 129 procedures performed in 95 patients with the CPVT was subjected to analyses in the study. Table 1 shows the patients' characterization by the technique used for ablation. Among the studied population, 72 patients had 1 procedure only, 15 had 2 procedures, 6 had 3 procedures, 1 patient had 4 procedures, and 1 had 5 procedures. The mean number of ablation procedures amounted to 1.3 times per patient.

Data collection

The following data was recorded for every patient: age, gender, AF duration and type, previous antiarrhythmic drugs, weight, height, any prior cardioversion, and comorbidities including hypertension, diabetes, hypothyreosis, thyrotoxicosis, heart failure, as well as stroke/transient ischemic attack. The following echocardiographic data was recorded: left ventricle ejection fraction (EF) and left atrium diameter. Ejection fraction less than 50% was regarded as low EF. For every ablation an average fluoroscopy and procedure time were recorded.

During ablation, the following anatomical factors were assessed: the presence of patent foramen ovale (PFO; if it could be used to introduce the catheter into the left atrium), and left-side or right-side localization of the CPVT recognized on the basis of venography or computed tomography (CT). Furthermore, the method of ablation was recorded in each case and the nature of the procedure was referred to as the 1st or the repeated procedure.

Procedure details

The objective of the procedure was to isolate all PVs. Other ablation regions were performed if the surgeon decided so. In some patients with high far-field potentials from the superior vena cava (VCS) or the inferior vena cava (VCI) (n = 50 and n = 4, respectively), the vein was isolated, too. In patients with persistent AF, with a short distance between PV lines or with transformation of AF into an atypical atrial flutter, additional lines were performed. All lines were carried out using CARTO (Johnson & Johnson, New Brunswick, USA) system, including left atrial roof (n = 10), posterior wall (n = 10) or the mitral isthmus (n = 8), right atrial isthmus (n = 13), coronary sinus (n = 8), complex fractionated atrial electrogram (CFAE) (n = 3), VCS VCI line (n = 1), and septum (n = 6).

In the presented study, fluoroscopy was used as the only imaging method in the subgroup of patients in whom Lasso electrode and ablation with 4-mm tip catheter were used for segmental PV isolation, using the method described by Haïssaguerre et al.[14] The majority of the procedures performed by the use of Haïssaguerre's method were done with the LocaLisa system used to reduce fluoroscopy. Details of this technique were previously presented.[14] The LocaLisa navigation system (Medtronic EP Systems, Minneapolis, USA) uses 3 low-amplitude, high-frequency current fields. The fields are generated in 3 axes over the patient's thorax and are dedicated to compute the position of an electrode in the thorax relative to a reference electrode. The procedure of segmental PV isolation using the LocaLisa navigation system was precisely described in the previous publication.[15] Briefly, the Lasso catheter (Johnson & Johnson, New Brunswick, USA) was located in every PV under the fluoroscopic guidance. The ablation procedure was performed by the use of the ablation catheter under the LocaLisa system and fluoroscopy. The PV isolation was proved by disappearance or dissociation of potential recorded from the Lasso catheter.[15]

CARTO XP (Biosense Webster, Johnson & Johnson, New Brunswick, USA) visualized only the ablation catheter. This catheter was used to create the left atrial map. The next atrial PV linear isolation was performed based on the method presented by Pappone et al.[16] Verification of the lines was done using the voltage map (reduction of the potentials in the isolated area <0.1 mV).

Table 1. Baseline demographics and clinical characteristics

Characteristics	Total group n = 95	CARTO XP n = 7	CARTO 3 n = 6	Lasso n = 16	PVAC n = 21	LocaLisa n = 42	Cryoballoon n = 3
Age [years]	54.1 ±10.1	54.6 ±7.4	58.8 ±8.2	53.3 ±10.9	55.3 ±9.9	52.9 ±10.8	56.3 ±8.1
Sinus rhythm maintenance, n [%]	31 (32.4)	2 (28.6)	2 (33.3)	2 (12.5)	10 (47.6)	13 (31.0)	2 (66.7)
Male gender, n [%]	59 (62.1)	5 (71.4)	3 (50.0)	8 (50.0)	10 (47.6)	31 (73.8)	2 (66.7)
Persistent AF, n [%]	23 (24.2)	6 (85.7)	3 (50.0)	1 (6.3)$^{\#}$	5 (23.8)$^{\#}$	7 (16.7)$^{\#}$	1 (33.3)
LA [mm]	42.4 (6.6)	45.9 (3.7)	44.0 (2.8)	41.8 (5.5)	43.1 (4.9)	41.9 (8.2)	35.7 (1.2)
EF [%]	60.1 (6.5)	58.1 (8.3)	57.3 (6.7)	61.2 (4.9)	60.1 (8.1)	59.9 (7.0)	66.3 (2.3)
CHA_2DS_2–VASc median (IQR)	1 (1–2)	1 (1–2)	1 (1–2)	1 (1–2)	1 (1–2)	1 (0–2)	0 (0–2)
Low EF, n [%]	4 (4.2)	1 (14.3)	0 (0)	0 (0)	1 (4.8)	2 (4.8)	0 (0)
CPVT right or bilateral, n [%]	72 (75.8)	6 (85.7)	1 (16.7)	11 (66.8)	17 (81)	35 (83.3)	2 (66.7)
Ablation in VCS ostium, n [%]	41 (43.2)	2 (28.6)	0 (0)	8 (50.0)	8 (38.1)	23 (54.8)	0 (0)
FOA, n [%]	12 (12.6)	0 (0)	0 (0)	0 (0)	5 (23.8)	7 (16.7)	0 (0)
History of the electric cardioversion, n [%]	48 (50.5)	6 (85.7)	5 (83.3)	7 (43.08)	11 (52.4)	18 (42.9)	1 (33.3)
History of the thyroid diseases, n [%]	21 (22.1)	2 (28.6)	1 (16.7)	4 (25.0)	4 (19.0)	10 (23.8)	0 (0)
Hypertension, n [%]	55 (57.9)	5 (71.4)	4 (66.7)	11 (68.8)	11 (52.4)	23 (54.8)	1 (33.3)
Diabetes, n [%]	8 (8.4)	0 (0)	2 (33.3)	2 (12.5)	2 (9.5)	2 (4.8)	0 (0)
COPD, n [%]	3 (3.2)	1 (14.3)	1 (16.7)	0 (0)	0 (0)	1 (2.4)	0 (0)
Previous stroke, n [%]	7 (7.4)	1 (14.3)	0 (0)	1 (6.3)	2 (9.5)	3 (7.1)	0 (0)
Number of clinical factors, mean ±SD	1.8 ±1.3	3 ±0.8	2.7 ±1.4	1.7 ±1.3	1.9 ±1.4	1.6 ±1.1	1.0 ±1.0
Follow up [months], mean ±SD	48.7 ±25.7	44.7 ±21.1*	19.0 ±4.1*$^{\$}$	82.9 ±24.2$^{\$}$	29.4 ±6.0*$^{\$}$	51.6 ±19.7*	30.7 ±13.7*
Procedure duration [h]	2.4 ±0.8	3.7 ±0.8	2.5 ±0.5$^{\#}$	2.5 ±0.6$^{\#}$	2.1 ±0.6$^{\#}$	2.1 ±0.6$^{\#}$	2.8 ±0.4
Fluoroscopy duration [min]	20.7 ±12.4	14.2 ±9.3*	15.7 ±5.7*	37.5 ±19.0	18.7 ±6.9*	19.7 ±11.0*	28.9 ±10.5

AF – atrial fibrillation; LA – left atrium; EF – ejection fraction; COPD – chronic obstructive pulmonary disease; FOA – foramen ovale apertum; VCS – vena cava superior; PVAC – pulmonary vein ablation producer with catheter; CPVT – common pulmonary vein trunk; IQR – interquartile range; SD – standard deviation; * $p < 0.001$ vs Lasso; $^{\#}$ $p < 0.001$ vs CARTO XP; $^{\$}$ $p < 0.001$ vs LocaLisa.

CARTO 3 (Biosense Webster, Johnson & Johnson, New Brunswick, USA) system is an upgraded version, allowing a more accurate visualization of the cardiac anatomy (e.g., to perform a quick anatomical map) and enabling the visualization of all catheters used. This PV isolation verification technology was used for the Lasso catheter.

Ablation with PV ablation producer with catheter (PVAC) (Medtronic, Carlsbad, USA) was described in most detail in the previous publication.[17] Shortly after a PV selective angiography a circular decapolar catheter is advanced over wire positioned selectively in every PV. After several radio frequency (RF) applications over all electrode pairs, the PVAC was rotated around the PV ostium to ensure a complete isolation of the vein. During the ablation of large common ostia, electrode pairs with suboptimal contact to the atrial tissue were deactivated, preventing ineffective energy delivery.[17]

Cryoballoon ablation methods were described in the previous publications.[18] Shortly after an initial mapping of PV using the Lasso catheter, the cryoballoon (Arctic Front – Cryocath, Medtronic, Mineapolis, USA) was introduced into the left atrium and positioned at PV ostia. Contact of the balloon with the ostial tissue was checked by contrast administration and temperature inside the balloon during cryoapplication. At every PV ostium 2 cryoapplications with good contact were performed. Subsequently, remapping of cryoapplications in all veins was performed with Lasso catheters. In the absence of PV potentials, the procedure was finished; otherwise, next cryoapplications were performed.[18]

Ablations with CARTO 3 system, cryoballoon, and PVAC catheter were considered as advanced methods, whereas those with CARTO XP, Lasso guided alone or with LocaLisa system were regarded as reference methods, potentially less effective because of hindered adaptation to the changed anatomy.

Follow-up

The details of follow-up procedures were presented elsewhere.[19] Briefly, in the 1st year after ablation, 24-h Holter monitoring was performed 3–5 times, and the patients were encouraged to visit their doctor or an emergency department if a cardiac arrhythmia occurred. The procedure was assessed as ineffective when an event of AF lasting at least 30 s was documented later than after 3 months of a blanking period following the procedure.[8] Long-term ablation effectiveness was assessed based on the telephone interview and patients' answers to the questionnaires, including 12-lead electrocardiography (ECG). The gathered data was further

supplemented with the data from cardiology outpatient clinics and the results of the Holter monitoring. Had there been more than 1 ablation, all treatments preceding the last treatment (also in patients with whom the contact was lost) would have been considered ineffective.

Informed consent was obtained from all patients. The study was approved by the Bioethical Commission (Wroclaw Medical University, Poland).

Continuous variables were expressed as mean and standard deviation (SD) and compared with analysis of variance (ANOVA) test with Bonferroni correction for a multiple comparison. Categorical variables were presented as numbers and percentages, and χ^2 test together with Fisher's exact test were both used to assess the differences.

The CHA_2DS_2–VASc score was calculated for each patient. The number of clinical factors was calculated for each patient as a sum of points. One point was given for the presence of each diagnosis in the patients' medical history: age above 65 years, age above 75 years, hypertension, diabetes mellitus, previous stroke, previous myocardial infarction, congestive heart failure, the presence of the permanent character of AF, previous electric cardioversion, thyroid diseases, and chronic obstructive pulmonary disease (COPD).

The logistic regression analysis was performed to find association between the AF recurrence, the advanced method of ablation and the number of clinical factors which may predict AF relapse.

The classification and regression tree analysis were used to find associations among the long-term outcome, the method of ablation and the clinical predictors of the AF recurrence. Specificity, sensitivity, and positive and negative predictive values for each model were calculated. The classification and regression tree analysis constitute non-parametric methods to establish predictors of the studied end-point. These methods were described by Breiman et al. and they consist in recursive partitioning of the study group for each possible point to find those dividing the entire group into subgroups which are differentiated the most regarding the evaluated events.[19,20] The classification tree is a graphical method used for presenting the classification rules. The number in the upper right corner of the rectangle is an information about which subgroup it was included to, based on the classification rules. In the case of the present study "0" means maintaining sinus rhythm and "1" is the AF recurrence. The number of factors associated with the AF recurrence was assessed for every patient.

Results

Demographics, clinical characteristics within the study group and subgroups selected on the basis of the 1st procedure performed were presented in Table 1. Table 2 pictures the data regarding demographics and clinical

Table 2. Demographics and clinical characteristics in groups selected on the basis of the procedure performer

Characteristics	Total group n = 129	CARTO XP n = 17	CARTO 3 n = 11	Lasso n = 19	PVAC n = 24	LocaLisa n = 52	Cryoballoon n = 6
Age [years]	54.7 ±10.2	57.5 ±6.8	59.9 ±6.6	54.2 ±10.4	54.4 ±10.8	52.5 ±11.1	59.8 ±7.1
Sinus rhythm maintenance, n [%]	44 (34.1)	4 (23.5)	5 (45.5)	2 (10.5)	12 (50.0)	16 (30.8)	5 (83.3)
Male gender, n [%]	84 (65.1)	11 (64.7)	7 (63.6)	9 (47.4)	13 (54.2)	39 (75.0)	5 (83.3)
Persistent AF, n [%]	27 (20.9)	8 (47.1)	3 (27.3)	1 (5.3)	5 (20.8)	9 (17.3)	1 (16.7)
Low EF, n [%]	6 (4.7)	1 (5.9)	0 (0)	0 (0)	2 (8.3)	3 (5.8)	0 (0)
CPVT right or bilateral, n [%]	96 (74.4)	12 (70.6)	5 (45.5)	14 (73.7)	19 (79.2)	41 (78.8)	5 (83.3)
Ablation in VCS ostium, n [%]	50 (38.8)	2 (11.8)	0 (0)	9 (47.4)	10 (41.7)	29 (55.8)	0 (0)
FOA, n [%]	14 (10.9)	0 (0)	1 (9.1)	0 (0)	5 (20.8)	8 (15.4)	0 (0)
History of the electric cardioversion, n [%]	66 (51.2)	12 (70.6)	6 (54.5)	9 (47.4)	13 (54.2)	23 (44.2)	3 (50.0)
History of the thyroid diseases, n [%]	30 (23.3)	5 (29.4)	5 (45.5)	4 (21.1)	4 (16.7)	12 (23.1)	0 (0)
Hypertension, n [%]	75 (58.2)	12 (70.6)	5 (45.5)	13 (68.4)	12 (50.0)	32 (61.5)	1 (16.7)
Diabetes, n [%]	10 (7.8)	0 (0)	3 (27.3)	2 (10.5)	2 (8.3)	3 (5.8)	0 (0)
COPD, n [%]	4 (3.1)	2 (11.8)	1 (9.1)	0 (0)	0 (0)	1 (1.9)	0 (0)
Previous stroke, n [%]	10 (7.8)	3 (17.6)	0 (0)	1 (5.3)	2 (8.3)	4 (7.7)	0 (0)
Number of clinical factors, mean ±SD	1.8 ±1.2	2.5 ±1.2	2.2 ±1.7	1.6 ±1.2	1.8 ±1.3	1.7 ±1.1	1.0 ±0.9
Follow up [months], mean ±SD	48.7 ±10.1	51.5 ±26.3*	21.0 ±9.0*#$	84.3 ±24.2	29.5 ±5.7*#$	52.2 ±18.9*	25.5 ±12.8#$*
Procedure duration [h]	2.4 ±0.8	3.6 ±1.0	2.5 ±0.5#	2.4 ±0.6#	2.2 ±1.1#	2.1 ±0.6#	2.8 ±0.4#
Fluoroscopy duration [min]	21.4 ±13.2	14.6 ±9.5*	15.7 ±9.5*	35.5 ±12.4	18.7 ±6.9*	19.0 ±11.1*	28.9 ±10.5#

AF – atrial fibrillation; LA – left atrium; EF – ejection fraction; COPD – chronic obstructive pulmonary disease; FOA – foramen ovale apertum; VCS – vena cava superior; PVAC – pulmonary vein ablation producer with catheter; CPVT – common pulmonary vein trunk; SD – standard deviation; * $p < 0.001$ vs Lasso; # $p < 0.001$ vs CARTO XP; $ $p < 0.001$ vs LocaLisa.

characteristics in groups selected on the basis of the procedure performed. The subgroups did not differ in terms of age and gender distribution. The number of clinical factors associated with AF relapse was significantly greater in the group treated with CARTO XP system than with LocaLisa. Procedure times were the longest during procedures with CARTO XP.

Procedure safety

Two events of transient phrenic nerve palsy during cryoablation were observed, while in 1 patient a mild stenosis of PVs occurred during the long-term follow-up (from 16 × 14 mm before the procedure to 9 × 6 mm thereafter). Patient with the PV stenosis presented with hemoptysis and tussis. These symptoms subsided after a few months. This patient had the ablation performed with LocaLisa.

No significant differences were found with reference to the overall complication rate.

Follow-up

During the follow-up of a median of 42 months (range 12–120 months), the sinus rhythm was maintained in 44 patients (43.6%). Among 129 procedures, 85 procedures (65.9%) were assessed as ineffective.

Clinical variables associated with atrial fibrillation recurrence

Thyroid diseases history was more prevalent in the group with AF recurrence than in the group without AF recurrence (29.7% vs 6.5%; $p < 0.02$, respectively). No differences in the occurrence of other clinical variables related to the AF recurrence were observed. However, it is worthwhile to mention that the number of clinical factors associated with recurring AF was significantly lower in patients without AF recurrence than in those with AF recurrence (1.4 ±1.2 vs 2.0 ±1.2; $p < 0.01$, respectively).

Multivariate analysis

The logistic regression analysis revealed that a lower number of clinical factors (odds ratio (OR) = 0.09, 95% confidence interval (95% CI) = 0.02–0.56; $p < 0.01$) and advanced ablation methods (OR = 3.1, 95% CI = 1.4–7.1; $p < 0.01$) were related to better long-term effectiveness of the given procedure.

The classification and regression analysis confirmed the significance of the advanced methods for good outcome, whereas the low EF and the presence of PFO were related to the AF recurrence (Fig. 1), with the specificity of that model being 0.85, sensitivity 0.90, and positive and negative predictive values being 0.87 and 0.69, respectively.

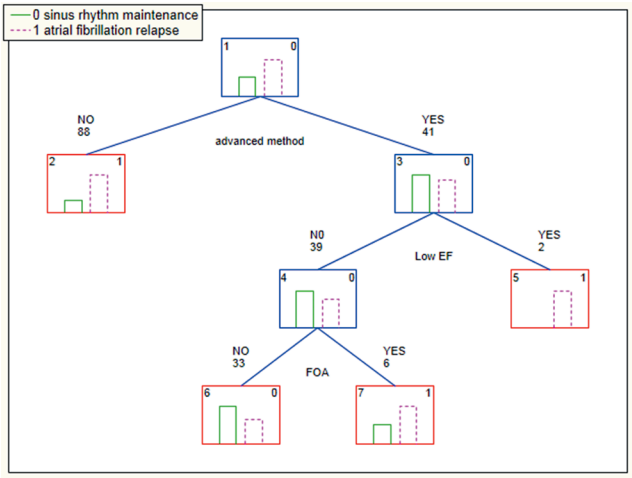

Fig. 1. Factors related to sinus rhythm maintenance

Discussion

The procedures of the PVs isolation are currently among the most effective therapeutic options for the AF treatment. The ablation effect on AF might be attributed to various mechanisms, including the elimination of the trigger, modification of the arrhythmogenic substrate, interruption of crucial pathways of conduction or rotors, or atrial denervation.

The PV isolation techniques differ with regards to the energy source (radiofrequency or cryoenergy), mapping system (electrophysiological or electroanatomical) and electrode shape (focal, ring or balloon). The effectiveness of these techniques may vary in the settings of the CPVT and normal anatomy.

Difficult anatomy of venoatrial junction may impede the effective implementation of the ablation procedure. Common pulmonary vein trunk is a quite frequently encountered variant of the PV anatomy. The size and shape of the veins ostia may be of particular importance for some of the techniques, whilst others may be more resistant to the associated difficulties.

Recent randomized studies comparing various methods of ablation techniques were performed in the setting of 4 independent PVs and demonstrated that the simplified strategy for PV cryoablation is inferior to PV isolation using open-irrigated radiofrequency catheters with electrophysiological and electroanatomical guidance.[21] It was also observed that a complete PV conduction block is critical to the success of AF ablation. Malmborg et al. reported that either cryoballoon and/or the circular multipolar duty-cycled radiofrequency-based PVAC proved to be comparably effective and safe in achieving acute PV isolation.[22] Other researchers have determined that among different procedures of catheter ablation there are no significant differences in the success rate between the 2 single procedures. Notwithstanding, the success rates are higher for combined methods than for single methods.[23]

The accuracy of the spatial localization by different advanced electroanatomical mapping systems seems to be comparable; however, in the case of the CPVT other factors may appear to be more important.

Furthermore, there are no established strategies for redo procedures after PV isolation. In the presented study, it was found that the advanced techniques are more effective than the older ones, regardless of whether used during the 1st or the repeated procedure. Contrary to the presented findings, Pokushalov et al. reported that when patients require a redo PV isolation ablation procedure for the recurrent paroxysmal AF, RF appears to be the preferred method relative to cryoablation.[24] However, the number of cryoballoon isolations in the presented study was too low to obtain conclusive results.

The clinical factors analyzed in the presented study overlap, but are not limited to, the parameters used in the CHA_2DS_2–VASc scale. Other factors assigned to the clinical picture include the following: the permanent character of AF, previous electric cardioversion, thyroid diseases, and COPD.[9]

The importance of clinical factors in predicting the long-term outcome of ablation procedure was confirmed in the study. The increase of the number of clinical factors associated with the reduced effectiveness of long-term ablation was associated with an increased risk of AF recurrence by 30%. The classification and regression tree analysis revealed that thyroid disease constituted the most important clinical factor. It is well known that thyroid diseases often have the 1st clinical manifestation as cardiac supraventricular arrhythmias. Thyroid hormones directly regulate the metabolism of myocardial cells, and thyroid diseases induce a diffuse atrial architecture disruption. This may predispose to ablation ineffectiveness.[25–27] The PV isolation procedure is targeted at removing the trigger off the PVs rather than treating the whole diseased atrial myocardium which constitutes the substrate for AF.

The presence of thyroid diseases may also represent the adverse effect of amiodarone therapy. Patients with a greater tendency to relapse could have been treated with amiodarone, which in turn could have induced thyroid diseases, further promoting AF.

The treatment with amiodarone may lead to thyroid dysfunction, which then reduces the effectiveness of ablation. Therefore, it should be considered whether in patients with paroxysmal AF, ablation should not take precedence over pharmacological treatment. A major problem in iatrogenic hyperthyroidism can particularly be the long time to get euthyroid.

Atrial fibrillation is a frequent rhythm in heart failure patients. It may exacerbate heart failure, which in turn may increase the susceptibility to AF occurrence. Heart failure is also a predictor of AF recurrence after ablation procedure, which was also determined in the presented study. An explanation for this observation may be the occurrence of changes in the myocardium of heart failure patients, which lead to the transformation of arrhythmia from trigger-driven to substrate-mediated. The risk of AF recurrence after catheter ablation is also increased in another setting of the atrial myocardium damage as sick sinus syndrome.[28]

Such anatomical factors as the localization of the CPVT and the presence of PFO were also included in the analysis because in the previous analysis we had found that these factors may have an impact on the effectiveness of the performed procedure. In the current analysis, contrary to the one performed earlier, only PFO presence was related to the lower effectiveness. The ablation methods used may have various effectiveness levels in the case of the right-side CPVT, which made it difficult to show the adverse significance of the localization of the CPVT.

There are no established strategies for the redo procedures after the PV isolation. The presented study indicates the higher effectiveness of the advanced methods both in the 1st and in the redo procedures.

Limitations

This study is subjected to several limitations. Firstly, the follow-up duration varies among the patients' groups based on methods that were used. However, even after taking into account a 5% annual increase in the risk of recurrence, the presented results indicate the higher effectiveness of newer ablation techniques.[29] Secondly, the limited number of patients and a single-center study design may produce a bias, thus these results may not be generalized.

The methods used for a long-term follow-up after AF catheter ablation are not as accurate as continuous ECG monitoring.[30] However, these methods allow us to find recurrences in the 2/3 of patients, although the immediate success rate was comparable with other studies.

Institutional experience over nearly 10 years may improve significantly; however, the total number of procedures was high, so the effect of a learning curve could affect only a small percentage of cases.

Conclusions

The long-term effectiveness of PV isolation in patients with AF and CPVT was higher when advanced ablation techniques were used.

The same group of ablation methods is more effective in treatment, regardless of whether it is applied during the 1st or the redo procedure.

Accumulation of the clinical risk factors was found to be the most tremendous predictor of AF recurrence, and this finding could be helpful in selecting patients with AF for an appropriate therapeutic strategy.

Thyroid disease seems to be the most significant clinical factor related to the AF recurrence after ablation.

Computed tomography scan before PV isolation and the performance of the ablation by experienced operator in the presence of CPVT should be recommended.

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Bridging anticoagulation in patients treated with vitamin K antagonists prior to trochanteric and hip fracture surgeries: The current practice

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Abstract

Background. The strategies of perioperative bridging anticoagulation in orthopedic surgical patients during oral anticoagulation (OAC) therapy with vitamin K antagonists (VKA) vary from center to center.

Objectives. The aim of this single-center study was to assess the risk of bleeding and thromboembolic events (TEs) in bridged patients on VKA who underwent orthopedic surgery due to trochanteric or hip fracture.

Material and methods. The retrospective study included 64 patients (mean age: 80 years) who received VKA for at least 3 months prior to orthopedic procedure. All subjects were bridged with enoxaparin (40 mg once a day). The control group (n = 69) comprised of age-, sex- and procedure-matched patients operated on for the same indications, but with neither a history of VKA therapy nor perioperative bridging anticoagulation.

Results. Severe postoperative bleeding occurred in 19 (29.7%) patients from the VKA group and in 13 (18.8%) controls (p = 0.16). Within the VKA group, intertrochanteric fractures (52.6%) and femoral neck fractures (47.4%) occurred more often in patients with bleeding than other lower extremity fractures (0%; p = 0.03). Severe adverse events (SAEs) were more common in the VKA group than in the controls (12.5% vs 1.5%; p = 0.01). Patients from the VKA group did not differ from the controls in the incidence of TEs (6.3% vs 8.9%; p = 0.31). No intrahospital mortality was documented.

Conclusions. Prophylactic administration of enoxaparin is a common strategy of bridging anticoagulation in a hospital setting. This approach does not seem to be associated with an increase in thromboembolic risk nor higher risk of bleeding in orthopedic patients who received VKA preoperatively.

Key words: anticoagulation, low molecular weight heparin, vitamin K antagonists, bridging therapy, trochanteric and hip neck fracture surgery

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Introduction

A large proportion of older persons from many countries receive oral anticoagulation (OAC) with vitamin K antagonists (VKA); one example is the UK, where VKA are prescribed to approx. 1% of older patients.[1,2] Noticeably, such individuals are more prone to osteoporosis, an established risk factor for femoral neck fracture.

Fixation of femoral neck and trochanteric fractures is a relatively common surgical procedure in older patients, associated with high comorbidity and mortality rates.[3–5] According to the literature, hip and trochanteric surgeries carry a 4% risk of perioperative mortality and a 3.2% risk of thromboembolism.[6] Despite the implementation of thromboprophylaxis, pulmonary embolism (PE) is still a common cause of perioperative mortality, accounting for approx. 10% of deaths among older orthopedic inpatients.[7] A retrospective analysis including a total of 3,082 patients who underwent hip, knee or spine surgeries documented major perioperative bleeding in 5.3% of the cases.[8] In another study, the incidence of thromboembolic events (TEs) and mortality rates in patients undergoing total hip or knee arthroplasties were estimated at 4% and approx. 0.7%, respectively.[9] A large proportion of patients being referred to an orthopedic treatment are at increased risk of venous thromboembolism (VTE), stroke or systemic embolism, due to the presence of atrial fibrillation (AF), mechanical heart valves or recurrent VTE; such individuals require long-term OAC therapy with VKA or new generation anticoagulants.

Appropriate anticoagulation treatment can be challenging in patients operated on in an emergency setting; while discontinuation of OAC may increase the risk of TEs, its maintenance may predispose to bleeding-related complications.[10] In a study of 1,884 patients with AF, in whom VKA treatment has been interrupted prior to an elective surgery or other invasive procedure, forgoing bridging anticoagulation was not inferior to perioperative bridging with low-molecular-weight-heparin (LMWH) in the prevention of arterial thromboembolism, while it decreased the risk of major bleeding.[11] Nevertheless, bridging therapy with LMWH should be applied to minimize thromboembolic risk during the anticoagulation-free interval, and in line with current guidelines, LMWH at a therapeutic dose is preferred in surgical patients at increased risk of bleeding and thromboembolic complications.[12] Perioperative administration of LMWH as a component of bridging anticoagulation may be associated with an increased risk of bleeding and severe adverse events (SAEs), such as intracranial hemorrhage with subsequent major disability or even death.[13–17] The ORBIT-AF study included a total 7,372 patients receiving OAC therapy; among them 665 individuals were given a short-acting anticoagulant to reduce the risk of TEs during a temporary discontinuation of OAC. In this study, bridging anticoagulation was associated with an increased risk of bleeding and other adverse events after the interruption of OAC.[18] A meta-analysis including a total of 7,118 bridged and 5,160 nonbridged patients demonstrated unequivocally that heparin bridging is associated with a 3–4% risk of major bleeding and 13–15% risk of overall bleeding complications in the perioperative period.[17]

To the best of our knowledge, bridging anticoagulation and its outcomes in Polish orthopedic inpatients receiving a long-term treatment with VKA has been a subject of only a few previous studies. The aim of this single-center study was to assess the risk of bleeding and TEs and the impact of bridging anticoagulation in patients on VKA who underwent orthopedic surgery due to trochanteric or hip fracture.

Material and methods

Patients

The retrospective study included all consecutive patients receiving VKA, who underwent surgical fixation of trochanteric or femoral neck fracture at the Department of Orthopedics, St. Lucas Hospital in Tarnów, Poland, in the period of 2012–2014. The study received the approval of the bioethics committee. A total of 4,453 patients were treated surgically for trochanteric or hip fractures during the study period, and individuals on VKA therapy represented 1.4% of this population. The VKA group included 24 (37.5%) patients with intertrochanteric fractures, 24 (37.5%) with femoral neck fractures, 2 (3.1%) with shank fractures, and 14 (21.9%) with ankle fractures. Only the patients who received bridging anticoagulation in line with the hospital protocol (n = 64) were included in the analysis. According to the protocol, anticoagulation therapy was discontinued on the day of admission, and enoxaparin (40 mg per day) was given 1 day prior to the orthopedic procedure and 1 day thereafter. All patients were informed about possible risks of discontinuation of OAC and implementation of bridging therapy beforehand, and gave their informed consent to this approach. The control group (n = 69) was comprised of age-, sex- and procedure-matched patients operated on for the same indications, but with neither a history of OAC with VKA nor perioperative bridging anticoagulation. Lower extremity fractures were diagnosed based on a physical examination, as well as pelvic and femoral radiograms.

Patients who received anticoagulation therapy with non-vitamin K or direct oral anticoagulants (NOACs) and/or individuals subjected to conservative treatment of the fracture were excluded from the study. Information about past and present comorbidities was extracted from patients' medical histories. Postoperative bleeding was classified as severe whenever the patient required a transfusion of at least 2 units of packed red blood cells. Severe adverse events were defined as major bleeding or serious cardiovascular events, such as myocardial infarction (MI),

stroke, VTE, or dyspnea after the surgery. The interruption of anticoagulation therapy was 2–7 days. Depending on the type of fracture, the study subjects underwent total hip arthroplasty or interlocking fixation of trochanteric fracture with the intramedullary GAMMA nail. In the case of total hip arthroplasty, the patient was placed on the nonfractured side; a straight incision, approx. 15 cm in length, was made, and either Bipolar or Exeter prosthesis (Stryker Howmedica, Kalamazoo, USA) with acrylic cement was implanted from a posterolateral approach. The mean duration of the procedure, defined as the time between the incision and placement of the last suture, was 70–80 min. Intertrochanteric fractures were treated by intramedullary stabilization with the GAMMA nail. This minimally invasive procedure was associated with only a mild bleeding. The mean time of the surgery was about 60 min.

Laboratory tests

Blood samples for laboratory testing were collected 12 h prior to the surgery and 8 h post-surgery. All laboratory tests were conducted at a local hospital laboratory using standardized assays.

Statistical analysis

Normal distribution of continuous variables was verified with the Kolmogorov-Smirnov test. Statistical characteristics of normally distributed variables are presented as means ± standard deviations (SD). Otherwise, the results are presented as medians (interquartile ranges (IQR)). Prior to statistical analysis, non-normal data was subjected to a logarithmic (log 10) transformation. Depending on the distribution type, the Student's t-test or the Mann-Whitney U test was used for intergroup comparisons of continuous variables. Distributions of categorical variables are presented as numbers and percentages, and were compared using χ^2 test. The results of statistical tests were considered significant whenever a 2-sided p-value was lower than 0.05. All calculations were carried out with STATISTICA v. 9.1 (StatSoft Inc., Tulsa, USA).

Results

Preoperative period

The study included 133 patients with trochanteric or femoral neck fractures (47 men and 86 women) with a mean age of 80 years. Baseline characteristics of the study subjects are summarized in Table 1. None of the controls had indications for VKA therapy. Indications for OAC in the VKA group included AF (n = 56; 87.5%), mechanical valve replacement (n = 7; 10.9%) and a previous VTE (n = 1; 1.6%). A total of 34 patients (53.1%) were treated with warfarin and 30 (46.9%) with acenocumarol. Subjects from the VKA group had higher body weight and body mass index (BMI) than the controls, and more often received aspirin, β-blockers, angiotensin converting enzyme (ACE) inhibitors, calcium channel blockers, and proton pump inhibitors. Moreover, they presented with significantly higher preoperative international normalized ratios (INR), activated partial thromboplastin time (APTT), red cell distribution width (RDW), and creatinine levels, as well as with significantly lower platelet counts and fasting blood glucose concentrations, than the controls. The study groups did not differ significantly in terms of the main diagnoses, comorbidities and medications (Table 1).

Postoperative period

During the postoperative period, severe bleeding occurred in 19 (29.7%) patients from the VKA group and in 13 (18.8%) controls (Table 2). Severe adverse events were more common in the VKA group than in controls (n = 8; 12.5% vs n = 1; 1.5%; p = 0.01). No significant intergroup differences were found in the incidence of cardiovascular complications, such as MI, stroke and VTE, as well as in terms of other complications (Table 2). Patients on VKA received more fresh frozen plasma units and required longer preoperative hospitalization. Within the VKA group, intertrochanteric fractures and femoral neck fractures occurred more often in patients with bleeding than with lower extremity fractures (intertrochanteric fractures: n = 10; 52.6% vs n = 14; 31.1%; femoral neck fractures: n = 9; 47.4% vs n = 15; 33.3%; other fractures: n = 0 vs n = 16; 34.6% in patients with hemorrhage and in patients without hemorrhage, respectively; p = 0.03) (Table 3). Patients from the VKA group who experienced perioperative hemorrhage did not differ from other subjects from this group in terms of their basic characteristics, medications and laboratory parameters (Table 3).

Discussion

The findings presented here demonstrate that patients operated on due to trochanteric or femoral neck fractures, both with prophylactic LMWH bridging and without it, were not at increased risk for bleeding and TEs.

Risk of bleeding is a major concern related to bridging anticoagulation. Recent evidence suggests that periprocedural bleeding-to-thrombosis ratio in bridged and non-bridged patients approximates 13:1 and 5:1, respectively, which implies that the former group is at a considerable risk of bleeding.[19] Indeed, according to literature, anticoagulation-related hemorrhage is associated with increased morbidity and mortality, which surpasses the benefits of perioperative bridging.[20,21] Thromboembolic events occur rarely during periprocedural period; in contrast, bleeding complications after implementation of bridging therapy

Table 1. Characteristics of the study subjects

Variable	Overall (n = 133)	VKA group (n = 64)	Controls (n = 69)	p-value
Age [years]	80 (72–86.5)	79.5 (72.25–86)	80 (68.5–87)	0.78
Male gender, n [%]	47 (35.3)	28 (43.8)	19 (27.5)	0.07
Body weight [kg]	70.0 (63.0–80.0)	72.0 (65.0–85.7)	69.0 (61.5–76.0)	0.03
Body height [cm]	165.7 (±8.0)	165.7 (±7.5)	165.8 (±8.5)	0.92
BMI [kg/m^2]	25.7 (23.2–28.4)	26.0 (23.5–30.0)	25.1 (23.0–27.5)	0.04
Current smoker, n [%]	2 (2.90)	0 (0.0)	2 (2.90)	1.00
Diagnosis				
Intertrochanteric fracture, n [%]	49 (36.84)	24 (37.50)	25 (36.23)	0.76
Femoral neck fracture, n [%]	48 (36.09)	24 (37.50)	24 (34.78)	–
Lower leg fracture, n [%]	7 (5.26)	2 (3.13)	5 (7.25)	–
Other fracture, n [%]	29 (21.80)	14 (21.88)	15 (21.74)	–
Indication for anticoagulation				
VTE, n [%]	1 (0.75)	1 (1.56)	0 (0.00)	0.48
AF, n [%]	56 (42.11)	56 (87.50)	0 (0.00)	<0.0001
Artificial heart valve, n [%]	7 (5.26)	7 (10.94)	0 (0.00)	0.005
Comorbidities				
CHD, n [%]	71 (53.38)	40 (62.50)	31 (44.93)	0.06
MI, n [%]	16 (12.03)	11 (17.19)	5 (7.25)	0.11
Previous stroke, n [%]	11 (8.27)	6 (9.38)	5 (7.25)	0.76
Arterial hypertension, n [%]	96 (72.18)	51 (79.69)	45 (65.22)	0.08
DM, n [%]	19 (14.29)	9 (14.06)	10 (14.49)	1.00
Hyperthyroidism, n [%]	8 (6.02)	6 (9.38)	2 (2.90)	0.15
Hypothyroidism, n [%]	7 (5.26)	3 (4.69)	4 (5.80)	1.00
CKD, n [%]	8 (6.02)	4 (6.25)	4 (5.80)	1.00
COPD, n [%]	6 (4.51)	2 (3.13)	4 (5.80)	0.68
Asthma, n [%]	4 (3.01)	1 (1.56)	3 (4.35)	0.62
Superficial thrombosis, n [%]	4 (3.01)	3 (4.69)	1 (1.45)	0.35
Previous gastric ulcer, n [%]	5 (3.76)	3 (4.69)	2 (2.90)	0.67
HF, n [%]	5 (7.81)	5 (7.81)	0 (0.0)	1.00
Medications				
VKA type				
Acenocumarol, n [%]	–	30 (46.88)	0 (0.0)	–
Warfarin, n [%]	–	34 (53.13)	0 (0.0)	–
LMWH				
Enoxaparin, n [%]	131 (98.50)	64 (100.00)	67 (97.10)	0.5
Nadroparin, n [%]	2 (2.90)	0 (0.00)	2 (1.50)	–
ASA, n [%]	106 (79.70)	61 (95.31)	45 (65.22)	<0.0001
β-blocker, n [%]	92 (69.70)	53 (84.13)	39 (56.52)	0.0006
ACEI, n [%]	104 (78.20)	56 (87.50)	48 (69.57)	0.02
ARB, n [%]	4 (3.01)	2 (3.13)	2 (2.90)	1.00
Aldosterone antagonist, n [%]	3 (2.26)	2 (3.13)	1 (1.45)	0.61
Calcium channel blocker, n [%]	18 (13.53)	13 (20.31)	5 (7.25)	0.04
Statin, n [%]	82 (61.65)	44 (68.75)	38 (55.07)	0.11
Fenofibrate, n [%]	20 (15.04)	12 (18.75)	8 (11.59)	0.33
Amiodarone, n [%]	2 (1.50)	2 (3.13)	0 (0.00)	0.23
Furosemide, n [%]	36 (27.07)	21 (32.81)	36 (27.07)	0.17
Metformin, n [%]	9 (6.77)	3 (4.69)	6 (8.70)	0.50
PPIs, n [%]	48 (36.09)	30 (46.88)	18 (26.09)	0.02

Table 1. Characteristics of the study subjects (cont.)

Variable	Overall (n = 133)	VKA group (n = 64)	Controls (n = 69)	p-value
NSAIDs, n [%]	6 (4.51)	5 (7.81)	1 (1.45)	0.11
Laboratory tests				
INR	1.43 (1.10–2.50)	2.59 (1.79–3.33)	1.10 (1.05–1.18)	<0.0001
APTT [s]	31.00 (27.65–37.85)	36.60 (31.35–44.36)	28.30 (25.60–30.80)	<0.0001
FBG [g/L]	3.20 (2.90–3.57)	2.96 (2.72–3.62)	3.23 (2.99–3.56)	0.02
WBC [10^3/μL]	10.20 (8.00–12.85)	10.30 (8.05–13.25)	10.20 (8.00–12.50)	0.67
RBC [10^6/μL]	4.05 (3.40–4.38)	4.04 (3.32–4.43)	4.06 (3.55–4.37)	0.60
HGB [g/dL]	12.10 (10.35–13.35)	11.90 (9.83–13.48)	12.30 (10.80–13.30)	0.35
HCT [%]	36.90 (31.60–39.90)	36.75 (30.43–40.33)	37.10 (32.35–39.90)	0.55
RDW [%]	14.30 (13.60–15.25)	14.50 (13.83–15.80)	13.90 (13.40–14.80)	0.02
PLT [10^3/μL]	198.00 (155.00–255.00)	176.50 (136.50–241.50)	212.00 (172.50–260.00)	0.01
Glucose [mmol/L]	123.00 (108.75–145.00)	122.00 (110.00–138.50)	127.00 (108.00–148.00)	0.52
Creatinine [μmol/L]	74.00 (58.25–94.75)	82.00 (63.00–108.00)	71.00 (54.50–91.00)	0.01
eGFR [mL/min]	80.00 (57.50–98.00)	78.00 (55.00–94.00)	80.00 (62.00–102.25)	0.28

ACEI – angiotensin converting enzyme inhibitors; AF – atrial fibrillation; APTT – activated partial thromboplastin time; ASA – acetylsalicylic acid; ARB – angiotensin receptor blockers; BMI – body mass index; CHD – chronic heart disease; CKD – chronic kidney disease; COPD – chronic obstructive pulmonary disease; DM – diabetes mellitus; eGFR – estimated glomerular filtration rate; FBG – fibrinogen; HCT – hematocrit; HF – heart failure; HGB – hemoglobin; INR – international normalized ratio; PPIs – proton-pump inhibitors; LMWH – low molecular weight heparin; MI – myocardial infarction; NSAIDs – non-steroidal anti-inflammatory drugs; PLT – platelets; RBC – red blood cell count; RDW – red blood cell distribution width; ST – stroke; VKA – vitamin K antagonist; VTE – venous thromboembolism; WBC – white blood cell count.

are far more common and this preventive measure does not seem to provide an evident antithrombotic benefit.[20] Nevertheless, various forms of bridging anticoagulation are still commonly used in patients qualified for invasive procedures.[20] In our study, average blood loss in bridged patients (3.11 g/dL of hemoglobin) tended to be greater than in nonbridged subjects, but the difference was insignificant. Blood loss in patients operated on due to intertrochanteric fracture or hip neck fracture was greater than in individuals with other types of lower extremity fractures. Probably, this was associated with the older age of patients with intertrochanteric and hip neck fractures, and with a larger extent of surgical procedures performed in this group. Altogether, our findings imply that prophylactic administration of enoxaparin to older patients qualified for orthopedic surgeries is not associated with increased risk of major bleeding. Nevertheless, irrespective of bridging anticoagulation or lack thereof, the risk of bleeding in this group is still high, as shown by a large proportion of our patients who required postoperative blood transfusions.

Beneficial effects of bridging in patients at increased risk of TE are unclear, and we still lack sufficient evidence in this matter from well-designed clinical trials. However, the results of observational studies suggest that implementation of bridging therapy is associated with a substantial decrease in the incidence of VTE events, even in high-risk populations.[20,22] Therefore, until adequate evidence from clinical trials becomes available, individualized bridging anticoagulation therapy still should be considered in patients with established risk factors for VTE, such as mechanical mitral valve, or acute or recent VTE. Evidence from retrospective studies suggests that bleeding-to-thrombosis profile of bridged patients with implanted mechanical valves, i.e., with an established risk factor for TEs, may be relatively favorable.[23,24] Whenever bridging therapy is deemed necessary, more conservative strategies should be considered, namely, low-dose heparin, administration of heparin solely in the postoperative period, delayed initiation of postprocedural heparin bridging, delayed onset of postprocedural heparin bridging, and early cessation of warfarin when a international normalized ratio (INR) value reaches 2.0 or more.[25–27] Early discontinuation of VKA and administration of enoxaparin, 40 mg once a day, are a preferred bridging strategy at our department. Such an approach may raise some controversies, especially in patients with mitral valve prostheses. Unfortunately, the subset of our patients who received VKA due to the implantation of mechanical valves was too small to conduct a subgroup analysis (n = 11). Although none of these subjects developed a TE episode during the follow-up period, it is still unclear whether bridging with higher doses of LMWH or nonfractioned heparin should be recommended for patients from this group prior to a major surgical procedure.

In line with current recommendations, surgical treatment of hip fractures in older patients should be implemented early, optimally within 24–48 h post-admission.[28] However, adherence to these guidelines can be quite challenging in the case of patients on VKA anticoagulation therapy; reversal of OAC to prevent excessive bleeding may cause a significant delay in a major orthopedic procedure, such as hip surgery.[29,30] Such a delay is associated with increased morbidity and mortality.[31] Vitamin K antagonists therapy can be reversed passively, by interruption of warfarin and waiting until INR returns to the reference range (<1.2),

Table 2. Postoperative characteristics of the study subjects

Variable	Overall (n = 133)	VKA group (n = 64)	Controls (n = 69)	p-value
Complications				
Hemorrhage, n [%]	32 (24.1)	19 (29.7)	13 (18.8)	0.16
PE/VTE, n [%]	4 (3.01)	4 (6.25)	0 (0.00)	0.05
MI, n [%]	1 (0.75)	1 (1.56)	0 (0.00)	0.48
ST, n [%]	1 (0.75)	1 (1.56)	0 (0.00)	0.48
SAEs, n [%]	9 (6.77)	8 (12.50)	1 (1.45)	0.01
Other complications, n [%]	1 (1.45)	4 (6.25)	5 (3.76)	0.20
Perioperative care				
PRBCs, n [%]	33 (24.81)	19 (29.69)	14 (20.29)	0.23
PRBCs [units]	2.00 (2.00–4.00)	2.00 (2.00–4.00)	2.00 (2.00–2.50)	0.44
FFP, n [%]	19 (14.29)	11 (17.19)	11 (17.19)	0.46
FFP [units]	2.00 (2.00–2.00)	2.00 (2.00–2.00)	1.50 (1.00–2.00)	0.009
Preoperative [days]	4.00 (2.00–5.00)	4.00 (3.00–6.00)	3.00 (2.00–4.00)	0.0009
Postoperative [days]	5.00 (4.00–7.00)	6.00 (5.00–8.00)	6.00 (4.00–7.00)	0.06
Postoperative laboratory tests				
INR	1.17 (1.06–1.39)	1.17 (1.06–1.39)	1.19 (1.13–1.25)	0.72
APTT [a]	29.30 (27.23–31.78)	30.40 (26.80–34.10)	29.55 (27.13–32.75)	0.36
FBG [g/L]	3.01 (2.60–3.30)	2.52 (2.37–2.98)	3.18 (2.98–3.46)	<0.0001
WBC [10^3/µL]	9.40 (7.33–12.20)	9.60 (7.10–12.40)	8.90 (7.45–11.55)	0.48
RBC [10^6/µL]	3.64 (3.26–4.03)	3.64 (3.24–4.13)	3.65 (3.27–4.02)	0.86
HGB [g/dL]	10.65 (9.63–11.90)	10.70 (9.70–11.90)	10.60 (9.60–12.00)	0.86
HCT [%]	32.95 (29.43–36.30)	33.00 (29.20–36.90)	32.90 (29.55–36.05)	0.53
RDW [%]	14.60 (13.50–15.90)	15.10 (13.80–16.20)	14.30 (13.30–15.30)	0.01
PLT [10^3/µL]	205.00 (162.00–278.75)	198.00 (147.00–284.00)	207.00 (172.50–270.00)	0.33

APTT – activated partial thromboplastin time; FBG – fibrinogen; FFP – fresh frozen plasma; HCT – hematocrit; HGB – hemoglobin; INR – international normalized ratio; MI – myocardial infarction; PLT – platelets; PRBCs – packed red blood cells; PE – pulmonary embolism; RBC – red blood cell count; SAEs – serious adverse events; ST – stroke; VTE – venous thromboembolism; WBC – white blood cell count.

or actively, by the administration of vitamin K, fresh frozen plasma, clotting factor concentrates, or a combination thereof. In line with current guidelines, prior to a major surgery, INR should be lower than 1.5.[32] Our findings confirm that this recommendation is followed strictly in clinical practice. Bleeding and neurological complications may be also associated with the insertion or removal of a spinal or epidural catheter in an anticoagulated patient and, therefore, warfarin therapy is an absolute contraindication to regional anesthesia.[33] In St. Lucas Hospital in Tarnów (Poland), regional anesthesia is given solely to patients whose INR is lower than 1.2. To the best of our knowledge, no specific guidelines regarding anticoagulation reversal in patients with hip fracture have been published thus far. In the case of patients scheduled for elective orthopedic surgeries of the hip, most orthopedic surgeons follow a "wait and watch" policy, with discontinuation of warfarin approx. 5 days prior to the procedure in order to decrease INR to a subtherapeutic level.[34] Similarly, no guidelines exist regarding bridging therapy in patients on long-term warfarin therapy who have been qualified for a major elective orthopedic procedure. However, administration of LMWH is recommended in high-risk patients as long as their INR remains at a subtherapeutic level. Decision on an anticoagulation strategy used in such group of patients should be made jointly by a hematologist, cardiologist, anesthesiologist, and orthopedic surgeon. In line with current guidelines, in patients subjected to major orthopedic surgeries, extended pharmacological prevention of TE with LMWH or another anticoagulant administered for up to 35 days post-procedure should be preferred over a short-term prophylaxis; thromboprophylaxis should be started no later than within the first 12 h post-surgery.[35,36] In our study, past history of VTE, if any, could be adequately documented on the basis of medical documentation.

This study is not free from potential limitations. Firstly, owing to the retrospective character of the analysis, a postoperative follow-up of patients after hip and trochanteric surgeries was quite short (up to 35 days) and we had no access to information on the incidence of TE or stroke after discharge. Therefore, it cannot be excluded that some patients with unstable anticoagulation might have experienced TE shortly after cessation of the bridging. Secondly, pharmacological thromboprophylaxis followed the same protocol in all patients and, therefore, we were unable to analyze the potential effects of its type, duration and

Table 3. Characteristics of bridged patients with and without hemorrhage

Variable	Bridged patients (n = 64)	Bridged patients with hemorrhage (n = 19)	Bridged patients without hemorrhage (n = 45)	p-value
Age [years]	79.50 (75.25–86.00)	84.00 (76.00–87.00)	78.00 (72.50–84.50)	0.13
Male gender, n [%]	28 (43.75)	7 (36.84)	21 (46.67)	0.59
Weight [kg]	72.00 (65.00–85.75)	72.50 (63.75–82.25)	72.00 (65.00–90.00)	0.76
Height [cm]	165.65 (±7.51)	164.78 (±6.42)	165.65 (±7.51)	0.56
BMI [kg/m^2]	26.09 (23.52–30.02)	26.85 (22.86–31.81)	25.93 (23.98–29.58)	0.82
Diagnosis				
Intertrochanteric fracture, n [%]	24 (37.50)	10 (52.63)	14 (31.11)	0.03
Femoral neck fracture, n [%]	24 (37.50)	9 (47.37)	15 (33.33)	–
Lower leg fracture, n [%]	2 (3.13)	0 (0.00)	2 (4.44)	–
Other fracture, n [%]	14 (21.88)	0 (0.00)	14 (31.11)	–
Comorbidities				
VTE, n [%]	1 (1.56)	0 (0.00)	1 (2.22)	1.00
AF, n [%]	56 (87.50)	19 (100.00)	37 (82.22)	0.09
Artificial heart valve, n [%]	7 (10.94)	0 (0.00)	7 (15.56)	0.09
CHD, n [%]	40 (62.50)	13 (68.42)	27 (60.00)	0.58
MI, n [%]	11 (17.19)	2 (10.53)	9 (20.00)	0.48
Stroke, n [%]	6 (9.38)	1 (5.26)	5 (11.11)	0.66
Hypertension, n [%]	51 (79.69)	14 (73.68)	37 (82.22)	0.50
DM, n [%]	9 (14.06)	3 (15.79)	6 (13.33)	1.00
Insulin, n [%]	7 (10.94)	3 (15.79)	4 (8.89)	0.42
Hyperthyroidism, n [%]	6 (9.38)	0 (0.00)	6 (13.33)	0.17
Hypothyroidism, n [%]	3 (4.69)	0 (0.00)	3 (6.67)	0.55
CKD, n [%]	4 (6.25)	2 (10.53)	2 (4.44)	0.58
COPD, n [%]	2 (3.13)	1 (5.26)	1 (2.22)	0.51
Asthma, n [%]	1 (1.56)	0 (0.00)	1 (2.22)	1.00
Superficial thrombosis, n [%]	3 (4.69)	1 (5.26)	2 (4.44)	1.00
Gastric ulcer, n [%]	3 (4.69)	2 (10.53)	1 (2.22)	0.21
Complications				
PE/VTE postoperative, n [%]	4 (6.25)	0 (0.00)	4 (8.89)	0.31
MI postoperative, n [%]	1 (1.56)	0 (0.00)	1 (2.22)	1.00
ST postoperative, n [%]	1 (1.56)	1 (5.26)	0 (0.00)	0.30
SAEs postoperative, n [%]	8 (12.50)	1 (5.26)	7 (15.56)	0.42
Other complications, n [%]	4 (6.25)	0 (0.00)	4 (8.89)	0.31
Perioperative care				
PRBCs, n [%]	19 (29.69)	19 (100.00)	0 (0.00)	<0.001
FFP, n [%]	11 (17.19)	5 (26.32)	6 (13.33)	0.28
FFP [units]	2.00 (2.00–3.00)	2.00 (2.00–3.00)	2.00 (2.00–2.50)	0.90
Preoperative hospital stay [days]	4.00 (3.00–6.00)	4.00 (3.00–5.00)	4.00 (2.50–6.00)	0.98
Postoperative hospital stay [days]	6.00 (5.00–8.00)	7.00 (5.00–9.00)	6.00 (4.00–7.50)	0.05
Medications				
VKA type	–	–	–	–
Acenocumarol, n [%]	30 (46.88)	10 (52.63)	20 (44.44)	0.60
Warfarin, n [%]	34 (53.13)	9 (47.37)	25 (55.56)	–
LMWH, n [%]	–	–	–	–
Enoxaparin, n [%]	64 (100.00)	19 (100.00)	45 (100.00)	–
Nadroparin, n [%]	–	–	–	–
LMWH preoperative [h]	10.5 (±4.0)	10.74 (±3.78)	10.4 (4.13)	0.76

Table 3. Characteristics of bridged patients with and without hemorrhage (cont.)

Variable	Bridged patients (n = 64)	Bridged patients with hemorrhage (n = 19)	Bridged patients without hemorrhage (n = 45)	p-value
LMWH postoperative [h]	21 (±8)	21.47 (±7.57)	20.80 (±8.25)	0.76
LMWH postoperative [days]	32.73 (±12.53)	36.47 (±14.36)	31.16 (±11.48)	0.24
Laboratory and laboratory-based characteristics				
Preoperative				
INR	2.59 (1.79–3.33)	2.22 (1.64–3.31)	2.75 (1.80–3.33)	0.54
APTT [s]	36.60 (31.35–44.36)	38.20 (31.30–47.80)	36.60 (31.35–44.36)	0.76
FBG [g/L]	2.96 (2.72–3.62)	2.90 (2.46–3.40)	2.99 (2.75–3.73)	0.29
WBC [10^3/µL]	10.30 (8.05–13.25)	10.10 (7.20–13.80)	10.30 (8.20–13.20)	0.71
RBC [10^6/µL]	4.04 (3.32–4.43)	3.40 (2.76–4.03)	4.20 (3.76–4.50)	0.0004
HGB [g/dL]	11.90 (9.83–13.48)	10.40 (8.30–12.20)	12.40 (10.50–13.70)	0.0044
HCT [%]	36.75 (30.43–40.33)	30.70 (25.50–36.70)	38.00 (31.95–41.90)	0.0026
RDW [%]	14.50 (13.83–15.80)	14.40 (14.00–15.10)	14.60 (13.75–15.85)	0.85
PLT [10^3/µL]	176.50 (136.50–241.50)	148.00 (127.00–243.00)	186.00 (151.00–240.00)	0.24
Glucose [mmol/L]	122.00 (110.00–138.50)	121.00 (109.25–143.00)	122.00 (110.00–137.00)	0.85
Creatinine [µmol/L]	82.00 (63.00–108.00)	73.00 (61.00–120.00)	85.00 (69.25–104.00)	0.37
eGFR [mL/min]	78.00 (55.00–94.00)	82.50 (56.75–116.00)	72.00 (54.00–92.00)	0.29
Postoperative				
INR	1.17 (1.06–1.39)	1.22 (1.04–1.36)	1.17 (1.06–1.41)	0.81
APTT [s]	30.40 (26.80–34.10)	30.40 (26.60–34.10)	30.35 (26.85–34.08)	0.99
FBG [g/L]	2.52 (2.37–2.98)	2.44 (2.22–2.99)	2.56 (2.38–2.99)	0.45
WBC [10^3/µL]	9.60 (7.10–12.40)	9.00 (6.30–12.40)	9.80 (7.45–13.03)	0.27
RBC [10^6/µL]	3.64 (3.24–4.13)	3.33 (2.79–3.55)	3.88 (3.44–4.23)	0.0013
HGB [g/dL]	10.70 (9.70–11.90)	10.00 (9.10–10.70)	11.10 (10.03–12.08)	0.0007
HCT [%]	33.00 (29.20–36.90)	30.60 (28.80–33.90)	34.75 (30.16–39.18)	0.0047
RDW [%]	15.10 (13.80–16.20)	15.50 (14.30–16.40)	15.05 (13.73–16.18)	0.36
PLT [10^3/µL]	198.00 (147.00–284.00)	175.00 (142.00–210.00)	222.00 (149.25–293.50)	0.08

ACEI – angiotensin-converting-enzyme inhibitors; AF – atrial fibrillation; APTT – activated partial thromboplastin time; ASA – acetylsalicylic acid; ARB – angiotensin receptor blockers; BMI – body mass index; CHD – coronary heart disease; CKD – chronic kidney disease; COPD – chronic obstructive pulmonary disease; DM – diabetes mellitus; eGFR – estimated glomerular filtration rate; FBG – fibrinogen; FFP – fresh frozen plasma; HF – heart failure; INR – international normalized ratio; PPIs – proton-pump inhibitors; LMWH – low molecular weight heparin; MI – myocardial infarction; NSAIDs – non-steroidal anti-inflammatory drugs; PLT – platelets; RBC – red blood cell count; SAEs – serious adverse events; ST – stroke; VKA – vitamin K antagonist; VTE – venous thromboembolism; WBC – white blood cell count.

anticoagulant dose on the outcome. Thirdly, due to the relatively small sample size, we did not conduct subgroup analyses, e.g., according to specific indications for VKA or comorbidities. Finally, none of our subjects received NOACs and, consequently, application of these agents in perioperative bridging of surgical orthopedic patients is yet to be established.

Conclusions

Periprocedural anticoagulation management in patients requiring urgent orthopedic procedures is a common issue and available evidence regarding the best practices in this matter is limited.

Perioperative bridging anticoagulation in orthopedic patients on anticoagulation therapy with VKA does not seem to be associated with an increase in thromboembolic risk nor with higher risk of bleeding.

Bridging therapy with LMWH in patients undergoing orthopedic procedures should be individualized to minimize thromboembolic and bleeding risks in the perioperative period.

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Low-density lipoprotein-decorated and Adriamycin-loaded silica nanoparticles for tumor-targeted chemotherapy of colorectal cancer

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Abstract

Background. Chemotherapy for colorectal cancer remains an unsatisfactory method of treatment and requires the development of more advanced drug delivery systems (DDSs). Among inorganic materials, silica nanoparticles (SLNs) have been considered a suitable candidate to be developed as versatile carriers for drug delivery and imaging applications. Low-density lipoprotein (LDL) is a widespread material that is responsible for cholesterol transport in plasma. The concept of employing LDL-modified nanoparticles for tumor-targeted drug delivery has been widely adopted.

Objectives. The objective of this study was to develop and test a new DDS for effective chemotherapy of colorectal cancer.

Material and methods. We successfully developed an Adriamycin (Adr)-loaded DDS based on LDL-modified SLNs (LDL/SLN/Adr). The tumor-homing property of LDL and the drug-loading capability of SLNs were combined to prepare LDL/SLN/Adr that can specifically deliver Adr to the cancer site to achieve effective chemotherapy of HT-29 colorectal cancer.

Results. In vitro analysis showed that LDL/SLN/Adr consisted of nano-sized particles and was capable of targeting the low-density lipoprotein receptors (LDLR) which were overexpressed in many cancer cell lines. As a result, LDL/SLN/Adr exerted better cytotoxicity than unmodified SLNs and free drugs. In vivo imaging and anticancer assays also confirmed the preferable tumor-homing and enhanced anticancer effect of LDL/SLN/Adr.

Conclusions. LDL/SLN/Adr might be a promising DDS for effective chemotherapy of colorectal cancer.

Key words: colorectal cancer, low-density lipoprotein, silica nanoparticles, Adriamycin

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Introduction

Inorganic materials have attracted the attention of researchers across the world, simply due to their extraordinary properties compared to their organic counterparts. The most commonly adopted ones, including calcium carbonate ($CaCO_3$) nanoparticles, gold nanoparticles and silica nanoparticles (SLNs), have been successfully applied in the biomedical field for more than a decade.[1–3] Among these inorganic materials, the introduction of SLNs has been considered a milestone, since SLNs have more advantages over other inorganic materials, such as ease of synthesis as well as convenient surface modification. On the other hand, high biocompatibility and biodegradability have made them a suitable carrier for biomedical applications.[4] Their large surface area and pore volume also guarantee high drug-loading capacity for a variety of drugs, ranging from hydrophobic to hydrophilic ones. In all, SLNs are suitable candidates to be developed as versatile carriers for drug delivery and imaging applications.[5]

A successful drug delivery system (DDS) for effective cancer therapy requires the capability to bypass the multiple extracellular and intracellular barriers to increase targeted drug accumulation in the tumor tissue, which is beneficial for reducing the unwanted side effects.[6] Two of the greatest challenges for common DDSs are escaping the capture of reticuloendothelial system (RES) and targeting the tumor tissue.[7] It has been demonstrated that SLNs without surface modifications can be recognized by the RES, retarded in the liver and finally excreted out of the body. Many researchers have worked to resolve this dilemma by employing polyethylene glycol (PEG) to encapsulate SLNs. By forming a tunable layer to seal the surface of SLNs, the in vivo interactions between SLNs and RES can be greatly reduced which is beneficial to the in vivo performance of SLNs.[8] However, PEG layer does not have the ability to recognize the abnormal upregulated receptors on the surface of cancer cells in order to guide the DDS to the wanted tumor tissue. The passive targeting of PEG-modified SLNs cannot meet the increasing demands in cancer therapy. As a result, endogenic materials capable of both escaping RES capture as well as targeting tumor tissues are widely recognized as a preferable way to reconcile this dilemma.

Low-density lipoprotein (LDL) is a widespread component responsible for cholesterol transport in plasma. As an endogenic component within the human body, LDL shows high biocompatibility and extremely low cytotoxicity.[9] On the other hand, it has been demonstrated that LDL has high affinity to LDL receptors (LDLR), a specific receptor which has been proved to be upregulated on the surface of many cancer cell lines, including breast cancer, prostate cancer and colorectal cancer.[10]

The concept of employing LDL-modified nanoparticles for tumor-targeted drug delivery has been widely adopted.[11] LDL-modified nanoparticles not only exert high biocompatibility and low cytotoxicity, but also have preferable tumor-targeting capabilities. These facts suggest that drug carriers with surface-modified LDL may be used as a suitable system for chemotherapeutic agents to neoplastic cells.[12,13] We developed LDL-modified SLNs with the aim to construct a DDS that is capable of delivering Adriamycin (Adr) specifically to the tumor site to achieve a better anticancer effect compared with unmodified SLNs and free drugs.

Material and methods

Material

Triton X-100, tetraethyl orthosilicate (TEOS) and *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAPS) were obtained from the Sinopharm Chemical Regent Co., Ltd (Shanghai, China). Adriamycin hydrochloride was supplied by Aladdin Bio-chem Technology (Shanghai, China). Plasma-derived LDL was obtained from Intracel (Frederick, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), coumarin 6 (C6) and 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR) were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals and reagents (unless stated otherwise) were from Sinopharm Chemical Reagent Co., Ltd, and of analytical grade.

Cell culture and animal model

Human colorectal cancer cell line HT-29 was purchased from American Type Culture Collection (ATCC, Manassas, USA). The cells were maintained in Dulbecco minimum essential medium (DMEM) (containing 10% volume ratio of endotoxin free fetal bovine serum (FBS), 2 mM glutamine and 100 U/mL streptomycin and penicillin) at 37°C in a humid atmosphere with 5% CO_2 and 95% air.

Male BALB/c nude mice (~18 g) were acquired from Shanghai Laboratory Animal Center, China, and were maintained in a specific pathogen-free (SPF) lab at the homothermal condition of 25 ±2°C with free access to food and water. All procedures were in strict compliance with our institute guidelines for the care and use of laboratory animals and approved by China Medical University Laboratory Animal Welfare and Ethical Committee (No. 20170115). The generation of HT-29 tumor-bearing nude mice model was originated from the previous report.[14] Briefly, 2×10^6 HT-29 cells were dispersed in 100 µL of phosphate buffer saline (PBS) and subcutaneously injected into the flank of each mouse.

Preparation of LDL/SLN/Adr

Amine-decorated SLNs (ASLNs) were first synthesized in a water-in-oil microemulsion with minor modification as previously reported.[15] In brief, a water-in-oil

microemulsion was prepared by mixing 1.8 mL of Triton X-100, 1.6 mL of n-hexanol and 7.5 mL of cyclohexane. The mixture was gently agitated for 0.5 h to obtain a transparent solution. Afterwards, 180 µL of TEOS together with 60 µL of AEAPS were added under agitation to allow for well dispersion within the microemulsion as precursors for silica formation. One hundred µL of ammonia solution (NH_4OH) was then added to initiate the polymerization process. The reaction was performed continuously under room temperature for 24 h, followed by the addition of 50 mL of ethanol to precipitate the ASLNs. The precipitate was washed with ethanol and water alternatively several times to remove the surfactant and unreacted reactants from the particles.

The ASLNs were resuspended in 10 mL of water with agitation. Adriamycin dissolved in water (5 mg/mL) was added and co-cultured with ASLNs for 6 h. Afterwards, the drug-loaded ASLNs was isolated under high speed of centrifugation (8,000 × g, 10 min, XPN-100; Beckman Coulter, Brea, USA). The remaining Adr in the supernatant was determined using fluorescence spectrophotometer (FluoroMax-4; HORIBA Scientific, Paris, France). The drug-loaded SLNs were resuspended in aqueous solution, to which LDL (1 mg/mL) was added and co-incubated with gentle agitation at room temperature for 12 h. Finally, the Adr-loaded DDS based on LDL-modified SLNs (LDL/SLN/Adr) was isolated from the solution using high speed centrifugation.

Drug loading content (DLC) was calculated according to the following formula:

$$\text{DLC (wt\%)} = \frac{(\text{weight of loaded drug}/}{\text{weight of LDL/SLN/Adr)}} \times 100\%$$

Particle size, distribution and zeta potential measurement

Characterization of nanoparticles concerning their size distributions, polydispersity index (PDI) and zeta potential were assessed at 25°C by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) methods using a Zeta plus zeta potential analyzer (SZ-100; HORIBA Scientific, Paris, France).

In vitro release experiments

The release behavior of Adr from LDL/SLN/Adr was investigated. LDL/SLN/Adr was diluted in PBS (pH 7.4 and 5.0, containing 0.1% Tween 80, w/v) and maintained at 37°C with gentle shaking (100 rpm). At predetermined time intervals, 1 mL of the solution was extracted and an equal volume of fresh medium was supplied. The extracted solution was centrifuged at 8,000 × g for 10 min to remove the nanoparticles and the supernatant was subjected to fluorescence measurement.

Cellular uptake of LDL/SLN/Adr

Fluorescent probe C6 was dissolved in ethanol (0.1 mg/mL) and loaded into nanoparticles along with drug loading. The internalization profile of Adr loaded amine-decorated SLNs (ASLN/Adr) and LDL/SLN/Adr in HT-29 cell line was assessed by monitoring the fluorescence signal of C6. HT-29 cells cultured in confocal dishes (Φ = 15 mm) with 60% confluence were treated with 1 mL of the serum-free medium containing free C6, C6 containing ASLN/Adr or C6 containing LDL/SLN/Adr at the C6 concentration of 350 ng/mL. After 2 h, 4 h and 6 h of incubation, the medium was discarded and cells were rinsed 3 times with PBS to remove the remaining nanoparticles. Afterwards, cells were treated with tyrosine to obtain monodispersed cell suspension. For quantitative determination of the fluorescence intensity of each group, the culture media were discarded, and the cells were harvested and subjected to flow cytometer (FCM) (Attune NxT; Thermo Fisher Scientific, Waltham, USA). The potential LDLR-mediated uptake of LDL/SLN/Adr was confirmed by competitive binding experiments. Then, all the cells were first treated with serum-free medium containing 200 µg/mL of LDL solution for 2 h. Afterwards, free C6, C6 containing ASLN/Adr or C6 containing LDL/SLN/Adr was added to the same medium to achieve the same C6 concentration as mentioned above. After the same procedure, the fluorescence intensity of each group was quantitatively determined by FCM and compared with LDL-untreated groups.

Cytotoxicity activity

To study the cytotoxicity of free nanoparticles and LDL/SLN/Adr, the HT-29 cells were detached using 0.5% trypsin, harvested, seeded into a 96-well plates, and allowed to grow overnight to reach a confluence of 70–80%. Then cells were incubated with fresh medium containing different samples: free Adr solution, drug-free LDL/SLN, ASLN/Adr, and LDL/SLN/Adr (Adr concentration was set at 0.1, 0.25, 0.5, 1.25, 1.5, 1.75, and 2 µg/mL). After different intervals of incubation, standard MTT assay was applied to evaluate the cell viability of all tested samples as reported previously.[16]

In vivo tumor-targeting of LDL/SLN/Adr

Mice with tumor volumes at about 100 mm^3 were recruited and randomly assigned to perform in vivo experiments. The DiR as a near infrared fluorescent probe was encapsulated into the nanoparticles similar to the C6 loading process. Afterwards, DiR-loaded ASLN/Adr and LDL/SLN/Adr were injected into the HT-29 tumor-bearing mice via tail vein at the DiR dosage of 10 µg per mouse. The in vivo real-time biodistribution of different nanoparticles at 2 h, 4 h and 8 h were recorded using in vivo imaging system (MIIS; Molecular Devices, San Jose, USA)

with filters set at excitation and emission at 720 nm and 790 nm, respectively. In order to confirm the LDLR-mediated targeting capability of LDL/SLN/Adr, the mice were first intratumorally injected with LDL (5 mg/kg) 1 h prior to the injection of DiR-loaded LDL/SLN/Adr, and then in vivo-imaged as mentioned above. The tissue distribution of nanoparticles at the end of the experiments was acquired by excising the tumor tissues as well as major organs from the sacrificed mice and subjecting them to ex vivo imaging using the same equipment.

In vivo antitumor efficacy

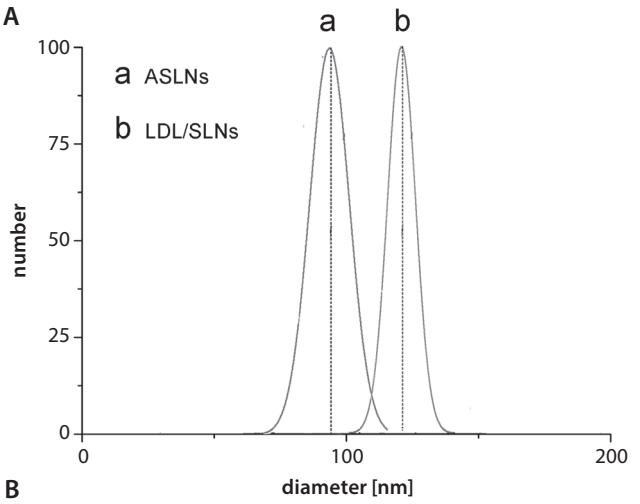
The in vivo antitumor efficacy of LDL/SLN/Adr was further confirmed by employing HT-29 tumor xenograft models. All mice were randomly divided into 4 groups (n = 5): 1. saline (control); 2. free Adr; 3. ASLN/Adr; 4. LDL/SLN/Adr. Protocols regarding administration route and dosing frequency were similar to the previous report with some modifications.[17] In brief, all formulations were administrated via tail vein (5 mg/kg Adr per mouse) once every 2 days 7 times. The body weights and tumor sizes of all treated mice were monitored and recorded before injection. Two days after the last injections, 3 mice from each group were randomly picked and sacrificed. Their tumor tissues were sliced and subjected to hematoxylin-eosin (H&E) staining, and images were taken with a microscope (LSM 510; Zeiss, Oberkochen, Germany).

Results

As shown in Fig. 1A, the ASLNs obtained by water-in-oil microemulsion were nano-sized particles with a diameter of about 92.7 nm. These particles were well-dispersed, with a relatively small polydispersion index (PDI) of 0.264. Compared with the particle size of ASLNs, LDL/SLNs demonstrated a slightly increased size of about 121.3 nm with a decreased PDI of 0.115. It can be seen in Fig. 1B that the originally prepared ASLNs are positively-charged particles with a surface charge of +29.8 mV. However, the surface charge was totally reversed after modification of LDL, with a negative charge of –17.5 mV being observed. According to the fluorescence analysis, the DLC for Adr was 8.6%, which is high enough for both the following in vitro and in vivo experiments.

It can be observed in Fig. 2 that the drug-release speed of Adr was slow in physiological conditions (pH 7.4) with less than 15% of the encapsulated drugs being leaked into the medium after 120 h of incubation. On the contrary, under the acidic condition (pH 5.0), the drug release of Adr was accelerated. The release rate of Adr was more than 3 time the speed of that in pH 7.4, with nearly 60% of the encapsulated drugs being released after 120 h of incubation.

As shown in Fig. 3, higher C6 fluorescence signals were observed at 2 h post-incubation in the cells of LDL/SLN/Adr



B

	Mean particle size (nm)	Zeta potential (mV)	PDI
ASLNs	92.7 ± 7.2	+29.8 ± 5.7	0.264
LDL/SLNs	121.3 ± 6.8	–17.5 ± 3.9	0.115

Fig. 1. Particle size distribution (A) of ASLNs and LDL/SLNs. Mean particle size, zeta potential and poly dispersion index (PDI) measurements (B) of ASLNs and LDL/SLNs

Data was shown as mean ±SD (n = 3); SLNs – silica nanoparticles; ASLNs – amine-decorated silica nanoparticles; LDL – low-density lipoprotein; SD – standard deviation.

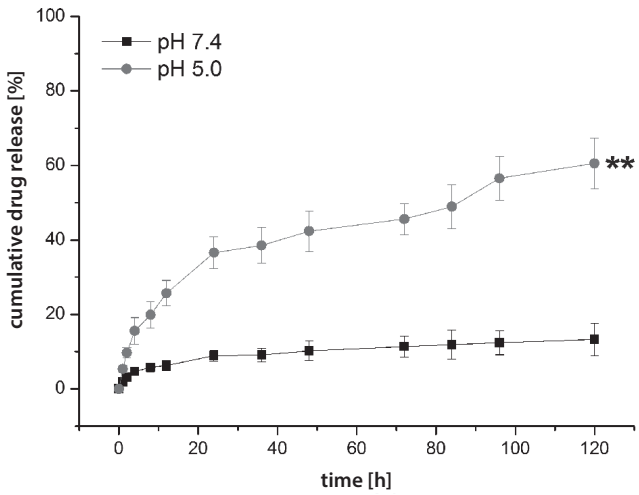


Fig. 2. In vitro drug release of LDL/SLN/Adr at different pH values (7.4 and 5.0)

Data was shown as mean ±SD (n = 3); ** $p < 0.01$ vs pH 7.4; Adr – Adriamycin; LDL/SLN/Adr – Adriamycin-loaded drug delivery system based on low-density lipoprotein-modified silica nanoparticles; SD – standard deviation.

group, compared to that of LDL-unmodified ASLN/Adr, as revealed by FCM results. This difference did not disappear but became more serious as incubation continued. It was also calculated from FCM data that the fluorescence intensity of LDL/SLN/Adr was approx. 1.56-fold higher than that of ASLN/Adr after incubation for 6 h. It was also noted that, during the whole incubation time, the intensity

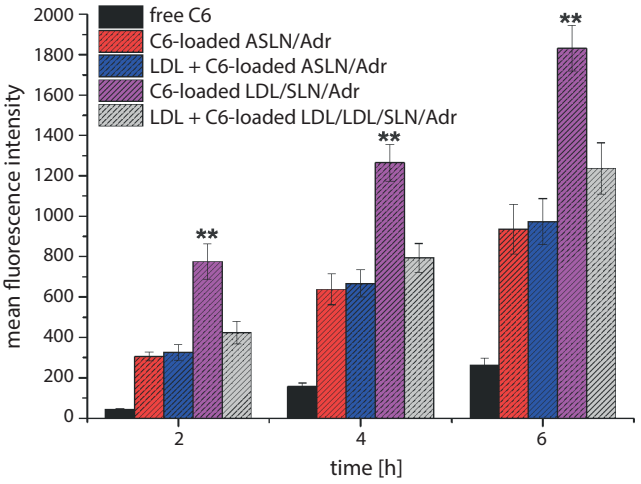


Fig. 3. In vitro quantitative flow cytometric analysis of free C6, C6-loaded ASLN/Adr and C6-loaded LDL/SLN/Adr with and without LDL pretreatment in HT-29 cells for 2 h, 4 h, and 6 h of incubation

Data was expressed as mean ±SD (n = 3); ** $p < 0.01$ vs LDL pretreated groups; Adr – Adriamycin; ASLNs – amine-decorated silica nanoparticles; C6 – coumarin 6; LDL – low-density lipoprotein; LDL/SLN/Adr – Adriamycin-loaded drug delivery system based on low-density lipoprotein-modified silica nanoparticles; SD – standard deviation.

of fluorescence signals in free C6-treated cells was much lower than the intensity of these signals in groups treated with DDSs.

As displayed in Fig. 4A, when treated by drug-free LDL/SLNs, no significance was found among all adopted concentrations as more than 90% of the cells survived the dosing concentrations. The following anticancer assay with drug-loaded nanoparticles showed some interesting results. It was clear that all Adr-containing formulations had anticancer effects on HT-29 cells and this effect was dose-related, as higher drug dosing would lead to more serious cell mortality. The cytotoxicity of cells treated with LDL-modified formulations was more severe than the cytotoxicity of the unmodified ones under the same conditions.

In Fig. 5A, it was clear that ASLN/Adr and LDL/SLN/Adr showed significant differences in targeting efficacy after in vivo administration and that the fluorescence intensity of tumor site in LDL/SLN/Adr-treated mice was stronger than that of ASLN/Adr at the same period. The difference in tumor targetability was also verified by using ex vivo imaging of the tumor tissues and main organs of the sacrificed mice (Fig. 5B). In detail, the fluorescence intensity of the tumor tissues in LDL/SLN/Adr-treated mice was 3.24-fold higher than the fluorescence intensity of ASLN/Adr-treated mice. Moreover, it was interesting to find that the fluorescence intensity of the livers showed an opposite result; it was 1.53-fold higher in ASLN/Adr-treated mice than the fluorescence intensity of LDL/SLN/Adr-treated mice.

As shown in Fig. 6A, although all the formulations can suppress tumor growth to some extent, the anticancer efficacy of LDL/SLN/Adr appeared to be most potent compared with others, since animals treated with LDL/SLN/Adr showed the smallest tumor volumes of 468 ±51 mm^3. In Fig. 6B, the body weight of mice treated with free Adr steadily decreased. However, no noticeable loss in body weight was observed in LDL/SLN/Adr-treated group. Hematoxylin-eosin staining assay and the representative pictures were displayed in Fig. 6C. It was observed that the cells in negative control group (saline) showed typical pathological characteristics of a tumor, such as large and irregularly shaped nuclei, which were closely packed with one another. Other drug-included formulations showed characteristics of cancer cell remission with tumor coagulative necrosis, intercellular blank and nuclei

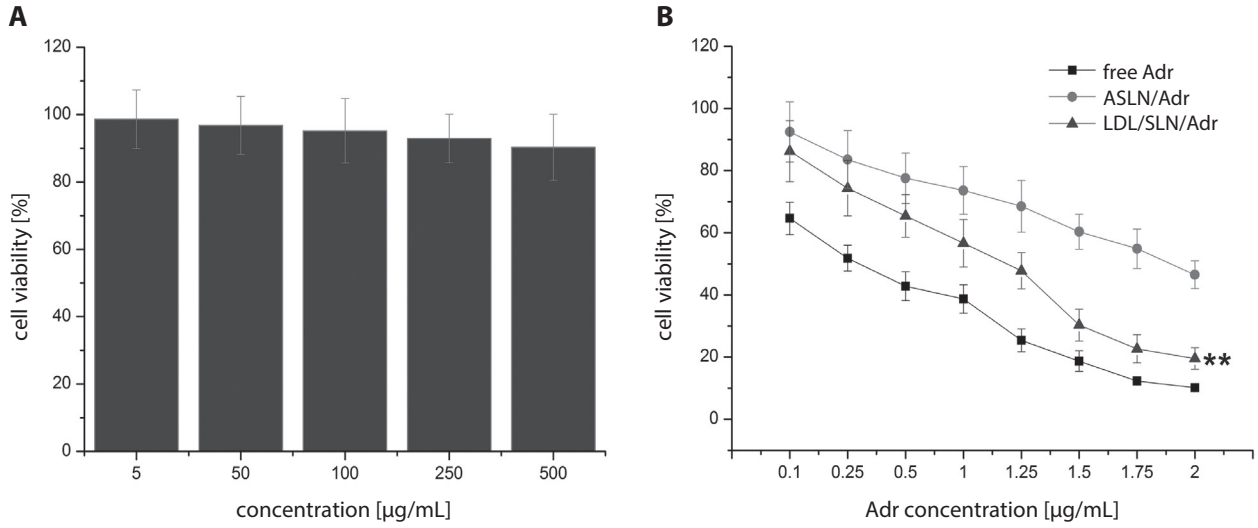


Fig. 4. (A) Cytotoxicity of free LDL/SLNs after 48 h incubation with HT-29 cells. (B) Cytotoxicity of free Adr, ASLN/Adr and LDL/SLN/Adr against HT-29 cells after 48 h incubation

Data was expressed as mean ±SD (n = 3); ** $p < 0.01$ vs ASLN/Adr; Adr – Adriamycin; LDL – low-density lipoprotein; SLNs – silica nanoparticles; ASLNs – amine-decorated silica nanoparticles; LDL/SLN/Adr – Adriamycin-loaded drug delivery system basen on low-density lipoprotein-modified silica nanoparticles; SD – standard deviation.

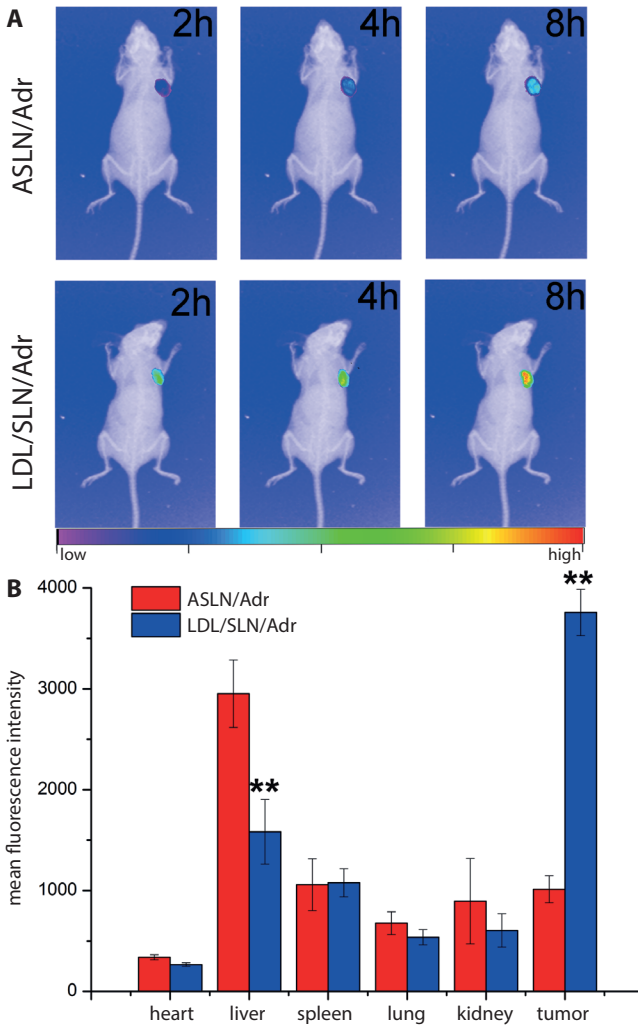


Fig. 5. (A) In vivo time-dependent tumor-targeting images after intravenous injection of (2 h, 4 h and 8 h) DiR-loaded nanoparticles in HT-29 tumor-bearing mice and (B) representative ex vivo mean fluorescence intensity of dissected tumors and major organs at 8 h post-injection

Data was expressed as mean ±SD (n = 3); ** $p < 0.01$ vs ASLN/Adr; Adr – Adriamycin; ASLNs – amine-decorated silica nanoparticles; DiR – 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide; SD – standard deviation.

fragmentation being observed. Compared with the other groups, the LDL/SLN/Adr group showed a massive cancer cell remission with the most promising antitumor ability.

Discussion

The ASLNs were surface-modified with free primary amine groups, which were capable of assembling into multifunctional DDSs with other molecules via either chemical reaction or physical interaction.[18] It has been reported that the water-in-oil microemulsion method can control the size of the particles using the generated water pools in the microemulsion. These pools are composed of a water core and a surfactant shell, which can define the reaction zone. On the other hand, the surfactant shell can also serve as a spacer to prevent the neighboring nanoparticles from aggregation.[19,20] The LDL was anchored to the surface of ASLNs via electrostatic adsorption. The attachment of LDL resulted in an increase in particle size of LDL/SLNs as compared with unmodified SLNs. The decreased PDI value of LDL/SLNs indicated that surface modification of hydrophilic LDL might further benefit the dispersion of LDL/SLNs, since it has been demonstrated by previous reports that hydrophilic surface modification, such as PEG, can increase the colloidal stability of the recipient.[21,22] On the other hand, the successful modification of LDL to the surface of SLNs was further proven by zeta potential measurement. The negatively-charged surface, according to the previous report, might be beneficial for the ability of the DDSs to bypass the recognition of many active components within the circulation system, and might ensure safe delivery of the drugs.[23]

Due to their large surface area and pore volume, SLNs were capable of loading a variety of drugs, ranging from hydrophobic ones to hydrophilic ones, with a relatively high loading efficiency. This is beneficial for SLNs to act as a co-delivery carrier for the loading of Adr.

One significant drawback of some currently available DDSs is that they are not able to meet the controversial requirements of drug delivery. In the 1st stage, while DDSs are circulating in the blood, it is beneficial to preserve the encapsulated drugs safely in the circulation system, since the leaked drugs might cause severe side effects to normal tissues and organs. However, upon reaching or entering the cancer tissue, DDSs should be able to accelerate their release rate in a different manner. In order to simulate the drug release profile of LDL/SLN/Adr in physiological and neoplastic conditions, drug release percentages of our system was monitored and recorded under PBS with different pH values. The pH 7.4 was mimicking the physiological condition while pH 5.0 was mimicking the intracellular condition of cancer cells, since all of the cancer cells have a relatively acidic pH conditions when compared with normal cells or blood conditions. The faster drug release of LDL/SLN/Adr in pH 5.0 than that in pH 7.4 could be explained by the fact that Adr is a weak base and its solubility might be increased in acidic conditions. It is beneficial to increase the drug release of the loaded Adr under acidic conditions. These results were encouraging since they revealed the potential of LDL/SLN/Adr to accelerate its drug release under acidic condition instead of in physiological condition. Since it has been reported that tumor tissue is composed of many highly active cells with high expression of many kinds of enzymes and capable of secreting various constituents, the drug-release speed of LDL/SLN/Adr was expected to be further enhanced as the decomposition of the carrier and competitive dissociation under such conditions will be more serious than that in PBS. This could be beneficial for drug release more intensive in neoplastic tissues than in normal areas to reduce the side effects of chemotherapy and increase the anticancer efficacy of the drugs.

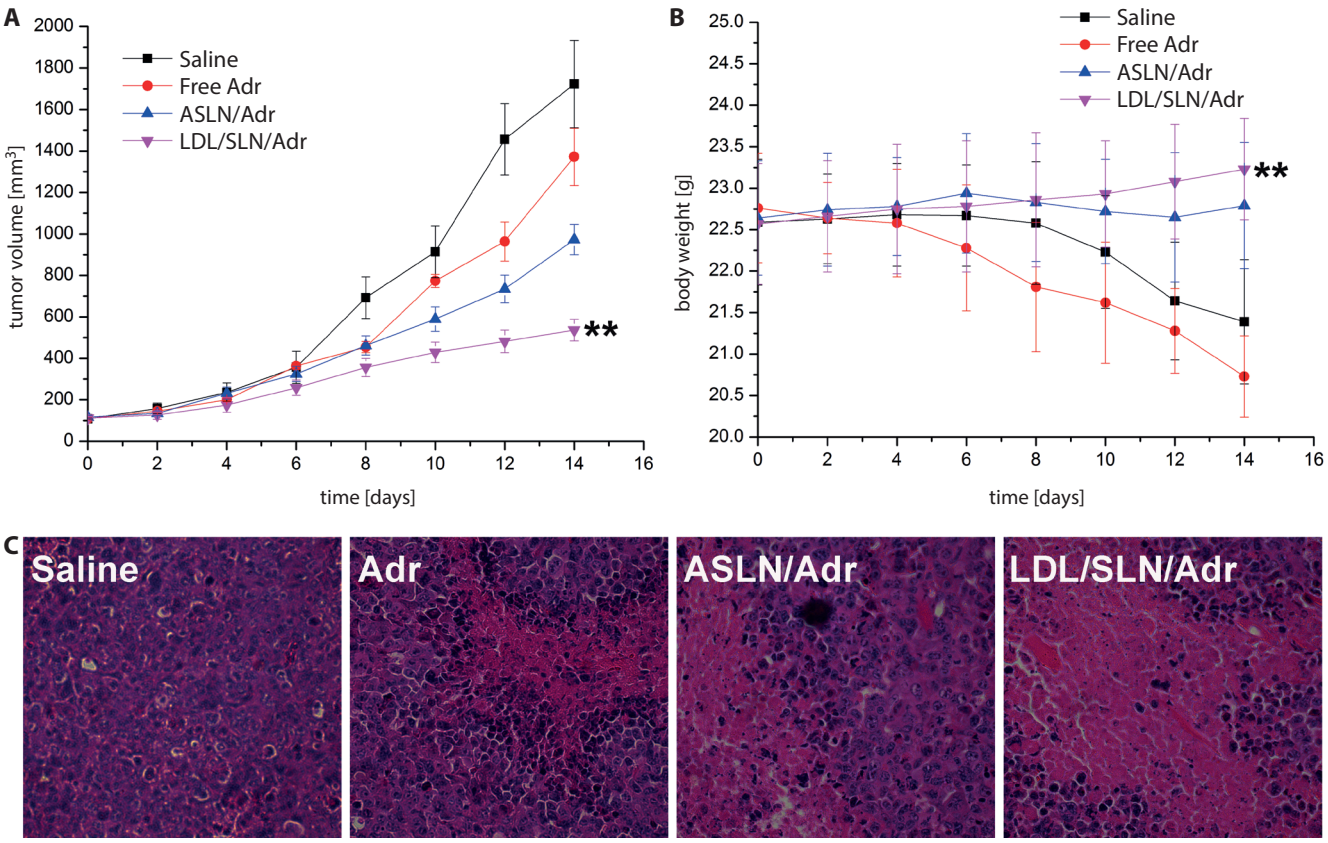


Fig. 6. The tumor volume (A, ** $p < 0.01$ vs saline), body weight (B, ** $p < 0.01$ vs free Adr) and H&E staining of tumor tissue (C) analysis of HT-29 tumor-bearing BALB/c nude mice after intravenous injection administration of different formulations

The measurement of tumor volumes and the injection of formulations were repeated every 2 days for 2 weeks. Dose: 5 mg/kg Adr per mouse. Data was expressed as mean ±SD (n = 5); Adr – Adriamycin; H&E – hematoxylin-eosin; SD – standard deviation.

It has been demonstrated by many previous articles that LDL anchored toward the surface of the DDS can target LDLR, which is excessively expressed in various cancer cells, including colorectal cancer.[24] In order to verify that our DDS is also capable of specifically targeting LDLR-overexpressed HT-29 cells, C6 was employed as both a fluorescence probe and a hydrophobic drug molecule to mimic the uptake profile of Adr. A quantitative analysis of the uptake behavior of different samples by FCM at different time points was also conducted. Results presented in Fig. 3 suggested that LDL/SLN/Adr can be more effectively internalized by HT-29 cells than its counterpart (ASLN/Adr). During the whole incubation time, the fluorescence signals intensity in free C6-treated cells was all much lower than the fluorescence signals intensity in the DDSs-treated groups. Such result could be explained by the fact that C6 as a drug molecule can be excreted outside the cells via P-glycoprotein, a common transporter associated with multi-drug resistance of cancer cells.[25] However, some evidence has shown that DDSs-based delivery can bypass and overcome such excretion effect to improve the anticancer efficiency of the loaded drugs. In addition, we also performed competitive uptake experiments to further confirm the LDLR-associated uptake of LDL/SLN/Adr. A great decline in fluorescence intensity of LDL/SLN/Adr group was observed at all time intervals after pretreatment of excess LDL, while the fluorescence intensity of ASLN/Adr group still remained at the same level. These results clearly concluded that LDL/SLN/Adr was internalized into cells via LDLR-mediated endocytosis.

In order to further explore and verify the in vitro Adr-delivery efficiency and anticancer efficiency of the well-designed LDL/SLN/Adr, MTT assay was employed to evaluate the cell viability and to demonstrate the results of cytotoxicity. Prior to the MTT assays of drug-loaded formulations, the cytotoxicity of drug-free LDL/SLNs were firstly conducted with nanoparticle concentrations ranging from 5 to 500 μg/mL to seek out whether the carriers we adopted in this study have cytotoxicity effects on HT-29 cells and to what extent it can influence the final results. Our results indicated that the LDL/SLNs we constructed had low cytotoxicity and were biocompatible, which shows a broad range of their potential application in the field of cancer therapy and other biomedical fields. Low-density lipoprotein modification can increase the cellular uptake of LDL/SLNs as proven by cellular uptake experiments, as the cytotoxicity of cells treated with LDL-modified formulations is more severe than the cytotoxicity of the unmodified ones under the same conditions, which was in accordance with cellular uptake assays.

Low-density lipoprotein modification on the surface of LDL/SLN/Adr was expected to aid the nanoparticles to bypass the RES and result in their increased accumulation at the tumor site. To verify this conjecture, we employed non-invasive near-infrared (NIR) optical imaging technique as well as NIR fluorescence probe-loaded nanoparticles to monitor the real-time tumor-targeting ability of the DDS. The distribution of DDS at the tumor tissue was reflected by monitoring the NIR fluorescence within the tumor site of HT-29 tumor-bearing nude mice for up to 8 h and is displayed in Fig. 5. The in vivo images at the tumor site after intravenous injection of DiR loaded nanoparticles at different time points were recorded. These results were consistent with the previous report that positively-charged DDSs can preferably be captured by the liver, while negatively-charged ones can reduce this dilemma.[23] These results indicated that LDL/SLN/Adr had stronger tumor-targeting ability than ASLN/Adr and that it showed preferable accumulation at the tumor tissue. They showed that LDL/SLN/Adr can act as a safe and effective DDS that delivers its payload to the targeted cells and achieves better anticancer effect.

With the aim to find the in vivo antitumor potential of LDL/SLN/Adr in HT-29 xenografted nude mice, LDL/SLN/Adr, ASLN/Adr as well as free Adr were assessed in regard to their ability to suppress tumor growth and influence body weight variation, with saline as a blank control. The observations suggested the enhanced tumor-homing property of LDL/SLN/Adr due to the modification of LDL. Moreover, the time- and formulation-dependent variations in body weight of subjected mice were recorded. Free drugs, especially Adr, were demonstrated to have strong system toxicity, which would result in body weight loss. As shown in Fig. 5B, the body weight of mice treated with free Adr steadily decreased, indicating that the health condition of mice was compromised, either due to tumor burden, side effects of free drugs or the combination of them. However, no noticeable loss in body weight was observed in LDL/SLN/Adr-treated group, suggesting that LDL/SLN/Adr could not only increase the anticancer efficacy of free drugs, but also reduce the safety risks. The H&E staining results also allowed for the same conclusion.

Conclusions

In summary, a tumor-targeting DDS composed of LDL and SLNs loaded with Adr was developed (LDL/SLN/Adr) to take advantage of the tumor-targeting ability of LDL and drug-loading property of SLNs for the safe and effective chemotherapy of colorectal cancer. Our experimental results indicated that nano-sized LDL/SLN/Adr with decent drug loading could preserve the encapsulated drugs under physiological condition, while unloading it in a faster way under acidic neoplastic conditions. On the other hand, LDL/SLN/Adr could increase the uptake ratio of drugs into HT-29 colorectal cancer cells compared with unmodified ASLN/Adr, possibly via the LDLR-mediated endocytosis. More importantly, LDL/SLN/Adr exhibited stronger anticancer activity in vivo with minimized toxic side effects and preferable tumor-suppression potential in HT-29 tumor-bearing nude mice model.

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The journey of the heart failure patient, based on data from a single center

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Abstract

Background. Care for patients with heart failure (HF) in Poland requires improvement.

Objectives. The aim of this study was to define the journey of the HF patient, taking into account the specialization of the hospital ward and further, highly specialized outpatient care.

Material and methods. Using the medical system CliniNET®, we analyzed 214 consecutive patients hospitalized due to HF (International Statistical Classification of Diseases and Health Related Problems – ICD-10: I50) in the period from September 1 to December 31, 2015, and also the data from post-discharge outpatient care in a 3-month period. To fairly compare the management of care and outcomes of patients hospitalized in the internal medicine (IM) ward and in the cardiac ward, propensity score matching was performed. The multivariate regression analysis was performed to determine the independent predictors of the hospital ward selection and the risk of rehospitalization due to HF and/or death.

Results. The majority of patients were hospitalized due to HF for the first time (72%) and in the cardiac ward (65%). For 55% of rehospitalized patients, the subsequent admission was within 3 months after initial discharge. The independent predictors of a higher risk of rehospitalization due to HF and/or death were ischemic heart disease, atrial fibrillation (AF), chronic kidney disease (CKD), mineralocorticoid antagonism (MRA) therapy, and hospitalization in the last year (for all, $p < 0.05$). Internal medicine ward patients differed from cardiac ward patients in: mode of admission (urgent 100% vs 83.5%; $p < 0.001$), length of hospitalization (median: 8 days vs 5 days; $p = 0.001$), death rate (24% vs 4.3%; $p < 0.001$), echocardiography (43% vs 98%; $p < 0.001$), and N-terminal prohormone B-type natriuretic peptide (NT-proBNP) measurements (43% vs 96%; $p < 0.001$). The burden of 5–9 accompanying diseases enhanced the choice of the cardiac ward ($p < 0.05$), while age and urgent mode of hospitalization decreased the chance of being referred to the cardiac ward ($p < 0.01$). Cardiac patients were more likely to receive β-blockers, diuretics, angiotensin receptor blockers (ARB), and MRA. Over 90% of cardiac ward patients were referred to cardiac ambulatory care after discharge from hospital, while among patients discharged from the IM ward, this rate was 60% ($p < 0.001$).

Conclusions. There were significant differences among the 2 wards in relation to the course of hospitalization and post-discharge outpatient care.

Key words: heart failure, hospitalization, cardiology, internal medicine

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Introduction

Nowadays, heart failure (HF) is a major health issue, as it is associated with high prevalence, high death rates and large consumption of healthcare resources.[1,2] Heart failure affects approx. 1–2% of adults in developed countries.[3] Its prevalence rises significantly with age and, according to the latest data, the morbidity will further increase.[4] Altogether, 1/5 of adult individuals will develop HF at some point.[5]

It is estimated that 30% of HF patients are readmitted to hospital within 60–90 days from initial hospitalization.[6,7] In European studies, reported HF rehospitalization rates ranged from 24% at 12 weeks to 44% after 1 year post-discharge and had poor prognosis.[8,9] In their study, Solomon et al. proved that the death rate in HF patients increased by 30% after the 2nd and 3rd readmission.[10] Approximately 10% of HF patients die 60–90 days post-discharge.[11,12] In Poland, 164 patients die every day due to HF, which amounts to over 60,000 deaths annually.[13]

According to the Organisation for Economic Co-operation and Development (OECD) 2015 report, Poland has the highest number of hospitalizations due to HF in the world.[2] The high numbers of hospitalizations for HF are a huge burden for healthcare systems. In 2012, the costs of HF patients' management reached 672 million PLN, of which 94% were hospitalization costs.[13] Analyzing the economic and social costs in 2012, Gierczyński et al. documented that 75% of HF patients were hospitalized at internal medicine (IM) wards and 22% at cardiac wards.[13] This fact became the basis of the hypothesis that there are differences related to the admission ward in terms of the characteristics of the hospitalized patient, diagnostic procedures, as well as in-hospital and post-discharge care.

The aim of this analysis was to assess the journey of the HF patient in Poland, based on the single-center experience, including the type of the admission ward and further cardiac outpatient care.

Material and methods

The study was designed as a questionnaire retrospective survey. It was conducted using questionnaire authoring, available in the electronic form. The standardized study questionnaires were designed to collect information on general characteristics of the patient group and to evaluate the HF patient management in the cardiac ward and the IM ward in the period from September 1 to December 31, 2015, in the Central Hospital of Medical University of Lodz, Poland, based on the available medical records. The survey was conducted using the medical system CliniNET®. The field studies dealt with the data on post-discharge outpatient care from the time of discharge to March 31, 2016. The questionnaire was constructed according to the applicable standards and rules concerning the collection of data through a survey. The questions concerned the demographics, etiology and history of HF, the results of laboratory tests, applied treatment, death during or after hospitalization, and data on post-discharge outpatient care in a 3-month period. The data in the registry was verified and entered by specially trained physicians.

At baseline, the study included 214 consecutive patients. The inclusion criteria were as follows: hospitalization with I50 diagnostic code in the International Statistical Classification of Diseases and Health Related Problems (ICD-10) classification, hospitalization in the cardiac or IM ward in the Central Hospital of Medical University of Lodz, Poland, and hospitalization in the period from September 1 to December 31, 2015.

The study excluded patients hospitalized in the cardiac or IM ward with a diagnostic code other than I50 in the ICD-10 classification and patients hospitalized in other period. The structure of the study sample is presented in Table 1. The analyzed wards were researched regarding the medical

Table 1. Characteristics of the study population

Age [years], mean ±SD	72.2 ±12.9
Males, n [%]	105 (49.1)
BMI [kg/m^2], median (IQR)	27.4 (24.28–31.2)[a]
SBP [mm Hg], median (IQR)	130 (115–140)[a]
DBP [mm Hg], median (IQR)	78 (70–85)[a]
Urgent mode of admission, n [%]	191 (89.2)
Arterial hypertension, n [%]	162 (75.7)
Coronary artery disease, n [%]	100 (46.7)
History of myocardial infarction, n [%]	54 (25.2)
CKD, n [%]	48 (22.4)
AF, n [%]	109 (50.9)
DM, n [%]	90 (42.1)
COPD, n [%]	30 (14.0)
Cancer, n [%]	15 (7.0)
ICD, n [%]	15 (7.0)
CRT, n [%]	7 (3.3)
LVEF [%], mean ±SD	42.2 ±16.8
NT-proBNP on admission [pg/mL], median (IQR)	3,356 (2,204–10,341)[a]
Hb [g/dL], median (IQR)	12.9 (11–13.9)[a]
Sodium [mmol/L], median (IQR)	138.1 (133–143.2)[a]
Creatinine [µmol/L], median (IQR)	98 (80.5–111.5)[a]
eGFR [mL/min/1.73 m^2], median (IQR)	61.4 (47.75–75.75)[a]
Fasting glucose [mmol/L], median (IQR)	6 (5.07–7.41)[a]
CRP [mg/L], median (IQR)	7.2 (2.9–21.8)[a]
Coronary angiography, n [%]	22 (10.3)

Data is presented as mean ± standard deviation (SD), median (interquartile range – IQR) or number (percentage). [a] Variables with non-parametric distribution. AF – atrial fibrillation; BMI – body mass index; CKD – chronic kidney disease; COPD – chronic obstructive pulmonary disease; CRP – C-reactive protein; CRT – cardiac resynchronization therapy; DBP – diastolic blood pressure; DM – diabetes mellitus; eGFR – estimated glomerular filtration rate; Hb – hemoglobin; ICD – implantable cardioverter defibrillator; LVEF – left ventricle ejection fraction; NT-proBNP – N-terminal prohormone B-type natriuretic peptide; SBP – systolic blood pressure.

records of the hospitalized patients with the final HF diagnosis.

The patients' medical history was taken, a physical examination was performed and basic laboratory results were assessed in all study patients on admission. The clinical symptoms of HF were classified according to the New York Heart Association (NYHA).[14]

Echocardiographic measurements were performed according to the guidelines of the American Society of Echocardiography (ASE) and European Association of Echocardiography (EAE).[15] A coronary arteriography was performed as needed according to the ASE/EAE and European Society of Cardiology (ESC) recommendations.[16] Heart failure with reduced ejection fraction (HFrEF) was diagnosed when the left ventricle ejection fraction (LVEF) measurement was less than 40%.[17] Heart failure with preserved ejection fraction (HFpEF) was diagnosed when the LVEF measurement remained greater than 50%.[17]

The following parameters were analyzed in this study:

– age, gender, body mass index (BMI), arterial blood pressure, coincidence of arterial hypertension, diabetes mellitus (DM), myocardial infarction, coronary artery disease, atrial fibrillation (AF), renal failure, chronic obstructive pulmonary diseases (COPD), cancer, coronary artery bypass graft (CABG), and electrotherapy history: cardiac resynchronization therapy (CRT)/implantable cardioverter defibrillator (ICD);

– HF etiology (ischemic vs non-ischemic: hypertension, valvular disease, congestive cardiomyopathy, myocarditis, toxic cardiomyopathy, tachyarrhythmic cardiomyopathy) and past history of HF;

– mode (urgent vs planned) and length of current hospitalization;

– selected laboratory results: hemoglobin (Hb), sodium, potassium, creatinine, estimated glomerular filtration rate (eGFR), C-reactive protein (CRP), fasting glucose, and N-terminal prohormone B-type natriuretic peptide (NT-proBNP);

– electrocardiography (ECG) variables: heart rhythm, heart rate (HR), left bundle branch block (LBBB);

– selected echocardiographic results, including LVEF;

– coronary arteriography results;

– drugs and doses of standard HF therapy prescribed at discharge: angiotensin-converting-enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB), β-blockers, mineralocorticoid antagonist (MRA), ivabradine, and diuretics;

– death during or after hospitalization;

– post-discharge outpatient care.

The collected data was subjected to hospital ward- and endpoint-related statistical analysis. The endpoints were as follows: HF rehospitalization and/or death.

Statistical analysis was conducted using the statistical package STATISTICA PL v. 10.0 (StatSoft Polska Sp. z o.o., Kraków, Poland). Continuous variables were first evaluated for normal distribution using the Shapiro-Wilk test. We also checked data distribution. The Mann-Whitney test was used to compare continuous variables. All continuous variables were expressed as mean, median, interquartile range (IQR, Q25–Q75), and standard deviation (SD); categorical variables were expressed as the number of observations (n) and the corresponding percentage (%). The χ^2 test was used to compare the qualitative data between the groups. Fisher's exact test for independence (in lower numbers) was used. The odds ratio (OR) and a 95% confidence interval (95% CI) were assigned. All statistical tests were two-sided. All variables significantly associated ($p < 0.05$) with the selection of a hospital ward and a higher risk of rehospitalization and/or death in the univariate model were included in the multivariate regression analysis to determine the independent predictors of the hospital ward selection and the risk of rehospitalization and/or death. Results were considered statistically significant at $p < 0.05$. All tables present only the variables which differ or have been selected in the context of the article.

The study design was approved by the Bioethics Committee of Medical University of Lodz, Poland (No. RNN/38/16/KE).

Data was collected and processed maintaining confidentiality of the patients and physicians participating in the study.

To adequately compare the management of care and outcomes of the patients hospitalized in the 2 wards, we had to create identical groups of patients in terms of the epidemiological data, frequency of comorbidities, etiology of HF, and clinical status on admission to hospital, by means of propensity score matching.

Results

The final study sample included 214 patients. Detailed characteristics of the study population are presented in Table 1. The studied cohort suffered the most common comorbidities including: arterial hypertension, AF, coronary artery disease, DM, and chronic kidney disease (CKD).

The majority of the studied patients were admitted in an urgent mode (n = 191; 89%) and hospitalized in the cardiac ward (n = 139; 65%). Comparing to cardiac ward patients, IM ward patients were older, more likely to have accompanying infection, as well as higher systolic blood pressure (SBP) and HR at discharge (Table 2). Internal medicine ward patients had less devices – ICD (1.3% vs 10%; p = 0.035) or CRT (0 vs 5%; p = 0.099) and history of myocardial infarction (10.7% vs 33%; p = 0.001).

According to the results of the univariate analysis, the significant variables for choosing the cardiac ward on admission were as follows: sex, age, mode of hospitalization (urgent vs planned), number of comorbidities, AF, and LVEF (>40% vs ≤40%). The multivariate regression analysis revealed the independent predictors of choosing the cardiac ward (Table 3). The burden of 5–9 accompanying diseases enhanced the choice of the cardiac ward more than 5-fold (Table 3), while age and urgent mode

Table 2. Comparison between hospitalizations in cardiac and internal medicine (IM) wards

Variables	Cardiac ward	IM ward	p-value
All patients, n [%]	139 (65.8)	75 (35.0)	–
Males, n [%]	76 (54.7)	29 (38.7)	0.591
Age [years], median (IQR)	72.0 (62.0–80.0)[a]	81.0 (72.0–86.0)[a]	<0.001
BMI [kg/m^2], mean ±SD	28.75 ±6.1	27.99 ±5.8	0.611
SBP [mm Hg], median (IQR)	126 (110–140)[a]	135 (120–140)[a]	0.007
DBP [mm Hg], median (IQR)	75 (66–80)[a]	80 (70–85)[a]	0.092
HR on admission [bpm], median (IQR)	76 (70–90)[a]	880 (70–100)[a]	0.168
HR at discharge [bpm], median (IQR)	70 (64–75)[a]	76 (70–80)[a]	<0.001
Comorbidities			
Underweight, n [%]	0	2 (4.4)	0.142
Overweight, n [%]	52 (38.8)	14 (31.1)	0.142
Obesity, n [%]	42 (31.3)	15 (33.3)	0.142
Coronary artery disease, n [%]	71 (51.1)	29 (38.7)	0.111
AF, n [%]	73 (52.5)	36 (48.0)	0.626
DM, n [%]	57 (41.0)	33 (44.0)	0.781
Arterial hypertension, n [%]	108 (77.7)	54 (72.0)	0.447
CKD, n [%]	27 (19.4)	21 (28.0)	0.207
COPD, n [%]	18 (12.9)	12 (16.0)	0.684
Cancer, n [%]	8 (5.8)	7 (9.3)	0.485
Infection, n [%]	20 (14.4)	33 (44.0)	<0.001
Current hospitalization			
Urgent hospitalization, n [%]	116 (83.5)	75 (100)	<0.001
Length of hospitalization [days], median (IQR)	5 (3–8)[a]	8 (5–10)[a]	0.001
Death in-hospital, n [%]	4 (2.9)	15 (20.0)	<0.001
Death post-discharge, n [%]	2 (1.4)	3 (4.0)	<0.001
Access to ambulatory care			
Referral to a cardiology outpatient clinic, n [%]	127 (91.9)	45 (60.0)	<0.001
Control visit in a cardiology clinic, n [%]	44 (35.5)	7 (18.9)	0.089
<1 month, n [%]	16 (36.4)	1 (14.3)	0.007
1–3 months, n [%]	9 (20.5)	0	0.007
unknown, n [%]	4 (9.1)	5 (71.4)	0.007
Pharmacotherapy			
ACEI, n [%]	87 (62.6)	42 (56.0)	0.427
ARB, n [%]	27 (19.4)	5 (6.7)	0.022
β-blockers, n [%]	124 (89.2)	55 (73.3)	0.005
MRA, n [%]	47 (85.5)	5 (50.0)	0.022
Ivabradine, n [%]	4 (26.7)	0	1.000
Diuretics, n [%]	120 (86.3)	52 (69.3)	0.005

Data is presented as mean ± standard deviation (SD), median (interquartile range – IQR) or number (percentage). A p-value <0.05 is considered statistically significant. [a] Variables with nonparametric distribution. A p-value <0.05 is considered statistically significant. ACEI – angiotensin-converting-enzyme inhibitors; AF – atrial fibrillation; ARB – angiotensin receptor blockers; BMI – body mass index; CKD – chronic kidney disease; COPD – chronic obstructive pulmonary disease; DBP – diastolic blood pressure; DM – diabetes mellitus; HR – heart rate; IM – internal medicine; MRA – mineralocorticoid antagonism; SBP – systolic blood pressure.

of hospitalization decreased the chance of choosing the cardiac ward (Table 3).

As compared to IM ward patients, cardiac ward patients were more likely to undergo echocardiography (98% vs 43%; p < 0.001) and the measurements of NT-proBNP concentration (96% vs 43%; p < 0.001). For the whole population, the median NT-proBNP concentration was high, with a median of 3356 pg/mL (IQR = 2,204–10,341). Based on the available echocardiography results (n = 168), the mean LVEF was 42.2 ±16.8%. Among patients with ejection fraction (EF) data, 44.04% had HFpEF and 55.96% had HFrEF. Based on the available ECG results (n = 212),

Table 3. Results of the multivariate regression models

Variables	Exp (B) – OR	95% CI for OR		p-value
Choice of the cardiac ward				
Sex: women vs men	0.83	0.33	2.09	0.689
Age [years]	0.93	0.91	0.96	<0.001
Number of comorbidities: 1–3 3–5 5–9	 1.52 2.15 5.46	 0.53 0.71 1.20	 4.31 6.45 24.85	 0.436 0.174 0.028
Mode of hospitalization: urgent vs planned	0.31	0.14	0.67	0.003
AF	1.25	0.51	3.06	0.631
LVEF: >40% vs ≤40%	0.90	0.35	2.34	0.832
Rehospitalization and/or mortality				
Ischemic heart disease	4.65	1.32	16.34	0.017
AF	5.24	1.81	15.13	0.004
Arterial hypertension	0.13	0.05	0.36	<0.001
Valvular disease	0.47	0.19	1.16	0.102
CKD	3.62	1.06	12.30	0.04
The number of comorbidities: 0–3 vs 3–8	0.37	0.11	1.27	0.115
MRA therapy	2.57	1.05	6.26	0.038
Mode of hospitalization: planned vs urgent	0.19	0.05	0.77	0.02
Hospitalization in the last year	64.17	7.29	564.75	<0.001
Hospital ward: cardiac vs IM	0.2	0.08	0.52	<0.001

A p-value <0.05 is considered statistically significant. AF – atrial fibrillation; CKD – chronic kidney disease; CI – confidence interval; IM – internal medicine; LVEF – left ventricle ejection fraction; MRA – mineralocorticoid antagonism; OR – odds ratio.

in 17.9% of the hospitalized HF patients (n = 38), LBBB with QRS duration >120 ms was registered in 12-lead ECG. Most of them were hospitalized in the cardiac ward (n = 31; 81.6%). About half of patients with LBBB (n = 20; 52.6%) had reduced EF (LVEF ≤ 35%).

Atrial fibrillation was observed in 50.9% of the hospitalized patients (n = 109) and more often in cardiac ward patients (52.5% vs 48%), although not statistically significantly. In AF patients, the median baseline HR on admission was 80 bpm (IQR: 60–106; HR_{max} – 160 bpm) and at discharge 70 bpm (IQR: 60–80; HR_{max} – 120 bpm).

Ischemic etiology of HF was present in 31.3% of patients (n = 67). Regarding non-ischemic etiology, arterial hypertension was present in 49% of patients (n = 105), 38.8% had valvular disease (n = 83), 7.5% had congestive cardiomyopathy (n = 16), and 8.4% had other etiology, namely, toxic cardiomyopathy or tachyarrhythmic cardiomyopathy.

Coronary angiography was performed in 10% of the cardiac patients (n = 22) and slightly more than a half of them (n = 13; 59%) showed no significant atherosclerosis in the coronary arteries; in 1 case, the myocardial bridge was observed, and in the other one, percutaneous coronary intervention and CABG surgery were performed. Some of the cardiac ward patients also underwent dobutamine stress echocardiography (DSE). Patients from the study group were also qualified for coronary computed tomography angiography (n = 6) and cardiac magnetic resonance imaging.

Most of the patients (n = 145; 67.8%) were hospitalized for 3–8 days, but significantly shorter in the cardiac ward than in the IM ward (Table 2). As opposed to cardiac ward patients, all IM ward patients were hospitalized immediately (Table 2). Most of the studied patients (n = 154; 72%) were hospitalized due to HF for the first time. For most of them (n = 93; 60.4%), it was hospitalization due to HF exacerbation, but for 61 patients (39.6%), it was hospitalization due to acute HF de novo. Additionally, 8 patients (5.2%) with acute HF de novo also had LVEF ≤ 35% during current hospitalization. Sixty patients were rehospitalized; for 18 of them (30%), the rehospitalization took place within 1 month after initial discharge and for 33 of them (55%) within 3 months after initial discharge. Twelve patients (20%) were readmitted to hospital after 6 months. For the majority of the rehospitalized patients (n = 50; 83%), current hospitalization was the 2nd or 3rd HF hospitalization.

Compared to the patients hospitalized for the first time, the rehospitalized patients were more likely to have AF,

LVEF ≤ 40%, ischemic etiology of HF, history of myocardial infarction, CKD, and 3–8 accompanying diseases (for all, $p < 0.05$). These patients had lower SBP (mean: 133.42 vs 126.24 mm Hg; $p = 0.032$) and diastolic blood pressure (DBP) (mean: 78.82 vs 75.00 mm Hg; $p = 0.04$) on admission, had more frequently at least 1 HF hospitalization in the past year (1.5% vs 40.1%; $p < 0.001$), their hospitalizations were longer (mean: 5.30 vs 7.56 days; $p < 0.001$), they were more frequently hospitalized urgently (77.6% vs 94.6%; $p = 0.001$) and admitted to IM ward (20.9% vs 41.5%; $p = 0.006$).

The characteristics of the studied patients related to rehospitalization and/or death are presented in Table 4. The mortality rate was higher for IM ward patients ($p < 0.001$), both during and after hospitalization (Table 2).

The univariate analysis presents the following significant variables for a higher risk of rehospitalization and/or death: ischemic heart disease, LVEF ≤ 40%, AF, arterial hypertension, valvular disease, CKD, number of comorbidities, MRA therapy, mode of hospitalization, hospitalization in the last year, and hospitalization in the IM ward. The independent predictors of a higher risk of rehospitalization

Table 4. Characteristics of the study population related to rehospitalization and/or death

Variables	No rehospitalization and/or death	Rehospitalization and/or death	p-value
All patients, n [%]	67 (31.3)	147 (68.7)	–
Age [years], mean ±SD	71.6 ±11.81	73.96 ±13.36	0.216
LVEF ≤ 40%, n [%]	17 (28.8)	62 (56.9)	0.001
HR on admission [bpm], mean ±SD	79.3 ±17.68	84.29 ±20.42	0.086
HR at discharge [bpm], mean ±SD	70.27 ±9.61	72.64 ±10.64	0.121
Comorbidities			
Number of comorbidities: 0–3, n [%]	54 (80.6)	81 (55.1)	0.001
Number of comorbidities: 3–8, n [%]	13 (19.4)	66 (44.9)	0.001
Coronary artery disease, n [%]	21 (31.3)	79 (53.7)	0.004
History of myocardial infarction, n [%]	8 (11.9)	46 (31.3)	0.004
AF, n [%]	25 (37.3)	84 (57.1)	0.011
DM, n [%]	24 (35.8)	66 (44.9)	0.272
Arterial hypertension, n [%]	57 (85.1)	105 (71.4)	0.047
CKD, n [%]	6 (9.0)	42 (28.6)	0.003
COPD, n [%]	5 (7.5)	25 (17.0)	0.098
Cancer, n [%]	5 (7.5)	10 (6.8)	1.00
Etiology of HF			
Ischemic heart disese, n [%]	8 (11.9)	59 (40.1)	<0.001
Hypertension, n [%]	46 (68.7)	59 (40.1)	<0.001
Valvular disease, n [%]	25 (37.3)	58 (39.5)	0.883
Congestive cardiomyopathy, n [%]	2 (3.0)	14 (9.5)	0.160
Other[a], n [%]	8 (11.9)	10 (6.8)	0.322
Current hospitalization			
Urgent hospitalization, n [%]	52 (77.6)	139 (94.6)	0.001
Length of hospitalization [days], mean ±SD	5.3 ±2.81	7.56 ±4.17	0.001
Hospitalization in the last year, n [%]	1 (1.5)	59 (40.1)	<0.001
Pharmacotherapy			
ACEI, n [%]	42 (62.7)	87 (59.2)	0.738
ARB, n [%]	15 (22.4)	17 (11.6)	0.064
β-blockers, n [%]	61 (91.0)	118 (80.3)	0.076
MRA, n [%]	36 (53.7)	99 (67.3)	0.078
Ivabradine, n [%]	2 (3.0)	7 (4.8)	0.815
Diuretics, n [%]	53 (79.1)	119 (81.0)	0.886

Data is presented as mean ± standard deviation (SD) or number (percentage). A p-value <0.05 is considered statistically significant. [a] toxic cardiomyopathy, tachyarrhytmic cardiomyopathy; ACEI – angiotensin-converting-enzyme inhibitors; AF – atrial fibrillation; ARB – angiotensin receptor blockers; CKD – chronic kidney disease; COPD – chronic obstructive pulmonary disease; DM – diabetes mellitus; HF – heart failure; HR – heart rate; LVEF – left ventricle ejection fraction; MRA – mineralocorticoid antagonism.

Table 5. Analysis of standard heart failure (HF) pharmacotherapy

Variables	Yes	<50% of target dose	≥50% of target dose	Target dose
ACEI, n [%]	129 (60.3)	67 (51.9)	37 (28.7)	25 (19.4)
ARB, n [%]	32 (15.1)	12 (37.5)	19 (59.4)	1 (3.1)
β-blockers, n [%]	179 (83.6)	87 (48.6)	74 (41.3)	18 (10.1)
MRA, n [%]	135 (63.1)	4 (3.0)	32 (23.7)	99 (73.3)

A p-value <0.05 is considered statistically significant. ACEI – angiotensin-converting-enzyme inhibitors; ARB – angiotensin receptor blockers; MRA – mineralocorticoid antagonism.

and/or death in the multivariate regression model are presented in Table 3.

At discharge, over 90% of cardiac ward patients and only 60% of IM ward patients were referred to outpatient cardiac clinics ($p < 0.001$). All LBBB patients had a cardiologist's consultation during the 3-month follow-up after the HF hospitalization, regardless of the hospital department (IM vs cardiac). Slightly over a half of cardiac ward patients (56.9%) and none of IM ward patients had their 1st ambulatory appointment in a period shorter than 3 months post-discharge (Table 2).

The majority of the hospitalized patients were under the optimal medical treatment of HF (ACEI/ARB, β-blockers, MRA). More than 80% of all hospitalized patients and more than 90% (n = 101) of AF patients were taking β-blockers. The percentage of patients receiving ACEI or ARB reached more than 75% (Table 5). However, cardiac ward patients more frequently than IM ward patients received β-blockers, diuretics, ARB, and MRA (Table 2). None of IM ward patients, and only 4 patients in the cardiac ward, received ivabradine (Table 2).

Despite the fact that β-blockers were prescribed most often, in the majority of cases, they were prescribed in <50% of the target dose. Only 10% of all studied patients received the target dose of β-blockers for HF. The population of AF patients, in the majority of cases (53.5%; n = 54), also received β-blockers in 50% or <50% of the target dose. The others (33.6%; n = 34) received β-blockers in >50% of the target dose and only 12.87% (n = 13) of AF patients were under the target dose of β-blockers. Otherwise, about half of AF patients (51.4%; n = 56) had therapy with digoxin as concomitant therapy.

Similarly, only 20% of patients received the ACEI target dose and less than 5% received the ARB target dose. In the studied population, only in MRA treatment, over 70% of patients reached the recommended target dose (Table 5). However, cardiac ward patients more frequently than IM ward patients received diuretics in the target dose (30% vs 7%; $p < 0.001$).

Discussion

The presented analysis is to our best knowledge the first analysis of Polish HF patients distinguishing between admission wards. No other project in Poland compared the in-hospital and post-discharge management of HF patients in relation to hospital admission wards – cardiac vs IM. This analysis showed differences in guideline implementation between the 2 studied hospital wards.

The HF population from our study is similar to the general Polish HF population analyzed in 2012, in terms of the mode of admission and median length of hospitalization.[13] However, in the 2012 report, 75% of HF patients were hospitalized in IM wards and 22% in cardiac wards.[13] In our study, these proportions are different, which is related to the high specialization of the cardiac department in HF. Similarly to the 2012 HF analysis, we found longer hospitalization in the IM ward than in the cardiac ward (median of 7 and 6 days in the 2012 report vs 7.56 and 5.30 days in our study, respectively). Also, similarly to the 2012 analysis, the majority of HF patients were admitted due to acute decompensation of HF (83% of all HF hospitalizations in 2012 vs 89% in the studied population).[13] Most of the studied patients (n = 154; 72%) were hospitalized due to HF for the first time. For 61 patients (39.6%), it was hospitalization due to acute HF de novo. Additionally, 8 patients (5.2%) with acute HF de novo had also LVEF ≤35% during current hospitalization. These patients, after 3 months of optimal medical treatment, should be reevaluated by a cardiologist, taking into account the ECG analysis and echocardiography measurement of LVEF, before the final decision on electrotherapy.

From the HF exacerbation subgroup, 54 patients also had LVEF ≤35% during current hospitalization. Taking into account 3 months of optimal pharmacological therapy for HF, the ECG analysis and ongoing clinical indications, the patients should be potentially qualified for electrotherapy. Additionally, in 17.9% of the hospitalized HF patients (n = 38), LBBB with QRS duration >120 ms was registered in 12-lead ECG. About half of patients with LBBB (n = 20; 52.6%) had reduced EF (LVEF ≤35%). This is an important group of HF patients in terms of qualifying for electrotherapy. According to the valid guidelines in the period of our study (the 2013 ESC guidelines on cardiac pacing and cardiac resynchronization therapy), a cardiac resynchronization therapy and defibrillator (CRT-D) should be considered in patients with LBBB with QRS duration of 120–150 ms (class of recommendation 1, level of evidence B), or >150 ms (class of recommendation 1, level of evidence A), with chronic HF and LVEF ≤ 35%, who

remain in NYHA functional class II, III and ambulatory IV despite 3 months of optimal medical treatment.[1,18]

In 2012, the overall death rate for HF patients in Poland was 11%.[13] In our short-term analysis of hospitalization and the 3-month period after discharge, the overall death rate was 11.2%. The mortality rate was higher for IM ward patients ($p < 0.001$), both during and after hospitalization (Table 2).

So far, European and Canadian studies have shown specialty-related differences in the management and prognosis of HF patients.[19–22] In countries such as Italy, Spain and Canada, the admission ward was related to a clear dissimilarity in the process of diagnosis and treatment.[19–22] In these studies, as well as in our analysis, IM ward patients were older, had more co-morbidities and their hospitalizations were longer.

According to the results of our analysis, HF management in Poland, compared to other European countries and Canada, seems to be similar in terms of differences between IM and cardiac departments. In a study from Italy, similar to our population, patients treated by cardiologists were more likely to be prescribed ACEI and β-blockers at discharge than IM ward patients (100% vs 74% and 41% vs 4%, respectively).[20] Patients receiving a follow-up by cardiologists were younger than IM ward patients (median: Canada – 71.7 vs 75.8 years; Italy – 70 vs 79 years, Spain – 72.5 vs 77.4 years, respectively).

Unlike in Poland, in other analyzed countries, there was no statistical significance in terms of in-hospital and post-discharge mortality in relation to the type of admission ward.

Post-discharge care in HF patients with regular ambulatory visits is strongly indicated.[1] Based on the new ESC guidelines, the HF patient should be examined by a general practitioner (GP) within 1 week of discharge and by the hospital cardiology team within 2 weeks of discharge.[1] However, none of the abovementioned European and Canadian studies focused on post-discharge care.[18–21]Another important observation from our study is that cardiac ambulatory care was more often recommended in the case of patients hospitalized in cardiac wards as compared to IM wards (90% vs 60%). In our study, the number of referrals of the cardiac patients to outpatient cardiac care did not differ significantly from the general HF population results in 2012, in contrast to IM patients. Compared to the 2012 analysis, in our study, more of all hospitalized patients were referred to ambulatory cardiac care after discharge (80% in our study vs 70% in the 2012 report).[13] In our study, all LBBB patients had a consultation with a cardiologist during the 3-month follow-up after HF hospitalization, regardless of the hospital department (IM vs cardiac). Compared to the general hospitalized HF population in this study, this was a very good result, because at discharge over 90% of cardiac ward patients and only 60% of IM ward patients were referred to an outpatient cardiac clinic ($p < 0.001$).

Another important issue is that patients hospitalized in the IM ward were more likely to have a worse baseline general condition, e.g., they more often had CKD (although not statistically significantly), had a significantly higher mean age (by almost 10 years) and were more often admitted urgently, e.g., due to infection.

Many variables of significance for cardiac events in HF are described in the literature. One of the most important is older age. In the elderly, the most common cause of hospitalization is HF. In people over 70 years of age, HF affects 1/10 of seniors and is the leading cause of death in this age group.[1,23–25] In seniors over 80 years of age, comorbidities also have an important prognostic value for annual prognosis in chronic HF.[25] In our study, a worse baseline general condition with comorbidities, such as ischemic heart disease, AF and CKD, are independent variables for longer hospitalization and/or mortality.

It should also be emphasized that there are differences in characteristics and diagnostic procedures performed in cardiac and IM wards. In IM departments, only 43% of patients underwent, at the same time, echocardiography with the measurement of LVEF and natriuretic peptides. In our study, in the majority of cardiac ward patients, opposite to IM ward patients, the echocardiography and NT-proBNP measurement were performed. It is worth noticing that biomarkers are crucial for the diagnosis and professional management of HF.[26] In other European studies, patients managed by cardiologists were also more likely than IM wards patients to undergo echocardiography (Italy: 89–92% vs 37–54.8%).[20,21]

Compared to our findings, in EURObservational Research Programme The Heart Failure Pilot Survey (ESC HF-Pilot), BNP/NT-proBNP measurements as well as echocardiography were performed much more frequently in patients hospitalized in our cardiac ward (96% vs 36.6% and 98% vs 75%, respectively). The results were different in the IM ward (43% vs 36.6%, and 43% vs 75%, respectively).[9] The studied HF population was burdened with comorbidities similar to those of the individuals from ESC-HF Pilot, most frequently coronary artery disease, hypertension, CKD, and DM.[27,28]

Although the ESC-HF Pilot population (n = 5118) differed significantly from our HF population in terms of the admission mode to a cardiac ward (for acute HF – 37% vs 83.5% and for chronic HF – 63% vs 16.5%, respectively), the median length of stay in a cardiac ward in ESC-HF Pilot was higher than in our study (8 vs 5 days, respectively).[27] It should be highlighted that ESC-HF Pilot included only patients hospitalized in cardiac departments. ESC-HF Pilot population was treated mainly with ACEI and β-blockers (80%). However, target doses were reached in 1/3–1/4 of the ESC-HF Pilot patients only. Even lower results of the target dose achievement were observed in our study (19.4% for ACEI and 10% for β-blockers).

The Polish population from ESC-HF Pilot was younger compared to our study (66 ±13.7 vs 72.2 ±12.9 years,

respectively).[29] Angiography confirmed that coronary artery disease was the main etiology of HF in Polish patients from the registry (39%). The analysis of data from our study showed that, as proven in the ESC-HF Pilot study, ischemic etiology of HF was very common among cardiac ward patients (51%).

The 3-month death rate for ESC-HF Pilot Polish patients was estimated at about 2.5%, while it was 3% in other European countries. Meanwhile, it is noteworthy that, in our study, the death rate was at a lower level than in the ESC-HF Pilot study – 2.9% in-hospital and 1.4% post-discharge.

A recently published global survey shows the implementation of guideline-recommended HF treatment.[30] In the QUALIFY survey, as well as in the cardiac HF population from our study, the majority of patients were treated with ACEI (65.7% vs 62.6%), β-blockers (86.7% vs 89.2%) and diuretics (83% vs 86.3%). Ivabradine has proven its efficacy in reducing hospitalizations for 26% in the SHIFT study.[31] In our analysis, it was observed that cardiologists prescribed this drug at discharge to a greater extent than internists, but still with a very low frequency (26.7% vs 0, respectively). In the QUALIFY survey, the number of patients treated with ivabradine was above 33.4%.[30]

The optimal HF therapy at discharge in our study and in the QUALIFY survey was poor. However, target doses were at a better rate in the QUALIFY survey than in the studied population – 27.9% vs 19.4% for ACEI and 14.8% vs 10.1% for β-blockers.[30]

Compared to the QUALIFY registry, the frequency of use of implantable devices, such as ICD or CRT, was also poor in both studies, but at a better rate in QUALIFY survey than in our study – 9.7%.[30]

A crucial observation from our study is that HF hospitalization in the previous 12 months was the most important risk factor for subsequent hospitalizations. The results of the QUALIFY survey showed that 30.4% of patients had a history of 2 or more HF hospitalizations.[30] In the ESC-HF Pilot study, 57% of HF population had a history of previous hospitalizations and, additionally, 24.75% of patients were rehospitalized in a 1-year follow-up.[27,29]

This study has some limitations that have to be acknowledged. Firstly, it should be noted that it was a retrospective analysis. Inclusion criteria were based only on diagnostic code I50 in the ICD-10 classification and on the data available in the medical system CliniNET®, not on medical assessment.

Other limitations are that the study included only a 3-month period of follow-up and analyzed the adherence score to guideline recommendations for only standard HF treatment according to the ESC recommendations (3 groups of drugs: ACE/ARB, β-blockers and MRA), and additional ivabradine and diuretics without digoxin or nitrates.

Moreover, we made a hospital ward- and endpoint-related statistical analysis, but it was carried out with the EF value (HFrEF vs HFpEF).

Despite the advances in clinical practice, as documented in our analysis and previous publications, there is still a place for improvement in terms of the diagnosis of HF, determination of prognosis and treatment selection.

In conclusion, the single-center study has shown that the management of HF patients differs significantly depending on the admission ward. The differences include diagnostic procedures, hospitalization, treatment, ambulatory care, and prognosis, showing the advantages of cardiac wards. The presented results of the journey of HF patients indicate the need for improvement in the field of HF care.

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The importance of fetuin-A in vascular calcification in children with chronic kidney disease

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Abstract

Background. The status of the cardiovascular (CV) system in children with chronic kidney disease (CKD) is significantly influenced by increasing stiffness of the arterial wall. This largely depends on the shortage of local and systemic inhibitors of soft tissue calcification.

Objectives. The aim of the study was to evaluate the role of fetuin-A in conjunction with other factors in the progressive hardening of the vascular wall in these children. We examined serum fetuin-A concentrations in relation to renal function, dialysis modality, and other clinical and biochemical markers promoting vascular calcification.

Material and methods. Twenty children on peritoneal dialysis (PD), 20 on hemodialysis (HD), 36 treated conservatively, and 26 healthy subjects were enrolled into a cross-sectional study. In all children, fetuin-A and numerous clinical and biochemical parameters were measured.

Results. The fetuin-A concentration was significantly lower in children on hemodialysis (HD) vs children on peritoneal dialysis (PD), conservatively treated subjects, and the control group. In sick children, fetuin-A concentration negatively correlated with dialysis vintage, PWV/ht, phosphate concentration, calcium phosphate product (CaxP), cumulative doses of calcium, and vitamin D_3. In the whole study population, fetuin-A negatively correlated with blood pressure (BP), pulse wave velocity indexed to height (PWV/ht), intact parathyroid hormone (iPTH), high sensitivity C-reactive protein (hsCRP), and cholesterol concentrations.

Conclusions. In children with CKD, the decreased concentration of fetuin-A is related to other vascular calcification risk factors. Serum fetuin-A concentration may play a role in the identification of vascular disease risk factors in this population.

Key words: children, chronic kidney disease, fetuin-A

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Introduction

The status of the cardiovascular (CV) system in children with chronic kidney disease (CKD) depends largely on the growth in vessel wall stiffness (atherosclerosis). Mönckeberg-type medial calcification leads to an exacerbation of arterial stiffness, resulting in a rise in systolic blood pressure (SBP) and a reduction in diastolic blood pressure (DBP), as well as in an increase in pulse pressure value (PPV), which has been recognized as an independent risk factor for death from CV causes.[1] In the course of progressive stiffening of the vessel wall, left ventricular hypertrophy, impairment of coronary blood flow and calcification of the heart valves are observed. All of these disorders lead to increased risk of myocardial infarction, arrhythmia and heart failure, and consequently, to sudden death.[2,3] In patients with CKD, additional risk factors are disturbed calcium metabolism, phosphate (P), high parathyroid hormone (PTH) level, elevated calcium phosphate product (CaxP), the number of episodes of hypercalcemia, and doses of calcium phosphate binders.[4,5] The promoting factors of the formation of calcifications are long-lasting hypercalcemia and hyperphosphatemia, but the severity of changes largely depends on genetic predisposition. Block et al. reported on the negative correlation between the degree of calcification of the vascular wall and bone turnover.[6] Similarly, other authors have shown that patients on dialysis who have had low levels of intact PTH (iPTH) and low alkaline phosphatase activity, the degree of calcification increases faster than in patients with high levels of iPTH.[4] In light of recent reports, it is considered that the calcification of blood vessels is an active process that is initiated by hyperphosphatemia and is associated with deficient endogenous inhibitors of calcification.[7,8] The basic structure responsible for this process is vascular smooth muscle cells (VSMC). Chronic kidney disease patients, especially patients on chronic dialysis, are deficient in systemic and local soft tissue calcification inhibitors.

The greatest interest of researchers is currently focused on fetuin-A (α2-Heremans-Schmid-glycoprotein), the main systemic inhibitor of calcification in soft tissues. Synthesized in the liver, it reaches a high concentration in extracellular fluid, including plasma (above 1 g/L). Fetuin-A shows a negative relationship with inflammatory markers. The results of experimental studies demonstrate its important role in the inhibition of vascular wall calcification. It has been shown that mice lacking the gene for fetuin-A have severe metastatic calcifications of the soft tissues.[9] The action of fetuin-A is performed through the binding of small calcium phosphate crystals, thus inhibiting their growth and deposition in tissues. It was found that in the presence of fetuin-A, a soluble complex called calcyprotein is formed, comprised of 8 molecules of fetuin-A (80% of the complex by weight), 1 molecule of matrix Gla protein (MGP) (2% by weight), 790 atoms of calcium, and 580 molecules of phosphate (18% by weight).[10] The fetuin-A is able to inhibit the activity of TGF-β and macrophages, and thereby to reduce the release of proinflammatory cytokines.[11] In the presence of calcium, it has an affinity for proteins located on the cell surface – annexin II and VI. Fetuin-A binds to them and enters the endosomes of smooth muscle cells, thus inhibiting their apoptosis. The fetuin-A entering into the matrix vesicles and apoptotic bodies prevents soft tissue mineralization. It has also been found that fetuin-A accelerates the phagocytosis of apoptotic bodies and inhibits VSMC function and bone morphogenetic protein 2 function.[12,13] Many investigators have shown that in patients with CKD, especially stage 5, fetuin-A is significantly lower than in healthy individuals, which can affect the increased mortality from CV disease in these patients.[14–17] Goodman et al. have studied this aspect of young adults (20–30 years of age) undergoing chronic hemodialysis (HD). Almost all patients (88%) presented calcifications in the coronary arteries, especially those who started dialysis early in childhood.[4] There have been only single reports of vascular calcification in children and adolescents with CKD.[14,18–21]

The aim of the study was to assess serum fetuin-A concentrations in children with CKD in relation to renal function, dialysis modality, and other clinical and biochemical markers promoting vascular calcification.

Material and methods

Seventy-six children with CKD, still asymptomatic of CV complications, were enrolled into the study. The patients were divided into 3 groups according to CKD stage and dialysis modality. Twenty-six age-matched children with primary nocturnal enuresis and normal kidney function served as controls. Clinical and demographic details of the study subjects are summarized in Table 1. The 1st group included 20 children on peritoneal dialysis (PD). Nocturnal intermittent peritoneal dialysis (NIPD) was performed in 12 patients and continuous cyclic peritoneal dialysis (CCPD) was administered in 8 patients. A Home-Choice machine was used (Baxter International, Inc., Deerfield, USA). Standard dialysis solutions with glucose concentrations of 1.5% and 2.3% and calcium concentrations of 1.25 mmol/L or 1.75 mmol/L were applied according to individual recommendations. The causes of CKD in children from the 1st group (PD group) included structural urinary tract abnormalities (n = 9), glomerulonephritis (n = 5), polycystic kidney disease (n = 3), hereditary nephropathy (n = 2), and hemolytic uremic syndrome (n = 1). Fifteen children remained on hypotensive medication, including angiotensin-converting enzyme inhibitors (ACEI) (n = 13), calcium channel blockers (n = 6) and β-blockers (n = 3). All of the subjects received calcium-containing phosphate binders and vitamin D metabolites in doses adjusted to their requirements.

The 2nd group (HD group) consisted of 20 children on maintenance HD. Dialysis sessions were performed

Table 1. Clinical characteristics of the chronic kidney disease (CKD) groups and the controls (two-by-two comparison, p-values are shown)

Group parameter	PD n = 20	HD n = 20	Pre n = 36	Controls n = 26	p-value
Age [year]	14.3 ±2.3	15 ±3.3	14.9 ±3.5	14.5 ±3.3	NS
Gender [male/female]	12/8	10/10	17/19	12/14	NS
Dialysis vintage [months]	12 ±11	19 ±16	–	–	p = 0.02
BMI [kg/m^2]	18.7 ±3.9	18.7 ±3.4	20.6 ±4.1	18.8 ±3.9	NS
SBP [mm Hg]	117 ±11[1,3]	128 ±13[1,2,4]	115 ±10[1]	100 ±9	p < 0.0001
DBP [mm Hg]	75 ±11[1,3]	82 ±10[1,2,4]	71 ±8[1]	64 ±6	p < 0.0001
PP [mm Hg]	42 ±9[1]	46 ±9[1]	44 ±8[1]	35 ±7	p < 0.0001
MBP [mm Hg]	86 ±12[1,3]	97 ±10[1,2,4]	86 ±7[1,3]	76 ±6	p < 0.0001
Cumulative dose of calcium [g/kg]	29.7 ±45.9	46.6 ±45.8[4]	9.67 ±12.5[3]	–	p = 0.0002
Cumulative dose of vitamin D_3 [µg/kg]	3.71 ±3.7	4.76 ±3.37[4]	2.02 ±4.41[3]	–	p = 0.0005
PWV/ht [m/s]	5.51 ±0.68[1,3]	6.07 ±0.86[1,2,4]	5.53 ±0.69[1,3]	4.85 ±0.62	p < 0.0001

PD – peritoneal dialysis; HD – hemodialysis; Pre – predialysis; BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; PP – pulse pressure; MBP – mean blood pressure; PWV/ht – pulse wave velocity indexed to height; NS – not significant.
[1] vs control group; [2] vs PD group; [3] vs HD group; [4] vs Pre group.

3 times a week (3–5 h) using polysulfone membranes. The blood flow ranged from 120 to 250 mL/min, and dialysate flow did not exceed 500 mL/min. The dialysis fluid was buffered with bicarbonate and the calcium content was 1.25 mmol/L or 1.5 mmol/L. The causes of CKD in this group were urinary tract abnormalities (CAKUT) (n = 12), glomerulonephritis (n = 6) and hereditary glomerulopathy (n = 2). Nineteen hypertensive children were treated with ACEi (n = 19), calcium channel blockers (n = 11) and β-blockers (n = 2). All of the patients received calcium-containing phosphate binders and vitamin D compounds.

The 3rd group (Pre group) included 36 children with CKD (stages 2–4) on conservative treatment. The causes of CKD were urinary tract malformations (n = 22), glomerulonephritis (n = 5), polycystic kidney disease (n = 3), hereditary glomerulopathy (n = 2), unknown cause (n = 2), hemolytic uremic syndrome (n = 1), and complications after chemotherapy for cancer (n = 1). Stage 2 CKD was detected in 13 children, stage 3 in 10 and stage 4 in 13. Twelve subjects were treated with ACE-I, 6 patients with angiotensin receptor blockers (ARB), 4 patients with calcium channel blockers, and 1 child received β-blockers.

All patients with stage 3 or 4 CKD as well as 5 children with stage 2 CKD received treatment with calcium-containing phosphate binders and vitamin D compounds.

Children under the age of 6 years and patients with diabetes or infection were excluded from the study. Informed consent for participation in the study was obtained from all of the parents and from children 15 years old or older. The research project was approved by the Wroclaw Medical University (Poland) ethics committee.

Selected biochemical and functional parameters in the study population were measured in order to assess the degree of vascular stiffness. In all patients and in the control group, the following biochemical parameters were determined: serum creatinine, albumin, acute phase proteins (high sensitivity C-reactive protein (hsCRP)), hemoglobin (Hb), lipid count (total cholesterol (TCL), low-density lipoproteins cholesterol (LDL-cholesterol), high-density lipoproteins cholesterol (HGL-cholesterol), and triglycerides (TGL)), as well as parameters related to calcium P metabolism (calcium, P and iPTH). The serum concentration of fetuin-A was also measured as a principal systemic inhibitor of soft tissue calcification.

As part of the assessment of vascular function, 24-h blood pressure (BP) was monitored with the evaluation of SBP, DBP, mean blood pressure (MBP), pulse pressure (PP), and pulse wave velocity (PWV).

The stage of CKD was diagnosed based on the The National Kidney Foundation Kidney Disease Outcomes Quality (NKFK/DOQI) recommendations and the calculation of glomerular filtration rate (GFR) was performed using the Schwartz formula.[22]

In dialysis patients, the type and duration of renal replacement therapy was taken into account. In each patient, based on the measurement of height and weight, body mass index (BMI) was determined according to this formula: BMI = body weight [kg]/height [m^2].

Blood pressure was measured using a sphygmomanometer or an electronic DIANA device (DINAMAP, Boston, USA) for 24-hour monitoring. Measurements were taken in a seated position with the appropriate cuff selected to cover the width of 1/3 of the length of the arm. Normal BP values were defined as values below the 90th percentile and below the upper BP limit (according to the nomenclature defined in the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents) and prehypertension was defined as values between the 90th and the 95th percentile.[23] For the evaluation of the results, percentile charts relating to gender, age and growth percentile from the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents, Third Report, were used.[24] The percentile growth charts we used

in our study were developed by the Institute of Mother and Child in Warszawa, Poland. Mean blood pressure and PP were calculated according to these formulas: MBP = DBP + [(SBP – DBP)/3] and PP = SBP – DBP. In each patient, the cumulative dose of oral calcium in the gastrointestinal tract, mostly taken in the form of calcium-containing phosphate binders, was calculated. The cumulative dose of the active form of vitamin D_3 which each patient had previously received was also calculated.

Blood samples were collected in the morning, under fasting conditions, while performing other routine tests. In order to determine the concentration of creatinine, albumin, Hb, acute phase protein (hsCRP), TCL, LDL-cholesterol, HDL-cholesterol, TGL, calcium, and phosphate, blood was simultaneously drawn and the serum parameters were assayed on the same day. For fetuin-A, blood was drawn into dry tubes, then centrifuged at 3,000 rpm for 15 min and the serum was frozen at –20°C until assayed. The blood used in iPTH testing was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged for 15 min at 1,000 rpm, within 30 min of sampling. After the separation of plasma from cellular components, the plasma was frozen and stored at –20°C until assayed. Serum creatinine, albumin, calcium, phosphate, lipid, and hsCRP levels, as well as a complete blood count, were assessed in Central Laboratory, University Teaching Hospital in Wrocław, Poland, with standard methods. The iPTH concentration was determined by IRMA using Duo PTH manual kits (Scantibodies Laboratory, Inc., Santee, USA) (standard laboratory normal value: 14–66 pg/mL). The measurement of fetuin-A was performed twice and the mean values were analyzed. Fetuin-A was determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (BioVendor, Brno-Řečkovice a Mokrá Hora, Czech Republic). The PWV study was performed according to the methodology we presented in an earlier work.[25]

Statistical analyses were performed using the STATGRAPHICS package (Centurion XV v. 15.2.06, StatPoint, Inc. Herndon, USA). The results were expressed as mean values ± standard deviation (SD) when a normal distribution of variables was obtained. The differences were then compared by an analysis of variance (ANOVA) test. In the case of non-normal distribution, a nonparametric Kruskal-Wallis test for median values was used. For the evaluation of the relationship between parameters with a normal distribution of variables, Pearson's correlation test was performed. Spearman's test was applied for data with non-normal distribution. Since the p-value in the ANOVA table was less than 0.05, there was a statistically significant relationship between the variables. A p-value of less than 0.05 in other tests was considered statistically significant.

Results

The study groups were not found to differ significantly with respect to age, sex or BMI. The concentration of fetuin-A was the lowest in the HD group and was significantly different from the concentrations of the PD group, the Pre group and the group of healthy subjects. In patients undergoing PD, fetuin-A concentration was significantly lower than in Pre group patients and in the healthy controls. The highest values of this calcification inhibitor were detected in the control group. Detailed data are shown in Table 2.

The highest BP, SBP, DBP, and MBP values were found in the group of children on HD. They varied significantly compared to the group of healthy children, to the PD group and to the Pre group. Pulse pressure was significantly higher in all groups of children with CKD in comparison to healthy peers, regardless of the stage of the disease or the methods of renal replacement therapy. The duration of PD was shorter than the time of chronic HD treatment. Children in the HD group received the highest cumulative dose (per kg of body weight) of both calcium carbonate and active metabolites of vitamin D_3. The difference was statistically significant in comparison to the children in the Pre group. The highest values of PWV/ht were noted in HD patients, and they differed significantly from the results obtained in all other groups. Pulse wave velocity indexed to height (PWV/ht) measurements were also significantly higher in the PD group and in the Pre group than in the control group. Detailed data are presented in Table 1.

Significantly lower albumin concentration and higher total cholesterol and triglyceride levels were observed in PD and Pre groups than in healthy controls. The highest concentration of LDL-cholesterol and total cholesterol, found in the PD group, differed significantly from the values recorded in other groups. In all CKD groups, Hb levels were lower than in the healthy subjects. The most pronounced decrease in Hb concentration was observed in the HD group. The lowest concentration of calcium and the highest of phosphate were found in the HD group. These values were significantly different from the other groups.

Table 2. Fetuin-A concentration in the study groups (two-by-two comparison, p-values are shown)

Group parameter	PD n = 20	HD n = 20	Pre n = 36	Control n = 26	p-value
Fetuin-A [ng/mL]	56.67 ±0.55[1,3,4]	40.73 ±0.96[1,2,4]	63.38 ±0.88[1,2,3]	98.56 ±0.95	p < 0.0001

PD – peritoneal dialysis; HD – hemodialysis; Pre – predialysis.
[1] vs control group; [2] vs PD group; [3] vs HD group; [4] vs Pre group.

Table 3. Biochemical characteristics of the chronic kidney disease (CKD) groups and the controls (two-by-two comparison, p-values are shown)

Group parameter	PD n = 20	HD n = 20	Pre n = 36	Controls n = 26	p-value
Hb [g/dL]	11.14 ±1.23[1]	10.19 ±1.34[1,4]	11.99 ±2.07[1,3]	13.3 ±1.19	p < 0.0001
Albumin [g/dL]	3.9 ±0.34[1]	4.0 ±0.35	3.9 ±0.68[1]	4.3 ±0.44	p = 0.009
Total cholesterol [mg/dL]	206 ±43[1]	174 ±33	203 ±100[1]	162 ±13	p = 0.0001
LDL-cholesterol [mg/dL]	143 ±44[1,3,4]	92 ±31[2]	105 ±69[2]	84 ±6.2	p < 0.0001
HDL-cholesterol [mg/dL]	52 ±15	46 ±9	50 ±10	49 ±4	NS
TGL [mg/dL]	179 ±75[1]	166 ±59	187 ±242[1]	86 ±6.5	p < 0.0001
Calcium [mg/dL]	9.71 ±0.79[3]	9.23 ±0.56[1,2,4]	9.69 ±0.63[1,3]	9.99 ±0.34	p = 0.0002
P [mg/dL]	5.48 ±1.81[1,3,4]	6.32 ±1.17[1,2,4]	4.52 ±0.97[2,3]	4.45 ±0.54	p < 0.0001
Ca × P [mg^2/dL^2]	53.5 ±19.4[1,4]	58.1 ±10[1,4]	43.6 ±8.9[2,3]	44.3 ±5.3	p < 0.0001
iPTH [pg/mL]	206 ±177[1,3]	444 ±524[1,2,4]	138 ±135[3]	30.7 ±4	p < 0.0001
hsCRP [mg/L]	2.97 ±0.45[3]	3.64 ±0.98[1,2]	3.3 ±0.65	2.5 ±0.15	p < 0.0001
Creatinine [mg/dL]	6.37 ±2.6[1,4]	7.6 ±3.4[1,4]	2.5 ±1.6[1,2,3]	0.68 ±0.11	p < 0.0001

PD – peritoneal dialysis; HD – hemodialysis; Pre – predialysis; TCL – total cholesterol; LDL-cholesterol – low-density lipoproteins cholesterol; HDL-cholesterol – high-density lipoproteins cholesterol; TGL – triglycerides; P – phosphate; CaxP – calcium phosphate product; iPTH – intact parathormone; hsCRP – high sensitivity C-reactive protein; NS – not significant.
[1] vs control group; [2] vs PD group; [3] vs HD group; [4] vs Pre group.

Table 4. Analyses of factors correlated with fetuin-A in CKD patients (PD + HD + Pre) (Pearson correlation test)

Variable	r	p-value
Fetuin-A dialysis vintage	r = –0.34	p = 0.002
P	r = –0.23	p = 0.03
CaxP	r = –0.27	p = 0.01
Cumulative dose of calcium	r = –0.22	p = 0.04
Cumulative dose of vitamin D_3	r = –0.26	p = 0.02
PWV/ht	r = –0.47	p < 0.0001

P – phosphate; CaxP – calcium phosphate product; PWV/ht – pulse wave velocity indexed to height; r – correlation coefficient.

Table 5. Analyses of factors correlated with fetuin-A in the whole study population (Pearson correlation test)

Variable	r	p-value
Fetuin-A SBP	r = –0.53	p < 0.0001
DBP	r = –0.44	p < 0.0001
PP	r = –0.33	p = 0.0006
MBP	r = –0.44	p < 0.0001
P	r = –0.32	p = 0.0009
Ca	r = 0.24	p = 0.01
CaxP	r = –0.28	p = 0.003
iPTH	r = –0.31	p = 0.001
Cumulative dose of calcium	r = –0.35	p = 0.0002
Cumulative dose of vitamin D_3	r = –0.41	p < 0.0001
Total cholesterol	r = –0.21	p = 0.03
LDL-cholesterol	r = –0.22	p = 0.02
TGL	r = –0.23	p = 0.01
hsCRP	r = –0.32	p = 0.0009
PWV/ht	r = –0.39	p < 0.0001

SBP – systolic blood pressure; DBP – diastolic blood pressure; PP – pulse pressure; MBP – mean blood pressure; P – phosphate; CaxP – calcium phosphate product; iPTH – intact parathormone; LDL – low-density lipoproteins cholesterol; TGL – triglycerides; hsCRP – high sensitivity C-reactive protein; PWV/ht - pulse wave velocity indexed to height.

The values of CaxP in children with CKD on dialysis treatment were significantly higher than in the control group and in patients treated conservatively (Pre group), but did not differ regarding dialysis modality. The highest values of iPTH levels were found in the HD group; they differed significantly in relation to other groups. The CRP concentration was also highest in the HD group and was significantly different from those observed in the control group and the PD group. There were no significant differences in CRP levels between the Pre group and the control group or the PD group. Detailed data is shown in Table 3. In the total population of children with CKD (PD + HD + Pre), we found statistically significant negative linear correlations of fetuin-A levels with PWV/ht, dialysis duration, P, CaxP, and the cumulative doses of calcium and vitamin D_3. Detailed data is presented in Table 4.

In the entire study population, we observed similar correlations regarding fetuin-A among all patients. In addition in the whole analyzed population, we found a negative linear correlation between fetuin-A and blood pressure values (SBP, DBP, PP, and MBP), iPTH, hsCRP, total cholesterol, LDL-cholesterol, and TGL. Detailed data is presented in Table 5. In the control group, there were no statistically significant correlations with fetuin-A. Assessing the impact of a single HD session on serum fetuin-A concentration, a significant increase in the concentration of fetuin-A after HD was found. Detailed data is presented in Table 6.

Table 6. The impact of a single hemodialysis (HD) session on serum fetuin-A concentration

Serum	Before HD	After HD	p-value
Fetuin-A [ng/mL]	40.73 ±0.96	52.41 ±0.58	p < 0.0001

Discussion

The complex pathogenesis of vascular injury in the course of CKD also involves factors that promote and inhibit calcification of soft tissues. While the influence of stimulating factors such as calcium, P, PTH, vitamin D_3, and calcium phosphate-binding formulas on this process is well-known, less has been documented about the role of calcification inhibitors. Fetuin-A, which creates about 50% of the total plasma pool of calcification-inhibiting compounds, has been the subject of interest in patients with CKD over the past few years. In this study, the concentration of fetuin-A was significantly lower in all examined children with CKD compared to the control group, but the biggest decrease was observed in HD group. This is consistent with the vast number of reports on the subject in adults.[15,16,26,27] The explanation for the reduced levels of fetuin-A may be the status of chronic inflammation, which in patients with end-stage renal failure is the most severe causative factor.

The influence of inflammation on the concentration of serum fetuin-A is supported by the research of Dervisoglu et al., who showed a negative correlation between fetuin-A and the concentration of proinflammatory cytokines.[28] In a group of adults on HD, a relationship was also found between low levels of fetuin-A and higher mortality from CV causes. On the other hand, Shroff et al. presented different results in children: an increase in circulating levels of fetuin-A in children undergoing dialysis compared to healthy peers.[29] Lower concentrations of fetuin-A were observed only in patients with heart valve and coronary artery calcifications. But even in these cases, concentrations of fetuin-A were higher than those observed in the control group. Nevertheless, it should be noted that the average time on dialysis was nearly half as long in their study as in our population, which is probably not without significance.

Additionally, a negative correlation between serum fetuin-A and dialysis duration has also been demonstrated by other authors. According to Shroff et al., elevated levels of fetuin-A in children undergoing dialysis could be regarded as an initial phase of a systemic response to proinflammatory and hypercalcemic agents. The authors suggest that a prolonged stimulation of such factors can reduce the compensation mechanisms of the body and can lead to a reduction in the concentration of natural inhibitors of calcification.[29] On the other hand, Ziółkowska et al. examined 28 children on dialysis (either on HD or PD) and 43 healthy children, and found no significant difference in the concentration of fetuin-A between the 2 groups.[30]

The results of research on the levels of fetuin-A in PD patients are also ambiguous: different authors found both higher levels and the same levels in comparison to adult patients on HD.[31,32] In our study population, we demonstrated significantly lower concentrations of this calcification inhibitor in children on PD than in patients treated conservatively. However, compared to children on HD, the values were significantly higher. This can be explained not only by the shorter dialysis duration, but also by the less severe disorders of calcium-phosphate metabolism compared to children on HD. The negative correlation between the concentration of phosphate, CaxP, iPTH, and fetuin-A in the group of all patients with CKD seems to confirm this hypothesis. In children treated conservatively, fetuin-A level was the highest, although it was still lower than in the control group. These results do not support the observations made by Schaible et al., who did not show such differences.[14] In one of the few studies on pediatric kidney transplantation, Van Summerenet al.[32] found a significant decrease in the concentration of fetuin-A, in contrast to the observations of Schaible et al.[14] It should be emphasized that the reports on predialysis patients and PD are limited and include heterogeneous groups regarding the amount of procalcemic factors, chronic inflammation and comorbid conditions, which partly explains these differences. Also, one cannot rule out genetic factors which, as shown by studies of the German authors, may individually affect the synthesis of these inhibitors.[33] Also, it is important that fetuin-A is not only an inhibitor of calcification, but that it also plays other roles within the body. It acts as a negative acute phase protein which inhibits the overproduction of proinflammatory cytokines and modulates the activity of the insulin receptor, affecting insulin resistance.[34]

Regardless of these discrepancies, there is much evidence that fetuin-A plays a protective role against vascular calcification and increasing vascular stiffness. This is confirmed by both studies in animal models and by clinical data in patients with CKD, showing a negative correlation between fetuin-A concentration and the presence of calcifications in the coronary arteries, carotid artery intima-media thickness, and pulse wave acceleration.[35–40] Fetuin-A proved to also be an independent predictor of stiffness and vascular calcification in children after renal transplantation.[33] In our study, we demonstrated a significant negative correlation between serum fetuin-A and the values of PWV/ht, BP, calcium, and lipid metabolism. Ziółkowska et al. studied the influence of many factors responsible for the progression of blood vessel calcification – including indices of calcium and P metabolism, degree of bone turnover, and lipids in children at different stages of CKD, with and without vascular changes – and revealed that only fetuin-A was the differentiating factor.[30]

Our study has several limitations. Firstly, the sample size, particularly subgroups, may be insufficient to show

significant differences. Our patients were recruited from 3 of the pediatric nephrology centers which treat children from the whole southern region of Poland. Further studies with larger sample sizes are necessary, especially in light of new therapeutic methods which enable the lowering of vascular stiffness in CKD pediatric patients.

It can be concluded that the reduced serum fetuin-A concentration which is particularly pronounced in dialyzed children and its relationship with other progressive arterial stiffness risk factors indicate a significant role of this calcification inhibitor in the development of CV complications in children with CKD.

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Correlations between the expression of hTERT and α and β splice variants in human brain tumors

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Abstract

Background. Astrocytomas are diffusible infiltrative and aggressive brain tumors that are extensive and heterogeneous clusters of neoplastic growths in the central nervous system (CNS). Meningioma tumors are commonly benign but may demonstrate an invasive pattern with frequent recurrences. Human telomerase reverse transcriptase (hTERT) is an unfavorable prognostic factor for several types of cancers, and there are controversies about its role.

Objectives. In the present study, we investigated the relative expression of hTERT splice variants in 2 groups of brain tumors compared to non-tumor samples.

Material and methods. The mRNA of 40 brain tumor samples and 4 control samples was extracted; mRNA expression of hTERT α-deletion and β-deletion variants, as well as the wild type isoform, was quantified using quantitative reverse transcription polymerase chain reaction (RT-qPCR).

Results. The α-deletion variant was significantly expressed in primary benign meningeal tumors ($p = 0.01$). The results indicate a positive correlation between the relative expression of hTERT mRNA transcript and α-deletion and β-deletion variants in both groups of tumors (meningiomas and astrocytomas). A strong association between the expression of the full-length splice variant and the β-deletion variant was observed in astrocytoma tumors ($p = 0.045$). The most significant correlations were found between the hTERT full-length and β-deletion variants in high-grade meningiomas ($p = 0.018$, correlation coefficient (CC) $= 0.964$) and grade II astrocytomas ($p = 0.015$; CC $= 0.580$). In addition, in low grades of both types of tumors, the hTERT full-length variant and especially the α-deletion variant were the predominant isoforms. The over-expression of hTERT and β-deletion variants in high grades of these tumors was statistically significant. Our findings indicate that α-deletion and β-deletion isoforms are associated with high levels of full-length hTERT mRNA in both groups of brain tumor patients.

Conclusions. Changes in the splicing pattern of hTERT splice variants in brain tumors and their correlation with pathological alterations in cells could be applied as diagnostic or prognostic biomarkers, or possibly as targets for cancer therapy. However, the function and biological role of hTERT splice variants remain to be clarified.

Key words: meningioma, astrocytoma, human telomerase reverse transcriptase, α-deletion splice variant, β-deletion splice variant

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Astrocytomas are aggressive brain tumors that are lethal in their malignant form. Most of them show high rates of invasion that lead to recurrences of the disease.[1] The World Health Organization (WHO) assigned 4 grades of astrocytoma in 2007, but the recent update of the WHO classification uses both histology and molecular genetic alteration to define central nervous system (CNS) tumor diagnoses.[2–4]

Meningiomas are commonly benign, slow-growing extra-axial tumors that originate from arachnoid cells.[5] This type of neoplasia comprises 34% of primary brain and CNS tumors. In the 2007 WHO classification of tumors of the CNS, the 3 grades of meningioma are defined by histologic criteria predicting the risk of recurrence.[6] The 2016 update of the WHO classification made notable changes, including the addition of brain invasion as a criterion for atypical meningiomas.[4] Malignant meningiomas frequently relapse after a short time, regardless of total resection. The molecular basis of this variation is unknown, but gene expression variations may predict patient outcomes more accurately than pathological measurements. It would be beneficial to identify prognostic markers for these tumors.[7]

The ends of eukaryotic chromosomes are made of tandem-repeated short sequences associated with specific proteins, called telomeres. The length of telomeres is gradually shortened with each cell division.[8] Telomere shortening can be used as a biological clock that represents the progression of a cell to the end of its replicative lifespan. Most of the cell division stops when the cells reach the threshold of senescence.[9] Experimental data demonstrates that cells that escape replicative senescence have adopted a strategy to counteract the loss of telomeric repeats.[10] Immortal cells have solved the problem of truncated telomeres using telomerase.[11] Telomerase is a ribonucleoprotein enzyme complex that synthesizes the telomeric sequence (TTAGGG) at chromosomal ends, compensating for the progressive loss of DNA that occurs during replication.[12] This enzyme is composed of 2 core subunits of an RNA (hTR) as a template for the synthesis of new telomeric repeat sequences and a catalytic subunit with reverse transcriptase activity (human telomerase reverse transcriptase [hTERT]).[13]

Telomere shortening and telomerase activity have been found to be strong markers of cellular malignancy in most human tumors, including brain tumors.[14–16] Reactivation of telomerase seems to be related to unlimited cell proliferation and cancer progression.[17] The enzymatic activity of telomerase is subject to strict regulation.[18] The transcriptional and post-transcriptional regulation of hTERT is complicated and is not fully understood.[19] Its reactivation in immortalized cells is associated with the increased growth potential needed for malignancy.[20] High hTERT expression levels directly correlate with poor clinical outcomes in most cancer types.[21] Studies on hTERT deletion splicing transcripts, rather than the overall hTERT transcripts, may improve our understanding of telomerase regulation.[22] The most widely studied variants involve splicing at 2 main sites: the α splice site, which produces a 36-bp inframe deletion within the conserved reverse transcript motif A; and the β site, which results in a 183-bp deletion and non-sense mutation that truncates the protein.[23] A quantitative assay for each deletion transcript can accurately quantify the alternative splicing variant expression and assess its correlation with clinico-pathological parameters.[24] Splice variants that are found predominantly in tumors have clear diagnostic value and may provide potential drug targets.[25] At present, not much is known about the functionality of the hTERT β-deletion variant and it is unclear whether spliced hTERT is important in determining tumor genesis.[26,27] The α-deletion variant of hTERT with negative telomerase regulatory properties is expressed in very low amounts in cell culture systems, which is an issue that remains to be clarified.[28] There is considerable heterogeneity regarding hTERT splicing patterns among various primary and metastatic lesions.[29] Few studies have directly examined the differences in hTERT splice variants in various classes of CNS tumors, and the biological role of the isoforms has not been investigated at all. The aim of this study was to compare the expression of hTERT splice variants, including both functional and deleted variants, in 2 groups of brain tumors of different grades and in a group of healthy control samples.

Material and methods

Samples and patients

The tumor samples – 20 meningiomas and 20 astrocytomas – were taken at Isfahan Medical University Hospital (Alzahra, Iran). All the patients had been diagnosed pathologically with astrocytoma and meningioma, and clinical pathological details were determined using the 2007 WHO classification criteria. The control samples were obtained from 4 patients who had underwent resections of normal brain tissue for purposes other than intracranial malignancy treatment. To store the samples for the analysis of telomerase, the surgical specimens were immediately frozen in liquid nitrogen and stored at –80°C until use. The study was performed according to the instructions of the Ethics Committee of Isfahan University of Medical Sciences, Iran, and informed consent was obtained from the patients.

RNA extraction and cDNA synthesis

RNA was extracted from the frozen tissues and TRIzol Reagent (Invitrogen, Carlsbad, USA) was used for total RNA extraction. All the preparations and handling of RNA were carried out in a laminar flow hood under RNase-free conditions. The isolated RNA was resolved in 60 mL of RNase-free water, and the quality and quantity of the extracted RNA samples were determined

by spectrophotometry. Then, the isolated RNA (from 100 ng tissue) was transcribed to cDNA using a first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. Total RNA was used to synthesize a total volume of 20 µL cDNA, which was then stored at a temperature of –70°C.

Primers and reverse transcription polymerase chain reaction procedure

Four high-quality specific primers were designed for hTERT and α-deletion and β-deletion splice variants. *GAPDH* was considered the internal control gene (Table 1). The RNA expression of the hTERT variants was measured using quantitative reverse transcription polymerase chain reaction (RT-qPCR). A RealQ Plus 2x Master Mix Green kit (Ampliqon A/S, Odense, Denmark) containing SYBER Green dye was used. Polymerase chain reactions were set up in a total volume of 10 µL per reaction, and 1 µL of cDNA was poured into a 9 µL reaction mixture. All the reactions were done in triplicate using an ABI Step One Plus system (Applied Biosystems Corp., Foster City, USA). To normalize the data, RNA of the internal control gene (*GAPDH*) was used.

Table 1. Sequence of reverse transcription polymerase chain reaction (RT-PCR) primers used for human telomerase reverse transcriptase (hTERT) mRNA variants

Primer	Sequence	Expected amplicon size (bp)
hTERT	F: ACGGCGACATGGAGAACAA	214
	R: CACTGTCTTCCGCAAGTTCAC	
α-deletion	F: GTACTTTGTCCAGGACAGGCT	194
	R: GGAGGTCTGTCAAGGTAGAGAC	
β-deletion	F: GCTGTACTTTGTCAAGGTGGA	195
	R: ACTGGACGTAGGACTTGGCT	

Statistical analysis

The real-time PCR data was examined using the $2^{-\Delta\Delta CT}$ relative quantization method. IBM SPSS v. 20.0 software (IBM Corp., Armonk, USA) was used for the data analyses. The data distribution was determined by the one sample Kolmogorov-Smirnov test. Comparisons of transcript expression levels in the patients and healthy controls were made using the nonparametric Kruskal-Wallis test. Correlations between all the variants in different grades of samples were evaluated by the independent sample t-test. The independent sample t-test was also used to determine the expression levels in meningiomas compared to astrocytomas. Spearman's correlation coefficient (CC) was used to find correlations between the levels of expression of the different transcripts in different grades of tumors. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Clinical characteristics of the patients

The samples consisted of 20 meningiomas (80% from female patients (F) and 20% from male patients (M)) and 20 astrocytomas (60% F and 40% M). According to 2007 WHO classification criteria, 12 of the meningioma cases were histologically diagnosed as grade I, 5 were atypical grade II and 3 were malignant (anaplastic) grade III. Among the astrocytomas, 15 cases were diagnosed as grade II, 4 cases as grade I and 1 as grade III. The clinicopathologic characteristics of the patients are listed in Table 2.

Table 2. The clinicopathologic characteristics of 40 patients affected with brain tumors

Characteristics	Meningioma (non-neuroepithelial)	Astrocytoma (neuroepithelial)
Mean of age ±SD	58.6 ±4.5	40.8 ±3.1
Gender		
M	4	8
F	16	12
Grade		
I	12	4
II	5	15
III	3	1
IV	–	–
Mean tumor size [cm] ±SD	6 ±1.6	4 ±1.2
Total	20	20

SD – standard deviation; M – male; F – female.

Comparison of expression

Quantitative reverse transcription polymerase chain reaction showed that 5 out of the 40 cases (12,5%: 1 astrocytoma and 4 meningiomas) did not express any splice variants. In the comparison of different grades of meningiomas, the grade III meningioma tumors exhibited the highest expression of full-length hTERT variant and the β-deletion variant. The α-deletion isoform was the dominant variant in grade I meningioma tumors. In grade I, II and III meningiomas, the full-length hTERT variant showed the highest level of expression. In grades I, II and III astrocytomas, the β-deletion variant exhibited the highest level of expression.

In 1 patient with a grade III astrocytoma (anaplastic oligoastrocytoma), there was no difference between the expression of full-length hTERT variant and the β-deletion variant.

Expression of the full-length human telomerase reverse transcriptase transcript

According to our results, the full-length hTERT transcript, which encodes active hTERT protein, is expressed at a high level in tumors. Human telomerase reverse transcriptase expression was significantly higher in meningiomas

(n = 16; mean = 26.1 ±3.1; p = 0.003) than in astrocytomas (n = 19; mean = 24 ±4.2; p = 0.23). In astrocytoma tumors, increased expression of the full-length hTERT variant in higher grades is associated with increased expression of the β-deletion variant. Our results showed that generally all astrocytomas and meningiomas showed full-length hTERT mRNA expression as the most predominant variant in the majority of tumor samples, but the expression of the full-length transcript in high grades of tumors was found to be higher than in low grades (Fig. 1).

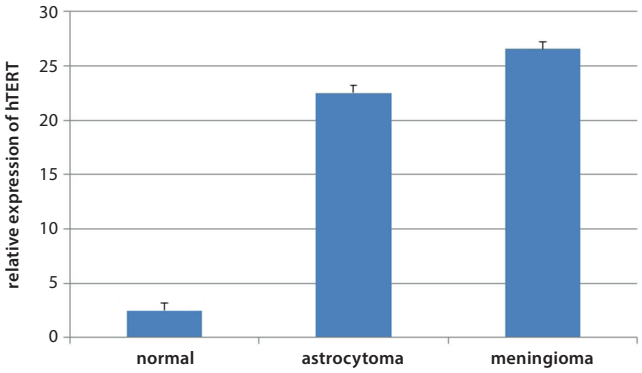

Fig. 1. The full-length human telomerase reverse transcriptase (hTERT) transcript and 2 common isoforms (α-deletion and β-deletion) were detected in patients with brain tumors and compared with healthy controls. The data revealed the functional transcript of hTERT was expressed at higher levels in the meningioma patients

Expression of other human telomerase reverse transcriptase splice variants

Our results showed diversity in the relative expression of hTERT transcript variants. The β-deletion transcript was the most abundantly expressed splice variant in astrocytoma patients. Both full-length hTERT variant and β-deletion splice variants were the most abundant transcripts in high grades of meningiomas and astrocytomas. Expression of the α-deletion variant was not significant in high grades of either astrocytomas or meningiomas compared to the β-deletion variant (Fig. 2). However, the

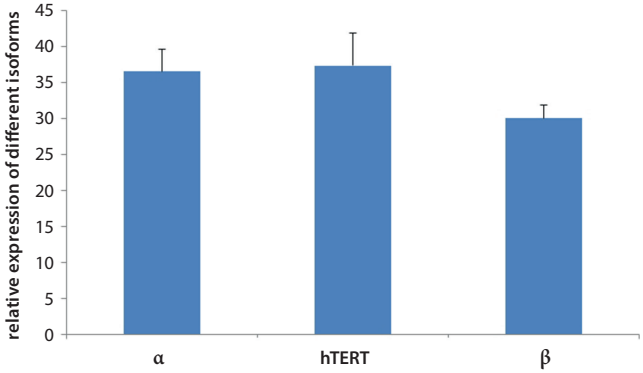

Fig. 2. The β-deletion transcript was the main transcript in the astrocytoma patients. Expression of the α-deletion variant is significantly lower than other splice variants in the astrocytoma patients

hTERT – human telomerase reverse transcriptase.

expression of this transcript was dominant in low grades of meningiomas (I and II) and grade I of astrocytomas (p = 0.01; mean = 31.1 ±4.3). No appreciable difference was observed between mRNA expression of full-length hTERT variant and the α-deletion variant in the astrocytoma samples (p = 0.97). Expression levels of the α-deletion transcript were statistically significant in meningioma patients (p = 0.01).

Correlations in expression between different isoforms

Our results indicated a positive correlation between the relative expression of hTERT mRNA transcripts and α-deletion and β-deletion variants in both meningiomas and astrocytomas (p = 0.018; CC = 0.0964). In lower grade meningiomas, a significant correlation was found between α-deletion isoforms and the β-deletion variant (p = 0.022; CC = 0.0614). A strong positive correlation between the expression of hTERT and the α-deletion variant was clearly detected in lower grade meningiomas but not in the higher grades (p = 0.031 and CC = 0.859). A positive correlation was found between the expression of α-deletion and β-deletion variants in grade I meningiomas (p = 0.022; CCs = 0.614) and astrocytomas (p = 0.016; CC = 0.910), but this correlation was not observed in the higher grades of the tumors. Expression of the β-deletion isoform showed a direct correlation with hTERT mRNA expression in grades II and III of astrocytoma (p = 0.045). We did not find any correlation between the expression of hTERT and β-deletion variants in grades I or II of meningioma. There was also no correlation between the expression of hTERT and β-deletion variants in grade I astrocytomas.

The most significant correlations were found between full-length hTERT and the β-deletion variant expression in grade III meningiomas (p = 0.018; CC = 0.964) and in grade II astrocytomas (p = 0.015; CC = 0.580) (Table 3).

In grade III meningiomas and grades II and III of astrocytoma tumors, expression of β-deletion variants was dominant, which correlated with the expression of hTERT (p = 0.018; CC = 0.964). The levels of expression of the full-length hTERT transcript significantly correlated with β-deletion variants in both meningiomas and astrocytomas.

Discussion

Amplification of the hTERT telomerase catalytic subunit is associated with a poor prognosis in intracranial and primary tumors.[30] An assessment of hTERT expression patterns, including both the wild type and the deletion variants in different CNS tumors is presented for the first time in this article. In addition, correlations between the expression of the wild type and 2 common deletion

Table 3. Correlations between the expression of human telomerase reverse transcriptase (hTERT) (full-length) and other isoforms in the 2 groups of brain tumors of different grades

Variant			Grade of meningioma				Grade of astrocytoma			
			all	I	II	III	all	I	II	III
hTERT	α	CC	0.453*	0.455	0.859*	0.390	0.655**	0.811*	0.412	–
		p-value	0.022	0.080	0.031	0.031	0.005	0.048	0.07	–
		N	20	12	15	3	20	4	15	1
	β	CC	0.741**	0.481	0.313	0.964*	0.673**	0.496	0.580*	–
		p-value	<0.001	0.067	0.304	0.020	0.001	0.019	0.015	–
		N	20	12	15	3	20	4	15	1
Alpha	β	CC	0.605**	0.614*	0.325	0.133	0.872**	0.910*	0.815**	–
		p-value	0.002	0.022	0.291	0.430	<0.001	0.010	<0.001	–
		N	20	12	15	3	20	4	15	1

* significant at level 0.05; ** significant at level 0.01; CC – correlation coefficient; N – number of patients.

variants in different grades of meningioma and astrocytoma have not previously been investigated.

Most previous studies investigated only telomere length or telomerase activity and did not assess differences in the expression of hTERT splice isoforms between benign and malignant brain tumors.[15,31] It has been shown that hTERT mRNA is expressed in 100% of glioblastomas, regardless of whether they show positive or negative telomerase activity.[32] Human telomerase reverse transcriptase mRNA expression has been found not only in neoplastic regions but in normal tissues as well, and the expression patterns of hTERT mRNA are consistent with increases in the severity of histopathologic changes.[33] The upregulation of hTERT mRNA in liver cancers and in the early stages of tumor progression are principally concomitant with the β-deletion variant.[11] In general, after the wild type transcript, the β isoforms are the most abundant and highly expressed.[34] Apparently, cell stress conditions cause changes in splicing variant foms.[35] In some cell lines, the loss of polymerase activity upon differentiation can illustrate a variation in the splicing patterns of the β-deletion variant.[36] In this study, we compared the expression of hTERT splice variants. In addition, we showed that the variance of expression of hTERT mRNA isoforms can have a determinant effect on the mechanisms of tumorigenesis in the human brain. Variations in hTERT expression can determine the mechanism of tumorigenesis. Our findings indicate a surprisingly high degree of variation in the proportions of the expression of hTERT splice variants in 2 groups of patients with brain tumors. High levels of the full-length hTERT transcript and 2 common deletion isoforms (α-deletion and β-deletion) were detected significantly more frequently in the patients with brain tumors compared to healthy controls. Our data revealed that the functional full-length hTERT variant was expressed at higher levels in the meningioma patients compared to the controls and astrocytoma patients (Fig. 1,2). High expression of the full-length hTERT splice variant had prognostic implications for meningioma patients: the data analysis suggested that there is an association between the quantification of hTERT expression and tumor progression. All of the meningeal tumor samples expressed hTERT mRNA, and as the degree of tumor increases, the rate of expression also increased (Table 4), which is in agreement with a study by Falchetti et al., in which hTERT expression was related to the MIB-1 and Ki-67 proliferation indexes and with the recurrence rate of high-grade meningiomas.[37]

With the progression of grade I tumor cells to higher grades, including II and III, there was an increase in the expression pattern of hTERT splicing, favoring β-deletion and full-length wild type isoforms, but the dominant form in grade II and III of meningioma and astrocytoma was the β-deletion variant (Fig. 3). This might explain the heterogeneity of neuroepithelial tumor cells.[26,33] In our study, a higher level of β-deletion isoform expression in high-grade astrocytomas was observed. The higher ratio of this isoform as compared to the full-length isoform indicates that there might be a higher proportion of nonsense mutations in malignant astrocytoma patients compared to the control samples (Fig. 1). The high expression of the β-deletion isoform in high grades of brain tumors suggests that unlimited cell proliferation and aggressiveness are dependent on the expression of the β-deletion isoform, which is consistent with the results of previous studies.[33,38]

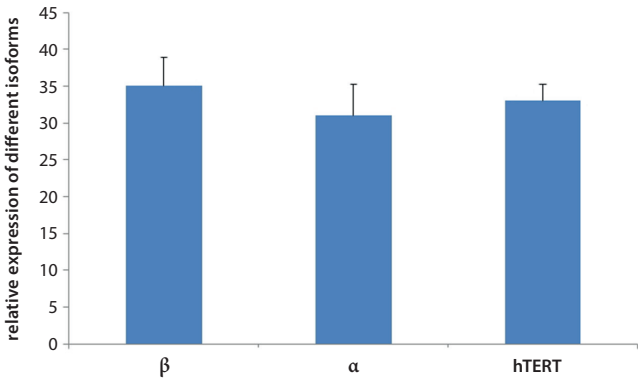

Fig. 3. The expression pattern of different splice variants in the meningioma patient showed high expression of human telomerase reverse transcriptase (hTERT) and α variants in benign tumors

Table 4. Relative expression patterns of human telomerase reverse transcriptase (hTERT) splice isoforms in different grades of brain tumors

Tumor type/ grade	hTERT	α-deletion variant	β-deletion variant
Astrocytoma			
Grade III	+++++	–	++++++
Grade II	++++	+	+++++
Grade I	+++	++++	++
Meningioma			
Grade III	++++++	+	++++
Grade II	+++++	+++	+++
Grade I	++++	+++++	+

+ level of expression; – lack of expression.

A relatively low percentage of the α-deletion splice variant, a negative inhibitor of telomerase activity, is overexpressed in both normal and benign tumor cells.[23,39] The α-deletion variant is expressed in low-grade meningioma and astrocytoma tumors that show the highest positive correlation with the expression of β-deletion variants and it is mostly absent in high-grade tumors.[26,33] The α-deletion isoform is the least abundant in meningiomas as well as astrocytomas (Fig. 2). This transcript is always detected in cells and tissues expressing hTERT.[36,40] Our data analysis showed that the differences between α-isoform expressions and hTERT transcript in low-grade meningiomas are statistically significant. In low-grade meningiomas and astrocytomas, the most abundantly expressed splice variant was the α-deletion variant, which probably has a dominant negative effect over the hTERT full-length variant (Table 4).

The fact that there is considerable heterogeneity in the expression of hTERT splice variants in brain tumors generates many questions about programs of alternative splicing and the role of each transcript in molecular changes in the field of tumorigenesis. Our results show that the β-deletion variant is more important in patients with astrocytomas, while full-length hTERT mRNA is more important in patients with meningiomas. Our findings indicate that α-deletion isoforms are associated with full-length hTERT mRNA levels in grades I and II meningiomas, in addition to grade I astrocytomas. Increased expression of the β-deletion variant was generally equal to or slightly higher than the expression of the full-length hTERT variant in grade III meningiomas and grades II and III astrocytomas. In low grades of brain tumors, the expression of the α-deletion variant was dominant while the expression of full-length hTERT variant and the β-deletion variant in high-grade tumors were statistically significant. Apparently, an ongoing process favoring the expression of the β-deletion variant in high grades, abating the expression of the functional transcript and variations in the expression pattern of hTERT splice variants with increasing tumor grade is associated with a poor prognosis.

In conclusion, changes in the splicing pattern of hTERT splice variants in brain tumors and their correlations with pathological changes in cells could be used as diagnostic or prognostic biomarkers, or as possible targets for cancer therapy. However, the function and biological role of hTERT splice variants remain to be clarified.

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Toll-like receptors TLR-2, TLR-4, TLR-7, and TLR-9 in tumor tissue and serum of the patients with esophageal squamous cell carcinoma and gastro-esophageal junction cancer

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Abstract

Background. Stimulation of toll-like receptors (TLRs) has been linked to the development of esophageal and gastric cancers.

Objectives. The aim of the study was to evaluate the clinical significance of tissue expression and serum concentration of TLR-2, TLR-4, TLR-7, and TLR-9 in patients with esophageal squamous cell carcinoma and gastro-esophageal junction adenocarcinoma.

Material and methods. The study group consisted of 97 individuals: 32 with esophageal squamous cell carcinoma, 27 with gastro-esophageal junction cancer, and 38 age- and gender-matched controls. The mRNA expression and protein concentration of TLRs in tissues and sera were measured with reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) tests.

Results. In esophageal cancer patients, mRNA expressions of TLR-2, TLR-4 and TLR-7, and protein concentrations of all TLRs were significantly higher in tumor than in control tissue ($p < 0.05$). In esophageal cancer patients with lymph node metastasis, a tendency toward higher protein concentrations of tumor TLR-4 was observed. In gastro-esophageal junction adenocarcinoma subgroup, only the mRNA expression of TLR-7 and protein concentrations of TLR-4, TLR-7 and TLR-9 were significantly higher in tumors than in normal mucosa ($p < 0.05$). Protein concentration of TLR-9 was significantly higher in tumors of gastro-esophageal junction cancer with lymph node metastasis and depth of tumor invasion. Diagnostic potential of serum TLR-4 as a marker of gastro-esophageal junction cancer presence was reported.

Conclusions. We demonstrated differences in the expression patterns of TLRs between esophageal squamous cell carcinoma and adenocarcinoma of gastro-esophageal junction, and showed circulating TLR-4 to be a potential marker of gastro-esophageal junction cancer.

Key words: biomarkers, toll-like receptors, gastro-esophageal cancer

Introduction

Esophageal squamous cell carcinoma (ESCC) and gastro-esophageal junction adenocarcinoma (GEJA) are characterized by one of the lowest indicators of 5-year survival rates in Eastern Europe (6%), Western Europe and the USA (14–18%).[1,2] Poor prognosis of patients with ESCC and GEJA is related to the fact that they are diagnosed in the advanced stage, when the presence of lymph node and/or distant metastases is observed.[1,2] The pathogenesis of GEJA remains unclear. However, the natural behavior of GEJA and its therapeutic modalities are often similar to those of esophageal adenocarcinoma, as both cancers may develop through metaplasia-dysplasia sequence due to the accumulation of genetic alterations.[3] As such, the decision whether GEJA is classified as esophageal or gastric cancer depends on whether the primary tumor extends to esophagus.[4]

Earlier studies have connected the influence of alcohol, nicotine, acidic gastric contents, and bile from the duodenum with cellular damage of esophageal and gastro-esophageal junction epithelium.[3,5,6] The transformation of the epithelial cells results in the loss of epithelial wall integrity and changes in esophageal microbiome.[6] The bacterial products stimulate toll-like receptors (TLRs) in the epithelial and inflammatory cells, inducing persistent innate immune responses, the expression of proinflammatory cytokines and the generation of reactive oxygen species (ROS).[6,7] Additionally, TLRs may be activated by endogenous ligands from dead or damaged host cells. They contribute to an abnormal increase of inflammatory response, which may lead to the disturbance of cellular homeostasis and the induction of metaplasia-dysplasia sequence.[3,8] The deregulation of innate and adaptive immune response may play an important role in the development of both ESCC and GEJA.

Toll-like receptors can be either extracellular (such as TLR-1, TLR-2, TLR-4, TLR-5, TLR-6) or intracellular (such as TLR-3, TLR-7, TLR-8, and TLR-9). Extracellular TLRs are located at the plasma membrane, where they recognize macromolecules exposed on the surface of pathogens. Intracellular TLRs are located in endosomes or lysosomes and they detect viral and bacterial nucleic acids, which plays an important role in host immune responses.[5,7–9] Recently, the ability of TLRs to heighten the immune response has been exploited for the treatment of cancer disease.[10]

An overexpression of TLR-3, TLR-4, TLR-5, TLR-7, and TLR-9 in ESCC tumors has been demonstrated both on mRNA and, qualitatively, on protein level. The potential usefulness of their evaluation as prognostic markers for ESCC has been suggested.[3,11] However, the quantitative analyses of TLRs concentrations have not been previously performed. Also, there is no data concerning TLR-2 mRNA expression in ESCC. With regard to adenocarcinomas, the expression of TLRs has been evaluated in adenocarcinomas of esophagus and stomach, but not in the area of gastro-esophageal junction.[12,13] A potential correlation between tissue expression and circulating levels of TLRs has not been determined, either.

Therefore, the aim of the present study was to evaluate mRNA copy numbers for TLR-2, TLR-4, TLR-7, and TLR-9, and their protein concentrations in ESCC and GEJA tumors as compared to patient-matched normal tissues with reference to the disease advancement and circulating levels.

Material and methods

Patients' characteristics

The study population consisted of 97 individuals: 59 cancer patients and 38 healthy individuals. Cancer patients (45 male, 14 female, median of age: 62 years, 95% confidence interval (CI) = 58–67 years) were admitted to the Department of Gastrointestinal and General Surgery of Wroclaw Medical University (Poland) from 2010 to 2015 for curative resection of upper gastrointestinal tract tumors. Patients with any systemic illness, prior radio- or chemotherapy and stage IV cancer (distant metastases) were excluded from the study. Preoperative evaluation was conducted by physical examination and imaging techniques, such as ultrasonography, computed tomography and magnetic resonance. There were 32 patients with histologically confirmed ESCC and 27 patients with histologically confirmed GEJA. In the present study, all adenocarcinomas of gastro-esophageal junction extended into the esophagus and, in line with the 7th edition of the Union for International Cancer Control (UICC) TNM classification, were classified as esophageal cancers.[4] Resected tumors were staged pathologically using the UICC TNM classification.[4] There were 8 patients with stage I cancer (3 with ESCC and 5 with GEJA), 14 patients with stage II (8 with ESCC and 6 with GEJA), and 37 with stage III (21 with ESCC and 16 with GEJA).

Sera of 38 apparently healthy blood donors (27 male, 11 female, median age 59 years, 95% CI = 55–63 years) from the Lower Silesian Center of Blood Donation and Therapeutics, Wrocław, Poland, were used as a reference in the analysis of circulating TLRs. The control group was age- and gender-matched to the study group ($p = 0.129$ and $p = 0.890$, respectively).

The study was planned according to the ethical standards detailed in the Declaration of Helsinki, as revised in 1983. The study protocol was approved by the Medical Ethics Committee, Wroclaw Medical University, Poland (signature numbers: KB 28/2011 and 203/2014). Informed consent was obtained from all participants.

Collection and preparation of samples

Tissue samples

Fresh samples of tumor and normal mucosa, taken approx. 10 cm from the tumor, were collected after the

resection and divided into 2 groups of samples: the first one, subsequently used for transcriptional analysis, was soaked in RNAlater (Ambion, Inc., Austin, USA) and stored at –80°C; the second one, subsequently used for protein analysis, was rinsed with 0.9% NaCl and stored at –45°C.

To determine TLRs protein concentration, tissue samples were homogenized in 10 mM Tris-HCl with 150 mM KCl and 1 mM EDTA, pH 7.4 buffer (proportion 1:2 w/v), using FastPrep-24 homogenizer (2 min, 4.0 m/s). The homogenates were centrifuged at 14,500 × g, 10 min, 6°C, and the supernatants were collected and stored at –45°C.

RNA extraction, quantitation and quality assessment

Tissue samples (30–40 mg) were homogenized in TRIzol Reagent (Invitrogen Life Technologies, Waltham, USA), using Fastprep 24 Homogenizer (MP Biomedical, Solon, USA), and total RNA was extracted using the phenol-chloroform method. Isolated RNA was purified using RNeasy Mini Kit (Qiagen, Valencia, USA) with DNase treatment, in accordance with the manufacturer's instructions. Purified RNA was quantified with Nano Drop 2000 (ThermoScientific, Rockford, USA). Its purity was assessed by calculating 260:280 and 260:230 ratios. The integrity of RNA was evaluated using the Experion platform incorporating LabChip microfluidic technology and Experion RNA StdSens analysis kits (BioRad, Hercules, USA).

Blood samples

Samples of peripheral blood were collected into sterile vacuum tubes from healthy controls and cancer patients (prior to surgery) after overnight fasting. Blood was clotted (30 min, room temperature) and centrifuged (1,500 × g, 10 min, room temperature). Obtained sera were stored at –45°C.

Analytical methods

Determination of protein concentrations

Concentrations of TLRs in tissue homogenates and sera were determined using enzyme-linked immunosorbent assay (ELISA) tests (Cloud-Clone Corp., Houston, USA). Detection ranges and minimum detectable doses were respectively: 0.312–20 ng/mL and 0.117 ng/mL for TLR-2; 0.312–20 ng/mL and 0.133 ng/mL for TLR-4; 0.780–50 ng/mL and 0.290 ng/mL for TLR-7; 0.625–10 ng/mL and 0.236 ng/mL for TLR-9. In the case of tissue samples, TLRs concentrations were adjusted to total protein level, measured using the Bradford method and BioRad Protein Assay (BioRad) with bovine serum albumin as a standard. Concentrations of TLRs were expressed as µg per g of total protein content.

cDNA synthesis

According to the manufacturer's instruction, 0.5 µg of RNA was transcribed by Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Negative transcription controls were performed and tested for all samples. All incubation steps were carried out in C1000 thermocycler (BioRad).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) mixture consisted of: 2 µL of cDNA (diluted 1:5), 10 µL of SsoFast EvaGreen Supermix (BioRad), 1 µL of each 10 nM forward and reverse target-specific primer, and 6 µL of water. The list of primers (GeneSys Sp. z o.o., Wrocław, Poland) used in this study is shown in Table 1. Reactions were conducted in triplicates, using CFX96 RT-PCR system (BioRad) with the following cycling conditions: 95°C for 30 s, 95°C for 5 s, 61°C for 5 s, 40 cycles. Specificity of the product was confirmed by melting curve analysis (60–90°C with fluorescent reading every 0.5°C) and by electrophoresis in a high-resolution agarose (SeaKem LE agarose; Lonza, Basel, Switzerland) in TBE with SYBR Green (Lonza) detection. For each sample, the expression of TLRs was examined and normalized to the *GAPDH* reference gene, and relative induction was calculated using the $2^{(-\Delta\Delta Ct)}$ method.[14]

Table 1. qPCR primer sequences used for TLRs detection

Gene	Primer sequence
TLR-2	forward CTTCACTCAGGAGCAGCAAGCA reverse ACACCAGTGCTGTCCTGTGACA
TLR-4	forward CCCTGAGGCATTTAGGCAGCTA reverse AGGTAGAGAGGTGGCTTAGGCT
TLR-7	forward CTTTGGACCTCAGCCACAACCA reverse CGCAACTGGAAGGCATCTTGTAG
TLR-9	forward TGAGCCACAACTGCATCTCGCA reverse CAGTCGTGGTAGCTCCGTGAAT

qPCR – quantitative polymerase chain reaction; TLR – toll-like receptor.

Statistical analysis

Data distribution was analyzed using the Shapiro-Wilk normality test. Descriptive data was presented as medians (Me) and minimum–maximum (min–max) values. Independent samples were analyzed using the Mann-Whitney U test, the Kruskal-Wallis H test with post-hoc Dunn's test, and paired samples were tested by the Wilcoxon test. Chi-squared test was conducted in order to analyze the compliancy of the control group with the study group (variable "gender"). Receiver operating characteristic (ROC) analysis was used to test the diagnostic utility of serum TLR-4. All values of $p < 0.05$ were considered as statistically significant. The statistical analyses were performed using STATISTICA v. 12.0 software (StatSoft, Tulsa, USA).

Results

Pairwise comparison of toll-like receptors protein levels between tumor and adjacent normal tissue

In the total group of patients, relative protein concentrations of TLR-2, TLR-4, TLR-7, and TLR-9 were significantly higher in tumor tissue than in the corresponding control tissue ($p < 0.05$ for all) (Fig. 1).

When analyzed separately, protein content of all TLRs was significantly higher only in the tumor tissue of ESCC. In GEJA, tumor concentrations of TLR-4, TLR-7 and TLR-9 were significantly higher, but there was no difference in the concentration of TLR-2 protein between tumor and normal tissue (Table 2).

Tumor concentrations of TLRs in ESCC and GEJA were comparable, except for TLR-4, whose concentration was significantly higher in ESCC than in GEJA patients ($p = 0.00002$) (Table 2).

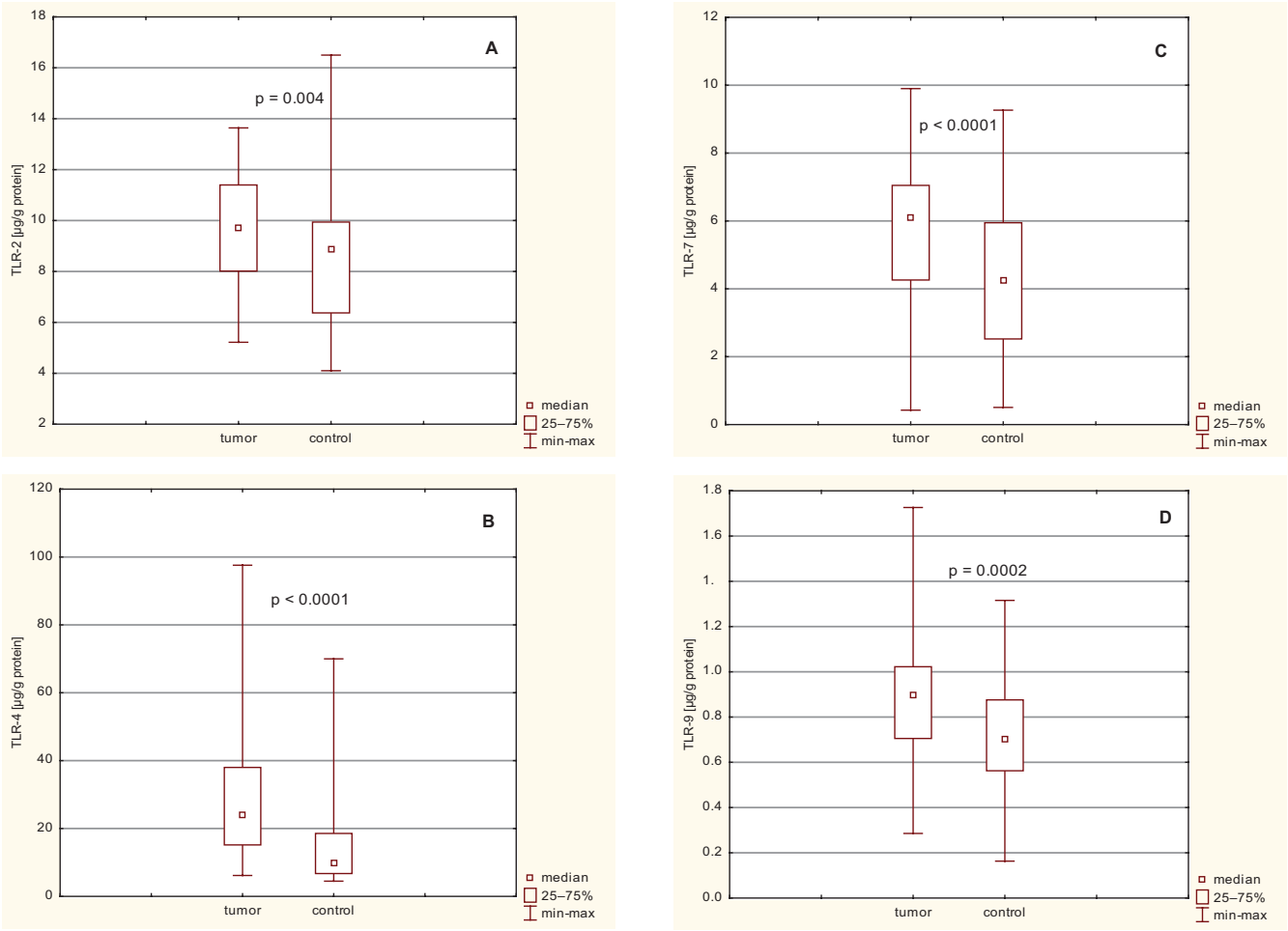

Fig. 1. Pairwise comparison of TLRs protein concentrations between tumor and adjacent control (nonneoplastic) esophageal tissue. Data was presented as relative TLRs concentrations adjusted to total protein content (µg/g protein). Figures A–D show results for TLR-2, TLR-4, TLR-7, and TLR-9, respectively

TLR – toll-like receptor.

Table 2. Pairwise comparison of protein concentrations of TLRs between tumor and control tissue in ESCC and GEJA subgroups of patients. Data in each subgroup was analyzed with the Wilcoxon test for paired samples

Toll-like receptor	ESCC (n = 32)			GEJA (n = 27)		
	tumor tissue – Me (min–max)	control tissue – Me (min–max)	p-value	tumor tissue – Me (min–max)	control tissue – Me (min–max)	p-value
TLR-2 [µg/g protein]	10.09 (6.76–13.64)	8.83 (4.95–16.50)	0.014*	8.80 (5.21–12.76)	8.94 (4.10–12.00)	0.084
TLR-4 [µg/g protein]	34.36 (10.57–97.57)#	9.86 (4.49–69.98)	<0.0001*	17.60 (6.16–30.27)#	11.20 (4.79–26.38)	0.012*
TLR-7 [µg/g protein]	6.10 (0.82–8.70)	3.98 (0.51–9.26)	0.001*	5.30 (0.43–9.90)	4.30 (0.53–8.00)	0.021*
TLR-9 [µg/g protein]	0.83 (0.45–1.73)	0.71 (0.40–1.25)	0.003*	0.93 (0.28–1.41)	0.66 (0.16–1.32)	0.016*

ESCC – esophageal squamous cell carcinoma; GEJA – gastro-esophageal junction adenocarcinoma; Me – median; * statistically significant; # protein concentration of TLR-4 in ESCC tumor vs protein concentration of TLR-4 in GEJA tumor, $p = 0.00002$ (the Mann-Whitney test).

Relations of tumor toll-like receptors concentrations to clinical and histopathological parameters

Tumor concentrations of TLRs were evaluated in relation to age, gender, stage of cancer, primary tumor progression, and lymph node invasion. In ESCC, a tendency toward higher protein concentrations of tumor TLR-4 in patients with lymph node metastasis was observed. No similar association for other TLRs was detected. Also, there was no relationship between TLRs and other clinic-pathological parameters (Table 3).

In GEJA patients, tumor protein concentration of TLR-9 was significantly higher in more advanced cancers (stage III, pT3/4 and pN1). No such association was observed for other TLRs. Protein content of TLR was not affected by patients' age or gender (Table 4).

Pairwise comparison of toll-like receptors mRNA expressions between tumor and adjacent normal tissue of cancer patients

The analysis of TLR expression on the mRNA level showed the up-regulated levels of all receptors in tumor tissues as compared to normal ones: by 3.9-fold for TLR-2, by 2.6-fold for TLR-4, by 3.6-fold for TLR-7, and by 2-fold for TLR-9 (Table 5). When analyzed separately, the expressions of TLR-2, TLR-4, and TLR-7, but not that of TLR-9, were significantly elevated in ESCC tumors. In GEJA, only the expression of TLR-7 was significantly higher in tumor tissue as compared to the normal one (Table 5).

Table 3. Relationships between clinical and histopathological parameters and protein concentrations of TLRs in tumor tissue of ESCC patients

Parameters	TLR-2 [µg/g protein]		TLR-4 [µg/g protein]		TLR-7 [µg/g protein]		TLR-9 [µg/g protein]	
	Me (min–max)	p-value	Me (min–max)	p-value	Me (min–max)	p-value	Me (min–max)	p-value
Gender		0.933		0.923		0.737		0.721
male (n = 23)	10.1 (6.9–13.3)		35.3 (12.0–86.6)		6.1 (0.8–8.7)		0.9 (0.5–1.7)	
female (n = 9)	9.7 (6.2–13.6)		30.9 (10.6–97.6)		6.1 (1.2–8.7)		0.8 (0.5–1.6)	
Age		0.122		0.624		0.597		0.821
<60 (n = 16)	9.2 (6.2–13.6)		32.9 (12.0–86.3)		6.1 (1.2–8.7)		0.8 (0.6–1.6)	
≥60 (n = 16)	10.7 (6.8–13.3)		35.8 (10.6–97.6)		6.3 (0.8–8.7)		0.9 (0.5–1.7)	
Stage (TNM)		0.936		0.451		0.721		0.706
I+II (n = 11)	9.4 (6.8–13.6)		24.3 (10.6–54.9)		6.3 (1.2–8.7)		0.8 (0.5–1.6)	
III (n = 21)	10.1 (6.2–13.3)		38.0 (12.0–97.6)		6.1 (0.8–8.1)		0.8 (0.5–1.7)	
Depth of tumor invasion (T)		0.899		0.644		0.737		0.232
1+2 (n = 9)	9.4 (6.2–13.6)		33.4 (13.2–54.9)		6.3 (1.2–8.7)		0.9 (0.5–1.8)	
3+4 (n = 23)	10.1 (6.9–13.3)		38.0 (10.6–97.6)		6.1 (0.8–8.1)		0.8 (0.6–1.7)	
Lymph node metastasis (N)		0.489		0.071		0.908		0.477
0 (n = 13)	9.4 (6.8–13.6)		23.3 (10.6–54.9)		6.2 (1.2–8.7)		0.8 (0.5–1.6)	
1 (n = 19)	10.1 (7.0–13.3)		38.3 (15.6–97.6)		6.1 (0.8–8.1)		0.9 (0.5–1.7)	

TLR – toll-like receptor; ESCC – esophageal squamous cell carcinoma; Me – median.

Table 4. Relationships between clinical and histopathological parameters and protein concentrations of TLRs in tumor tissue of GEJA patients

Parameters	TLR-2 [µg/g protein]		TLR-4 [µg/g protein]		TLR-7 [µg/g protein]		TLR-9 [µg/g protein]	
	Me (min–max)	p-value	Me (min–max)	p-value	Me (min–max)	p-value	Me (min–max)	p-value
Gender		0.230		0.925		0.119		0.453
male (n = 22)	8.6 (5.2–12.4)		18.0 (6.2–30.3)		4.6 (0.4–9.9)		0.9 (0.3–1.4)	
female (n = 5)	9.7 (8.0–12.8)		17.6 (6.4–23.2)		6.6 (5.2–8.2)		1.0 (0.7–1.0)	
Age		0.571		0.662		0.877		0.368
<60 (n = 9)	10.1 (6.6–12.4)		17.6 (8.9–26.6)		5.3 (1.2–9.9)		0.9 (0.7–1.2)	
≥60 (n = 18)	8.7 (5.2–12.8)		17.0 (6.2–30.3)		5.3 (0.4–8.2)		0.9 (0.3–1.4)	
Stage (TNM)		0.786		0.902		0.570		0.002*
I+II (n = 11)	10.0 (5.2–12.6)		15.5 (8.9–25.4)		4.8 (1.2–8.2)		0.7 (0.3–1.3)	
III (n = 16)	8.6 (5.7–12.8)		18.1 (6.2–30.3)		5.4 (0.4–9.9)		1.0 (0.9–1.4)	
Depth of tumor invasion (T)		0.559		0.811		0.614		0.013*
1+2 (n = 8)	9.9 (6.6–12.6)		17.6 (8.9–25.4)		5.2 (1.2–8.2)		0.9 (0.3–1.3)	
3+4 (n = 19)	8.7 (5.2–12.8)		18.3 (6.2–30.3)		5.4 (0.4–9.9)		1.0 (0.9–1.4)	
Lymph node metastasis (N)		0.786		0.902		0.570		0.002*
0 (n = 11)	10.0 (5.2–12.6)		15.5 (8.9–25.4)		4.8 (1.2–8.2)		0.7 (0.3–1.3)	
1 (n = 16)	8.6 (5.7–12.8)		18.1 (6.2–30.3)		5.4 (0.4–9.9)		1.0 (0.9–1.4)	

TLR – toll-like receptor; GEJA – gastro-esophageal junction adenocarcinoma; Me – median; * statistically significant.

Table 5. Relative expressions of TLRs genes in tumor tissue of cancer patients. Gene expression in tumor was normalized to gene expression in control mucosa. Analysis was conducted with the Wilcoxon test

Gene expression	Total group (n = 59)		ESCC patients (n = 32)		GEJA patients (n = 27)	
	Me (min–max)	p-value	Me (min–max)	p-value	Me (min–max)	p-value
TLR-2	1.41 (0.12–14.42)	0.0001*	4.77 (0.63–14.42)	0.0008*	1.36 (0.12–2.26)	0.110
TLR-4	1.28 (0.13–9.38)	0.011*	2.19 (0.15–9.38)	0.014*	1.25 (0.13–2.96)	0.216
TLR-7	2.14 (0.31–9.80)	0.0003*	2.69 (0.31–5.77)	0.006*	2.08 (0.38–9.80)	0.018*
TLR-9	1.23 (0.16–8.93)	0.048*	1.15 (0.25–8.93)	0.147	1.26 (0.16–4.57)	0.153

TLR – toll-like receptor; ESCC – esophageal squamous cell carcinoma; GEJA – gastro-esophageal junction adenocarcinoma; Me – median; * statistically significant.

Circulating levels of TLR-2, TLR-4, TLR-7, and TLR-9

Only the concentrations of circulating TLR-4 were higher, both in cancer patients and healthy controls, than the limit of detection of the assay. In the case of TLR-2, 82% of control samples and 25% of cancers yielded results that were below the detection limit. Similarly, 87% of cancers and all of controls in TLR-7 assay, and 75% of cancers and all of controls in TLR-9 assay were below the limit. Therefore, further analysis was limited to TLR-4.

We demonstrated significantly higher concentrations of serum TLR-4 in cancer patients in general than in healthy controls. Patients with GEJA had significantly higher circulating TLR-4 than these with ESCC (Table 6).

Circulating TLR-4 was significantly elevated in more advanced tumors of ESCC, but not in GEJA patients (stage III, pT3/4). A similar tendency was observed for lymph node metastasis (Table 7).

Diagnostic potential of circulating TLR-4 level in ESCC and GCC

To assess the strength of association and potential diagnostic utility of circulating TLR-4 as a marker of ESCC and GEJA, ROC analysis was performed. Circulating TLR-4 was significantly associated exclusively with GEJA presence with overall 79% accuracy, defined as area under ROC curve (AUC). At a cut-off of 1,338 pg/mL, TLR-4 as GEJA marker was characterized by 70% sensitivity and 78% specificity (Fig. 2).

Discussion

To our knowledge, this is the first study addressing the issue of TLR-2, TLR-4, TLR-7, and TLR-9 expression, either on mRNA and protein level, as well as their circulating levels in adenocarcinoma of gastro-esophageal junction. We showed that, similarly to ESCC, the protein expression

Table 6. Concentration of serum TLR-4 in cancer patients and healthy controls. Descriptive values are presented as median (min–max)

Toll-like receptor	Controls (n = 38)	Cancer patients			p-value	
		all (n = 59)	ESCC (n = 32)	GEJA (n = 27)	controls vs all cancers†	controls vs ESCC vs GEJA‡
TLR-4 [pg/mL]	1268 (1228–1389)**	1338 (1145–2546)	1276 (1145–2546)#	1367 (1195–2191)**#	0.011*	0.008*

ESCC – esophageal squamous cell carcinoma; GEJA – gastro-esophageal junction adenocarcinoma; * statistically significant; ** post-hoc Dunn's test for GEJA vs control, p = 0.002; # post-hoc Dunn's test for ESCC vs GEJA, p = 0.048; † the Mann-Whitney U test; ‡ the Kruskal-Wallis H test.

Table 7. Relationships between clinical and histopathological parameters and serum TLR-4 concentrations in ESCC and GEJA patients

Parameters	ESCC [pg/mL]			GEJA [pg/mL]		
	n	Me (min–max)	p-value	n	Me (min–max)	p-value
Gender			0.529			0.609
male	23	1275 (1145–1530)		22	1360 (1195–1568)	
female	9	1286 (1148–2545)		5	1437 (1261–2191)	
Age			0.451			0.354
<60	16	1271 (1234–1530)		9	1342 (1250–2191)	
≥60	16	1299 (1145–2548)		18	1394 (1195–1568)	
Stage (TNM)			0.019*			0.119
I+II	11	1263 (1145–1439)		11	1338 (1195–1568)	
III	21	1345 (1248–2545)		16	1416 (1250–2191)	
Depth of tumor invasion (T)			0.029*			0.110
1+2	9	1264 (1248–1530)		8	1340 (1195–1568)	
3+4	23	1439 (1145–2545)		19	1410 (1250–2191)	
Lymph node metastasis (N)			0.054			0.119
0	13	1263 (1148–1439)		11	1338 (1195–1568)	
1	19	1345 (1145–2545)		16	1416 (1250–2191)	

TLR – toll-like receptor; ESCC – esophageal squamous cell carcinoma; GEJA – gastro-esophageal junction adenocarcinoma: Me – median; * statistically significant.

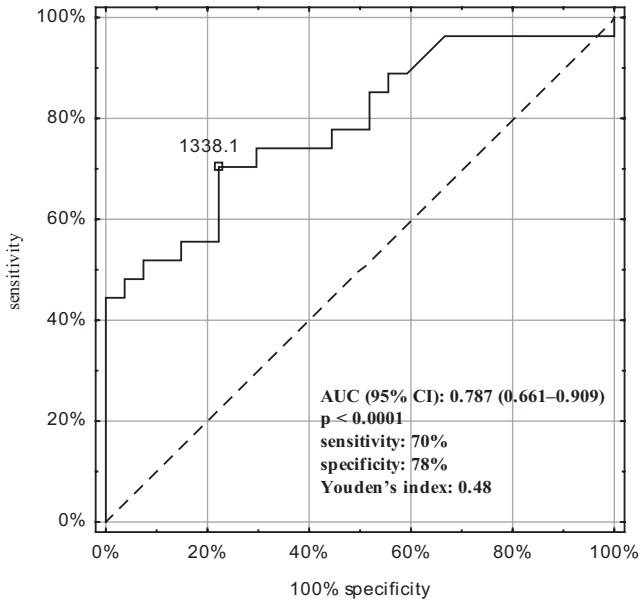

Fig. 2. ROC analysis of serum TLR-4 as a possible marker of GEJA presence. Calculated optimal criterion value (cut-off point) of serum TLR-4 concentration is 1,338.1 pg/mL

ROC – receiver operating characteristic; AUC – area under ROC curve; CI – confidence interval; TLR – toll-like receptor; GEJA – gastro-esophageal junction adenocarcinoma.

of TLR-4, TLR-7 and TLR-9 was up-regulated in GEJA tumors as compared to normal tissue. However, on the mRNA level, only the expression of TLR-7 was significantly higher. The observation of increased protein content of TLR-4 and TLR-9 in tumor tissue is not accompanied by equally pronounced up-regulation of their mRNA. This may be explained by enhanced accumulation of TLR proteins due to their increased stability.

Corroborating our findings on TLR-4 in GEJA, Huhta et al. showed increased TLR-4 protein expression in Barrett's esophagus.[12] TLR-4 immunoreactivity, both in the cytoplasm and nucleus, was correlated with the degree of dysplasia, its progression to esophageal adenocarcinoma, as well as with poor prognosis. Up-regulation of the receptor has also been reported due to *Helicobacter pylori* (HP), gastritis and subsequent metaplasia, dysplasia and gastric adenocarcinoma.[12,13,15,16] TLR-4 might contribute to neoplastic transformation by activating nuclear factor-kappaB (NF-κB) pathway and the production of proinflammatory cytokines and cyclooxygenase-2 in the epithelial and immune cells, as well as by mitochondrial ROS production.[6,9,12,17–19] Contrary to GEJA, TLR-4 in ESCC tumors was overexpressed both at protein and mRNA levels. Neither in ESCC nor in GEJA, protein expression of TLR-4 was correlated with TNM stage, except for a tendency toward higher protein content in ESCC with lymph node involvement. Also, Sheyhidin et al. reported an association of high mRNA and protein expression of tumor TLR-4 with the lymph node metastasis of ESCC.[11] They found that high TLR-4 expression in stromal mononuclear inflammatory cells was significantly associated with a risk of lymph node metastasis and poor prognosis of ESCC patients. Correspondingly, circulating levels of TLR4 in our ESCC patients were positively correlated with the disease advancement. The current study is the first one to assess the diagnostic usefulness of serum TLR-4. Its elevated levels were associated with GEJA presence with good accuracy, warranting its further evaluation as a potential GEJA marker.

We demonstrated overexpression of mRNA and an increase of protein concentration of TLR-7 in tumor of GEJA patients. However, no association between tumor TLR-7 concentration and clinic-pathological parameters was observed. Although a possible association might be obscured by the limited number of observations in subgroup analysis, analogous results were presented by Helminen et al., who evaluated TLR-7 expression in esophageal adenocarcinoma, using immunohistochemistry.[20] Much research points at a dual or controversial role of TLR-7 in cancer development.[20–23] Whereas Lin et al. showed down-regulation of TLR-7 gene expression in hepatocellular adenocarcinoma and hepatitis, Vaz and Andersson demonstrated its overexpression in pancreatic ductal adenocarcinoma.[21,23]

In summary, the role of TLR-7 expression and activation in adenocarcinomas remains unclear and requires further studies. In ESCC, TLR-7 was overexpressed both at mRNA and protein levels. Similarly, Sheyhidin et al. demonstrated a positive association between TLR-7 immunoreactivity and tumor grade.[11] Distinctive patterns of TLR-7 expression were observed in tumor cells and fibroblast-like cells in oral squamous cell carcinoma.[22] High expression of tumor TLR-7 associated with its low expression in stromal fibroblast-like cells has been reported to predict worse clinical outcome. It has been speculated that expression of TLR-7 in tumor cells may stimulate cancer development, whereas the expression of TLR-7 in fibroblast-like cells may play a protective role in oral squamous cell carcinoma.

We also demonstrated significantly higher protein concentration of tumor TLR-9 in GEJA, which corresponded with disease progression, depth of tumor invasion, and lymph node metastasis. Correspondingly, Kauppila et al. showed that an increase in tumor TLR-9 expression may contribute to the growth, metastasis and poor prognosis of patients with esophageal adenocarcinoma.[24] It has been suggested that changes in bacterial flora and apoptotic reactions in esophagus and gastro-esophageal junction can induce TLR-9 production, which stimulates the early steps of the cancer development.[24] However, the possible role of endogenous ligands for TLR-9 in the pathogenesis of esophageal adenocarcinoma requires further study. Herein, high protein concentration of TLR-9 was observed in ESCC tumors, whereas its gene expression did not differ significantly between tumor and normal mucosa. Previous qualitative studies demonstrated the predictive role of TLR-9 in esophageal squamous cell dysplasia and carcinoma, oral tongue squamous cell carcinoma, and cervical neoplasms.[11,25–28] Additionally, Mäkinen, et al. reported

that high expression of TLR-9 was associated with oral tongue squamous cell carcinoma recurrence.[17]

Our results are the first to demonstrate the high gene expression and protein concentration of TLR-2 in ESCC patients. Previous reports have described TLR-2 expression in cervical neoplasia and squamous cell carcinoma of cervix.[26,29] The predictive role of TLR-2 as a marker of invasive tumor growth has been shown in the early stage of oral tongue squamous cell carcinoma.[27] In the advanced stage of this cancer, nuclear TLR-2 expression level may be a marker of neck metastasis and tumor recurrence.[17] TLR-2 is considered as a promising marker in squamous cell carcinomas.

In conclusion, our results expand the current knowledge on TLRs in cancers of the upper digestive tract, adding information about cancers of gastro-esophageal junction. We demonstrated differences in the expression patterns of TLRs between ESCC and GEJA, and presented circulating TLR-4 as a potential marker of GEJA.

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Influence of different post-thaw culture time on the clinical outcomes of different quality embryos

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Abstract

Background. In thawed embryo transfer cycles, the most common method is to transfer the embryos after 2 h of culture. Clinical outcomes of frozen–thawed cleavage embryo transfer cycles regarding the embryos status and the culture time of frozen–thawed cleavage embryos were limited and did not elucidate all unclear issues.

Objectives. The objective of this study was to examine the clinical outcomes of frozen–thawed cleavage embryo transfer cycles according to the embryos status and the culture time (2 h or overnight).

Material and methods. In this retrospective study (5-year period), 1,654 frozen–thawed embryos were analyzed. Firstly, frozen–thawed cleavage embryos were divided into 2 groups according to their status as follows: with at least 1 optimal embryo and without optimal embryos. Secondly, both of them were divided into 2 groups according to the culture time (2 h or overnight). Age of the female, infertility factors, clinical pregnancy, implantation rate, and live birth rate were compared.

Results. There were no statistically significant differences in the pregnancy rate, the implantation rate, live birth rate, the miscarriage rate, and the ectopic pregnancy rate in each group. However, the implantation rate increased after 2 h of incubation (41.1%) compared to overnight incubation (36.0%) in the group with at least 1 optimal day-3 embryo ($p < 0.05$). The cancellation rate in the suboptimal day-3 embryos group (9.1%) was higher than in the group containing at least 1 optimal embryo (0.2%) for the long (overnight) culture ($p < 0.05$).

Conclusions. The implantation rate can be improved in the optimal day-3 embryos transferred after 2 h of culture, but not for suboptimal day-3 embryos. Some unnecessary transfers can be avoided after overnight culture because of no further cleavage of the embryos.

Key words: assisted reproductive technology, embryo transfer, vitrification, frozen–thawed, clinical outcomes

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Introduction

The first human pregnancy from a frozen embryo was achieved using a slow freezing protocol in 1983.[1] Since then, an increasing proportion of pregnancies are conceived after frozen embryo transfer (FET).[2,3] Embryo cryopreservation can help reduce multiple birth rate following assisted human reproduction and can also maximize the effectiveness of the in vitro fertilization (IVF) cycles.[4] Similarly, embryo cryopreservation is a crucial tool in cases of canceled embryo transfer (ET) due to ovarian hyperstimulation, endometrial bleeding, elevated serum progesterone levels on the day of triggering, preimplantation genetic screening (PGS), preimplantation genetic diagnosis (PGD), or any other unplanned events.[5–7]

In thawed embryo transfer cycles, the most common method is to transfer the embryos after 2 h of culture. One study suggested that a short post-thaw culture period is associated with higher implantation and live birth rates.[8] However, compared to studies without further development during culture, several papers showed that significantly higher pregnancy and delivery rate was observed after transferring frozen–thawed embryos that had undergone cleavage requiring an overnight culture after thawing followed by transfer of the developing embryo.[9,10] In addition, another study indicated that the pregnancy outcomes for embryos thawed and cultured overnight before transfer and those thawed and transferred on the same day are the same.[11] These studies focused only on comparing the clinical outcomes following frozen–thawed transfer of embryos with different post-thaw culture time. However, we wondered whether the post-thaw culture time should vary according to the embryo quality before freezing.

In this study, we retrospectively compared the clinical outcomes of 2-hour culture with overnight culture in good quality vs lesser quality embryos to explore if the embryos with different qualities should be treated differently after thawing.

Material and methods

Study design

This study was declared exempt by the ethics committee of the Guangzhou Women and Children's Medical Center, China. Due to the retrospective nature of this study, patients' informed consent was not needed. A total of 1,654 cycles of in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) frozen–thawed transfer cycles (vitrified-freezing) performed at our hospital from May 2010 to May 2015 were retrospectively analyzed. The causes of infertility before embryo transfer included female factors, male factors, combined factors, and unexplained infertility.

Morphology of day-3 embryos before vitrified-freezing were assessed according to the Istanbul consensus.[12] Embryos with 7–8 equally sized mononucleated blastomeres and <10% fragmentation were defined as optimal embryos. We divided the 1,654 cycles into 4 groups based on the quality of the embryos and the culture time before freezing. Group A1 (n = 321) had at least 1 optimal embryo on day 3 transferred after 2 h of thawing. Group A2 (n = 531) had at least 1 optimal embryo on day 3 cultured overnight (about 24 h) before FET. Group B1 (n = 196) had no optimal day-3 embryos transferred after 2 h of thawing. Group B2 (n = 541) had no optimal day 3 embryos cultured overnight before FET (Fig. 1).

Cleavage stage embryo cryopreservation and thawing

Vitrification was used for the cryopreservation of the embryos according to the manufacturer's protocol (ARSCI Inc., Longueuil, Canada). Embryo grading was done based on morphology: grade A – less than 10% fragmentation and equal blastomeres; grade B – 10–30% fragmentation and equal blastomeres; grade C – 30–50% fragmentation and/or unequal blastomeres; and grade D – more than 50% fragmentation.[13] Our strategy was to freeze the supernumerary embryos only if they exhibited a favorable grading with less than 30% fragmentation of the blastomeres (grade A and B). Embryo quality was evaluated again after thawing and before transfer. The evaluation criteria for morphological survival of these frozen–thawed embryos were as follows: more than 50% of the initial number of blastomeres intact and no signs of damage to the zona pellucida; otherwise thawing was considered to have failed.[13] All of the surviving embryos were cultured in G1/G2-Plus medium (Vitorlife AB, Västra Frölunda, Sweden) after thawing; parts of the embryos were transferred after 2 h of thawing. For the embryos undergoing overnight culture, only embryos with further cleavage of at least 1 cell were considered viable for transfer.

Endometrium preparation and thawed embryo transfer

The endometrium was prepared for transfer in one of two ways: hormone replacement therapy (HRT) or natural cycle (NC). Patients with regular ovulation were treated with FET in an NC. A transvaginal ultrasound was used to monitor follicular growth and endometrial thickness on day 10 of the menstrual cycle. If the endometrial thickness reached 8 mm or more, the cycle was considered suitable for FET. Frozen embryo transfer was performed in an HRT cycle if patients did not ovulate regularly. Oral estrogen 6 mg/day was commenced on the 3rd day of the menstrual cycle for 12 days. When the endometrial thickness reached 8 mm or more, injections of progesterone at 60 mg/day were administered for 3 days before FET, and the dosage was maintained after FET.[14]

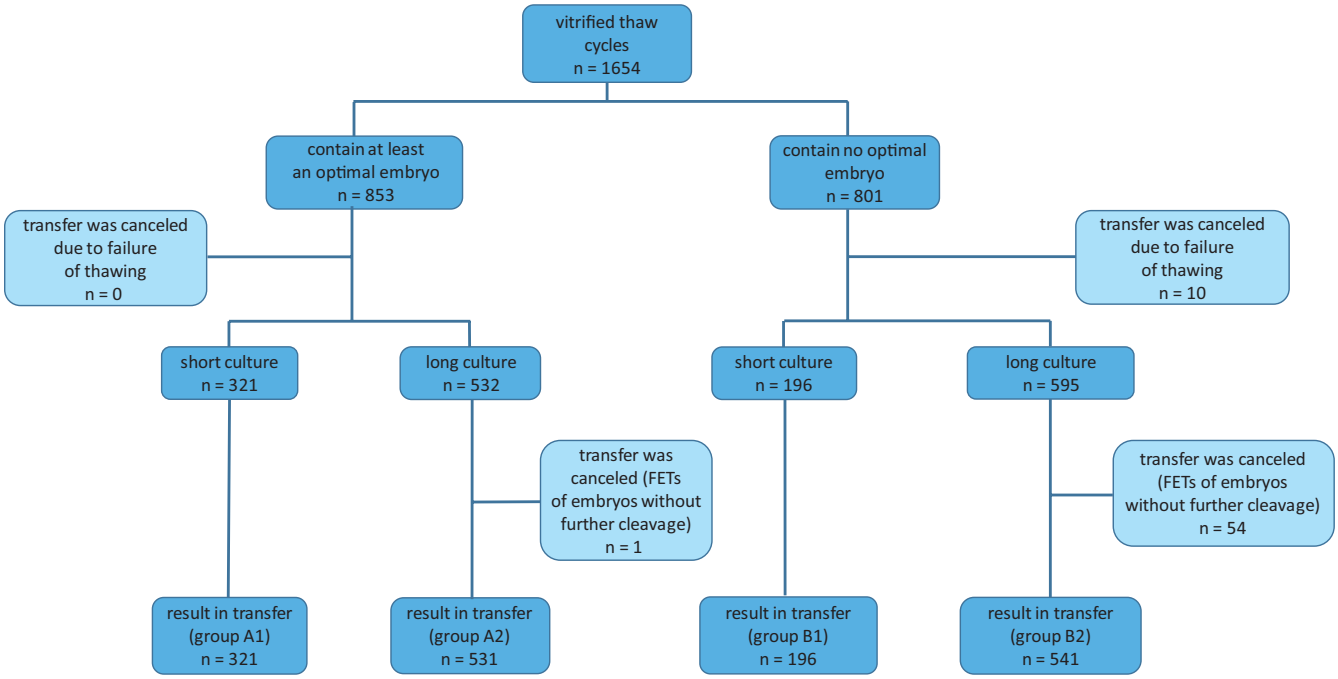

Fig. 1. Flow chart summarizing thawed embryo accountability of the study. The groups were divided by 2 aspects: the quality of the embryos and the culture duration after thawing

Pregnancy outcome

Clinical pregnancies (CP) were defined after observation of a gestational sac with or without a fetal heartbeat on ultrasound evaluation 4 weeks after FET. The number of sacs was taken as the number of successful implantations. Clinical miscarriage was defined when a pregnancy failed to progress after an intrauterine gestational sac had been detected with a pelvic ultrasonography. Live birth rate was defined as number of live births per number of FET. Ectopic pregnancy rate was also calculated.

Statistical analysis

Statistical analysis was performed using SPSS software v. 19 for Windows (SPSS Inc., Chicago, USA), applying parametric and nonparametric tests when appropriate. Continuous variables were expressed as mean ± standard deviation (SD) and analyzed using Student's t-test. Categorical variables were expressed as percentages and analyzed using χ^2 or Fisher's exact test depending on the sample size. Statistical significance was defined as p-values less than 0.05.

Results

Comparison of demographic characteristics

There was no significant difference between the groups regarding age, body mass index (BMI) as well as basal follicle-stimulating hormone (FSH), and estradiol (E2) levels ($p > 0.05$) (Table 1).

Analysis of immediate morphological survival and further cleavage

Out of a total of 1,654 cycles, 853 cycles contained at least 1 optimal embryo. Eight hundred and one cycles had no optimal embryos. Three hundred and twenty-one of the thawed cycles were transferred after 2 h of culture (group A1). In group A2, 1 FET cycle was canceled because no further cleavage was observed, so the total number of transfers was 531. In groups B1 and B2, 10 cycles were canceled because of thawing failure. In group B1, 196 FET cycles were transferred after 2 h of culture; in group B2, 54 FET cycles were canceled, because no further cleavage was observed for a total of 541 embryo transfers. The cancellation rate for group B (9.1%) was statistically significantly higher than in group A (0.2%) ($p < 0.05$) (Table 2).

Clinical outcomes

The clinical outcomes of 2 post-thaw cultures for optimal day-3 embryos (A1 vs A2) and suboptimal day-3 embryos (B1 vs B2) were compared. The pregnancy rate, live birth rate, miscarriage rate, and ectopic pregnancy rate were similar between the 2 groups but the implantation rate was higher after 2 h of incubation (41.1%) compared to overnight incubation (36.0%) ($p < 0.05$).

The pregnancy rate, live birth rate, miscarriage rate, ectopic pregnancy rate, and implantation rate of suboptimal

Table 1. Comparison of demographic characteristics of 2 post-thaw culture lengths for different quality of day-3 embryos

Parameter	At least 1 optimal embryo			No optimal embryo		
	short culture (n = 321)	long culture (n = 531)	p-value	short culture (n = 196)	long culture (n = 541)	p-value
Average age	32.03 ±4.63	31.74 ±4.62	NS (0.481)	32.89 ±4.60	32.60 ±4.76	NS (0.447)
EMT-BMI	21.10 ±2.88	20.89 ±2.68	NS (0.302)	21.06 ±2.39	20.82 ±2.62	NS (0.277)
Basic FSH	5.66 ±3.04	5.77 ±2.32	NS (0.559)	5.85 ±2.01	5.93 ±2.04	NS (0.639)
Basic E2	134.29 ±73.24	133.49 ±86.32	NS (0.893)	131.55 ±70.60	143.72 ±148.74	NS (0.271)

NS – not significant; EMT-BMI – body mass index; FSH – follicle stimulating hormone; Basic E2 – estradiol.

Table 2. Analysis of immediate morphological survival and further cleavage for different quality of day-3 embryos

Parameter	At least an optimal embryo (n = 853)	No optimal embryo (n = 801)	p-value
Failure of thawing rate [%]	0/853 (0)	10/801 (0.01)	0.001
Cancel rate without further cleavage [%] (only for long culture)	1/532 (0.2)	54/595 (9.1)	0.00

day-3 embryos were similar between 2 different post-thaw culture groups ($p > 0.05$) (Table 3).

Discussion

All optimal embryos survived after thawing; however, 10 transfers were canceled due to failed thawing in the groups with only suboptimal embryos. We hypothesize that optimal embryos may better cope with stress during the frozen–thawed procedure, so high-quality embryos may suffer less damage during the process of cryopreservation and low-quality embryos may suffer more blastomere damage during the frozen–thawed procedure.

At present, the most common frozen–thawed transfer cycle method is to thaw the embryos and transfer them after 2 h of culture. The blastomere survival after thawing only can be identified in 2-hour culture. Furthermore, some studies reported that the developing potential of the thawed embryos appears to rely upon the resumption of mitosis, which may require a longer culture, generally overnight.[15,16] Other studies also reported significantly better clinical outcomes after a longer culture time.[17,18] Other reports found that cleavage-arrested embryos at 24 h following thawing were nevertheless still able to show some signs of further cleavage up to 48 h after thawing and could be implanted, but these embryos may have had a higher rate of chromosomal aberrations and more frequently ended in clinical abortion.[19–21] In this study, there was only 1 canceled cycle in group A2 (at least 1 optimal embryo, overnight culture), but in group B2 (suboptimal embryos, overnight culture), 54 thawed cycles were canceled because no future cleavage was observed after overnight culture. This suggests that optimal embryos may have stronger development potential. If these 54 cycles had been transferred within 2 h of thawing, it may have resulted in an early pregnancy loss or fetal anomaly because of chromosomal aberrations and waste of resources. For the cycles without optimal embryos, it may be beneficial to prolong culture time (to overnight) after thawing in order to observe initial cleavage, so that some unnecessary transfers can be avoided.

We also compared the clinical outcomes of the 2-hour culture with the overnight culture to explore the specific effect of post-thaw culture time. We found that in the group with at least 1 optimal day-3 embryo, there

Table 3. Comparison of the clinical outcomes of 2 post-thaw culture times for different quality of day-3 embryos

Parameter	At least 1 optimal embryo			No optimal embryo		
	short culture (n = 321)	long culture (n = 531)	p-value	short culture (n = 196)	long culture (n = 541)	p-value
Clinical pregnancy rate [%]	189/321 (58.9)	285/531 (53.7)	NS (0.14)	68/196 (34.7)	206/541 (38.1)	NS (0.40)
Implantation rate [%]	280/682 (41.1)	391/1087 (36.0)	0.03	89/414 (21.5)	262/1115 (23.5)	NS (0.41)
Miscarriage rate [%]	23/189 (12.2)	30/285 (10.5)	NS (0.58)	14/68 (20.6)	34/206 (16.5)	NS (0.44)
Ectopic pregnancy rate [%]	11/189 (5.8)	18/285 (6.3)	NS (0.83)	5/68 (7.4)	11/206 (5.3)	NS (0.54)
Live birth rate [%]	155/321 (48.3)	237/531 (44.6)	NS (0.29)	49/196 (25.0)	161/541 (29.8)	NS (0.20)

NS – not significant.

was no significant influence of culture time on most clinical outcomes, but the implantation rate increased after 2 h of incubation compared with that of the overnight incubation group, which was consistent with the findings from a previous study.[8] The result of the increasing implantation rate after 2 h of incubation may be associated with the environmental impact on the potential of embryo development. Although embryo culture aims to mimic the fallopian tube and uterine environment, it always implies the induction of some related stress.[13,22,23] It thus seems reasonable to hypothesize that frozen–thawed suboptimal embryos may be less able to adapt to suboptimal environments such as the currently available culture systems. We also hypothesize that the suboptimal embryos may express oxidative stress in the cryopreservation medium, and higher oxidative stress levels are associated with lower implantation rates.[24] Therefore, we think that the implantation rate for optimal day-3 embryo transfers after 2 h of culture can be improved.

Conclusions

The conclusions from this study are based on retrospective data and have the inherent limitations of retrospective studies. For optimal quality embryos, 2 h of post-thaw culture appear to optimize clinical outcomes; however, for embryos of suboptimal quality, some unnecessary transfer of non-functional day-3 embryos can be avoided after overnight culture which allows for the identification of embryos that fail to undergo additional cleavage.

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Current vascular allograft procurement, cryopreservation and transplantation techniques in the Czech Republic

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Abstract

Background. Vascular allotransplantations are performed worldwide in selected patients suffering from vascular prosthesis infection or critical limb ischemia. Either fresh or cryopreserved vascular allograft may be used.

Objectives. In various points, we address several aspects (allograft procurement, cryopreservation and transplantation technique) of the program of vascular allotransplantations in the Czech Republic.

Materials and methods. Vascular grafts retrieval has been done within multiorgan harvests using no-touch technique. Very short time of cold ischemia is achieved due to close cooperation with Tissue Establishment where the following processing of cryopreservation is performed. Meeting all necessary quality criteria is a prerequisite for releasing grafts for clinical application. Standardized thawing protocol and surgical handling aims to minimize microfractures before implantation.

Results. Based on experimental and clinical work, the first validation of cryopreserved arterial and venous grafts for clinical use was performed between 2011 and 2013 in the Czech Republic. The developement of storage of vascular tissue in banks was stimulated in 2000–2010 by the issue of EU directives and national harmonized norms, aimed at assurance of high quality and safety of cells and tissues used for transplantations in humans.

Conclusions. There are several crucial moments affecting final quality, including graft retrieval within a multiorgan harvest, short ischemic time, cryopreservation, and thawing technique used. The recommended surgical handling during implantation may also affect results and graft-related complications.

Key words: tissue banking, cryopreserved vascular allograft transplantation, operative procedures, graft procurement, cryopreservation

Introduction

Vascular allotransplantations are performed worldwide in selected patients suffering from vascular prosthesis infection or critical limb ischemia. Either fresh or cryopreserved vascular allograft may be used. In this paper, all the aspects and the up-to-date state of the transplantation program of cryopreserved vascular allografts in the Czech Republic introduced in 2011 are presented.

In this country, there is a network of licensed surgical facilities performing vascular graft retrieval as a part of multiorgan recovery. These participating centers – Transplant Centers or Vascular Surgery Centers – were licensed for this activity by the State Institute for Drug Control (SUKL) as Procurement Establishments (PE) tightly connected by agreements with the licensed Tissue Establishment (TE) – Tissue Bank of the University Hospital Hradec Králové, Czech Republic. All the licenses were granted after providing proof of full compliance of practice in these facilities with the strict safety and quality requirements established by the UE Directives 2004/EC, 17/2006/EC and 2006/86/EC, and the national harmonized legal norms: Act No. 296/2008 Coll. (Human Cell and Tissue Act) and Decree of the Ministry of Health No. 422/2008 Coll.[1,2] Cryopreservation of collected grafts, subsequent storage at liquid nitrogen temperatures and quality control until the release of grafts for clinical application are the main duties of the tissue bank. This service is, however, accessible only for transplant centers involved in the program. Distribution of grafts is performed by a licensed company able to perform emergency and rapid transport of cryopreserved grafts in a vapor phase of liquid nitrogen to any destination in the Czech Republic.

Material and methods

Vascular graft procurement techniques

To meet the requirements of the SOP (standard operating procedure) provided by the PE with the aim to achieve the quality and safety of cryopreserved grafts, it is necessary to retrieve the blood vessels within multiorgan harvests.[3] The responsible person at each PE, an experienced vascular surgeon, guarantees that all surgical procedures are performed according to accepted SOP and all required documentation is maintained at the PE and/or sent to the TE as well. They are also responsible for the education and training of all surgeons included in the list of persons competent to perform graft recovery, and for reporting on any incidents of SAR (serious adverse reaction) and SAE (serious adverse event) that may occur in connection with the graft retrieval. If possible, it is necessary to perform perfusion through the internal iliac artery. Ideally, the artery is cross-clamped distally from the point of the harvested vessels. During a multiorgan harvest, a no-touch technique is routinely used when operating on the arterial system – aortic bifurcation ranging from renal arteries to superficial femoral arteries (10 cm) with side branches at least 1 cm long. For the collection of 1-sided arteries, harvest is started from the external iliac artery to the popliteal artery. The saphenous vein is collected in total length (Fig. 1). The spectrum of blood groups is harvested with a preference for type 0. The tissue bank also keeps unusual grafts in limited quantities: carotid bifurcation, the aortic arch with head arteries, inferior vena cava, and iliac vein bifurcation, preferentially of blood type 0. The grafts are replenished as needed. A very short time of cold ischemia (hours) is achieved due to close cooperation with the TE, where grafts are also processed during nights and weekends.

The vessels collected are immediately placed into a pre-cooled Celsior preservation solution (Genzyme, Cambridge, USA) supplemented with gentamicin and stored in sterile certified plastic jars (Medfor 250 mL; Medfor, Farnborough, UK). The jars are transported to the TE at the temperature of melting ice within 12 h after the harvest together with the documentation of the harvest and samples of the donor's blood to perform serology tests in the licensed diagnostic laboratories (Department of Clinical Microbiology and Department of Clinical Immunology of the University Hospital Hradec Králové – UHHK).

Cryopreservation

Vascular graft cryopreservation is performed using the SOP required by the TE (Tissue Bank of the University Hospital Hradec Králove) fully licensed by a national competent authority.[3,4] In the procurement and processing of vessels, only high-quality materials and drugs with approval for human use and meeting the requirements of the Directive of the European Parliament and Council No. 23/2004/EC are used.

After input control in the TE, the grafts are processed in a grade A clean room (according to the EU GMP classification) with a grade B background (Fig. 2 A,B) After decontamination using a modified van Katz[5] method, the vessels are put into double sterile disposable plastic bags (Eva Bags; Maco Biotech, Eckbolsheim, France) containing 50 mL of a pre-cooled 6% solution of hydroxyethyl starch with molecular weight of 130,000 Da (Voluven 6%; Fresenius Kabi, Bad Homburg vor der Höhe, Germany) and mixed with an equal volume of the pre-cooled cryoprotective solution (20% dimethyl sulphoxide; WAK ChemieMedical GmbH, Steinbach, Germany) (Fig. 2C). The samples of the solution for bacteriological and mycological tests are taken from the collection solution and from the final package. The plastic bags are closed using heat sealing. The bags closed into outer metal cassettes are put into the freezing chamber of the programmable freezer and frozen at a rate of 1 K/min to –90°C

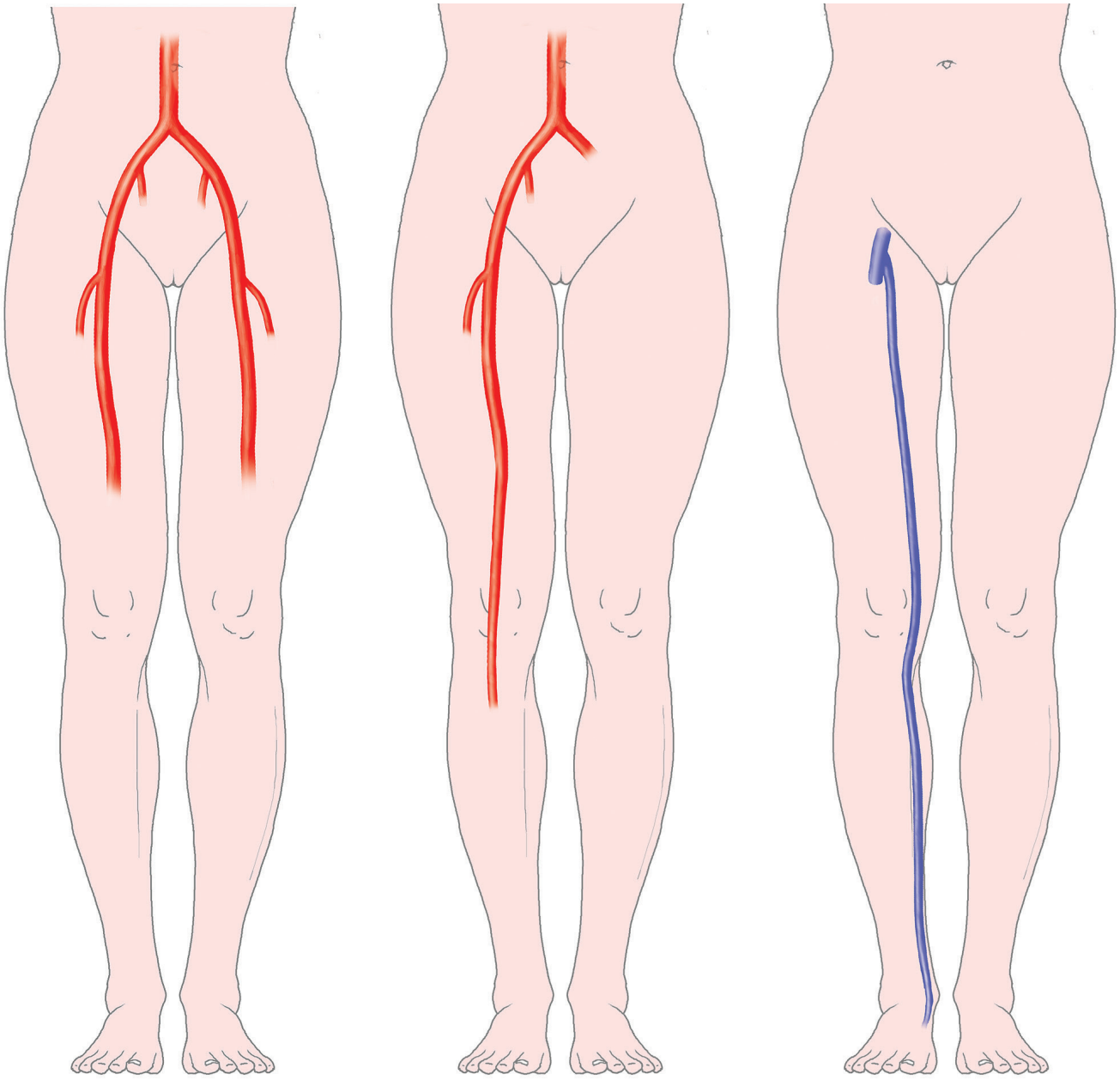

Fig. 1. Types of harvested vessels: There are 3 routinely harvested types of vessels – aortic bifurcation with iliac and femoral arteries, unilateral arterial graft and saphenous vein graft

(5 K/min to –150°C follows) (Fig. 2D). The grafts are stored until clinical use in the vapor phase of liquid nitrogen in the biological container equipped with an automatic filling system and continuous temperature monitoring (Fig. 2E).

Quality criteria for release of grafts for clinical application

The grafts can be released for clinical use by the responsible person from the TE only. The criteria for release are listed below:

- absence of contraindication for harvest in the clinical and anatomical diagnoses and patient's medical history;
- good quality of the harvested tissue reported by the responsible person of the PE;
- absence of laboratory signs of infection as determined by the serology tests of the donor;
- absence of contamination of recovered grafts by pathogenic bacteria, molds or fungi;
- proof of sterility at the output control;
- absence of serious deviations from the SOP during retrieval, transportation, processing, and storage of grafts.

Reporting of SAR and SAE is connected with procurement, distribution and transplantation of grafts and is another important feature of the quality assurance system. A register for recording all clinical results achieved in all centers using cryopreserved grafts was established

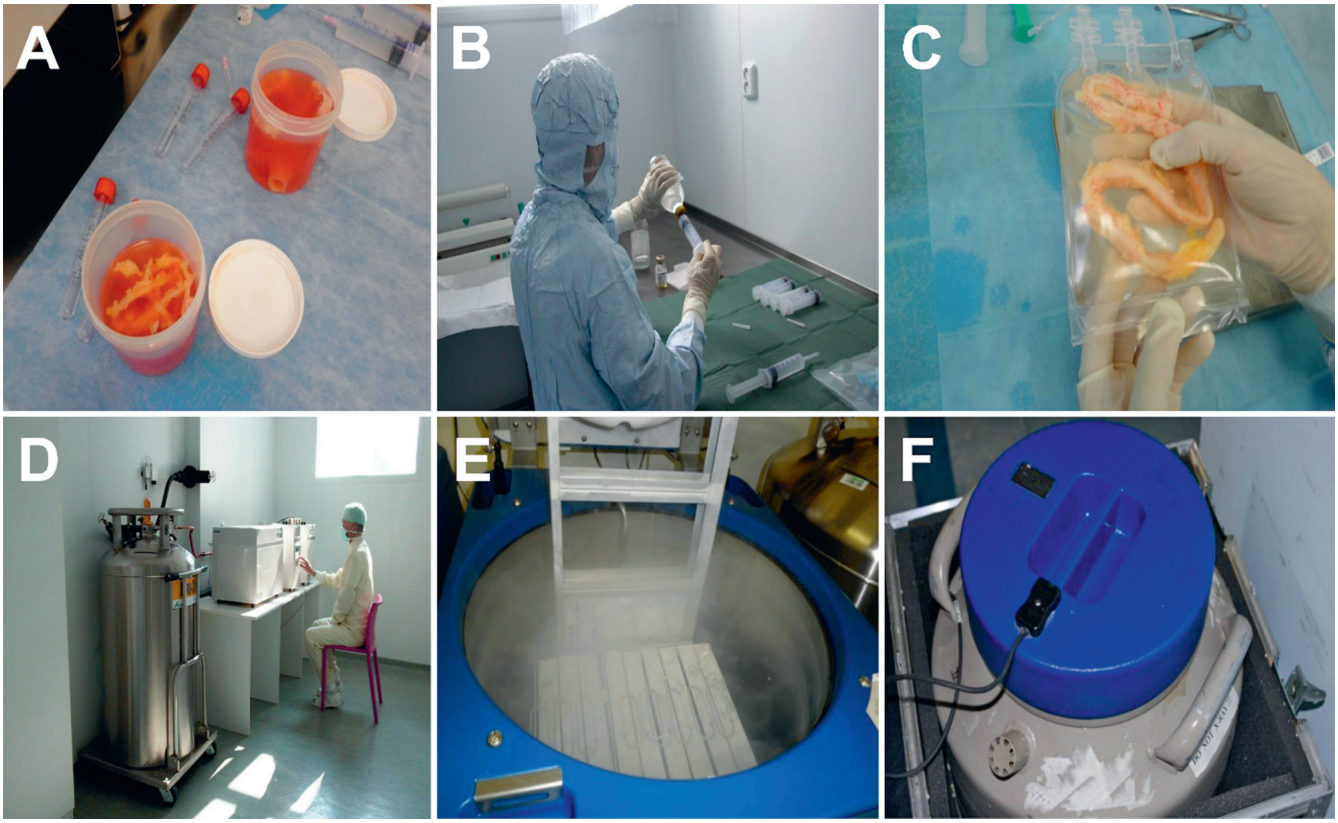

Fig. 2. Vascular allograft cryopreservation, storage and transport process

in the Institute for Clinical and Experimental Medicine in Prague, Czech Republic, as a tool for evaluation of long-term results of vascular transplantation in the Czech Republic.

Thawing

After removal from the storage container, the cassettes with bags are transported to the operating room in the vapor phase of liquid nitrogen in a special Dewar vessel-dry shipper (Fig. 2F). In the operating room, the cassettes are removed from the shipper and placed into a refrigerator with temperature rising from +2°C to +8°C within 2 h. If some ice is still present after removal from the refrigerator, thawing can be completed at room temperature. Immediately after the ice melts, the vessels are aseptically removed from the bags and stored in the pre-cooled preservation solution (Celsior; Genzyme) until implantation.

Surgical handling

The surgical technique of vascular transplantation requires that side branches of the grafts be treated with Prolene sutures (Ethicon, Somerville, USA), avoiding any ligation or clipping. Under no circumstances should the allografts be cross-clamped – only the native vascular system of the patient can be cross-clamped during the operation. Proximal anastomosis is performed first. Later, under arterial pressure, the correction of sutured side branches is performed if needed. Afterwards, the graft is passed through the prepared tunnel, avoiding any rotation. Distal anastomosis of the bypass is performed. The surgical wounds are extensively drained.[6]

Results

Clinical application

The first transplantation of a cryopreserved arterial allograft was performed in 2011. Five years of follow-up were uneventful (Fig. 3). A total of 87 cryopreserved vascular allografts were delivered for clinical application between 2011 and 2016 in the Czech Republic. A total of 59 saphenous vein grafts, 12 aortic bifurcations and 16 iliaco-femoro-popliteal (unilateral) grafts were used for bypass grafting. Our aim is to have all anatomical types of grafts in all blood groups (Table 1,2). A preference for grafts retrieved from donors with blood type 0 is evident, as an advantage in the case of a lack of anatomical types in TB stock.

Although the first experimental transplantations of vascular allografts or xenografts were performed more than 100 years ago[7] and the mechanisms of freezing damage and cryoprotection have been known since the middle of the last century,[8] some theoretical and clinical aspects of this surgical procedure still remain unsolved.

Some authors describe good results achieved using transplantation of fresh arterial grafts with immunosuppression.[9,10] Some research groups[11] point out the advantages of transplantation of cryopreserved grafts, such as the low probability of infection transmission and low immunogenicity leading to limited cellular and humoral rejection – in contrast to the acute rejection of fresh grafts which leads to progressive degeneration of elastic fibers and connective tissue and aneurysm formation if immunosuppression is not used. Other authors[12,13] are convinced that certain cryopreservation protocols are responsible for early ruptures of grafts that may occur even intraoperatively and are always associated with life-threatening complications. It seems, however, that such serious adverse events are less likely to occur if cryopreservation protocols based on equilibrium and slow freezing are used. Such procedures are followed by, e.g., the European Homograft Bank in Brussells (EHB)[14,15] and other centers, including our TE.[16,17]

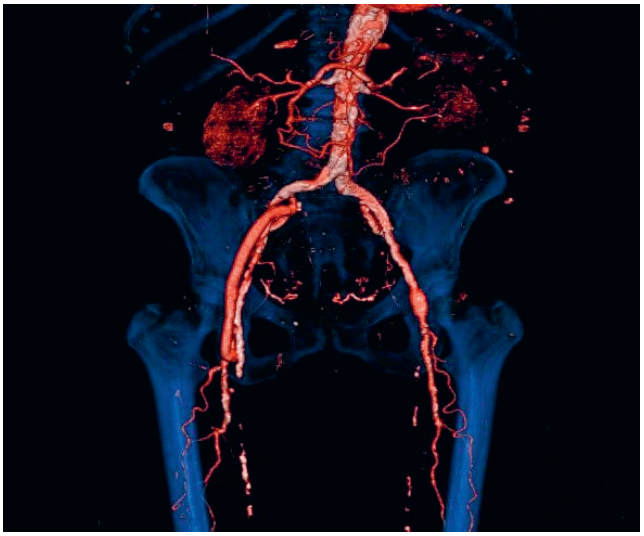

Fig. 3. First transplantation of cryopreserved arterial graft as a validation of the program was performed in 2011 in 81-year-old woman who presented with femorofemoral crossover prosthetic bypass virulent infection. Due to severe calcifications of abdominal aorta and common iliac artery an atypical iliofemoral bypass was performed

Table 1. Distribution of blood groups in venous cryopreserved grafts

Blood group	2011–2013	2014	2015	2016	Total	Rate [%]
0	2	4	6	3	15	54
A	0	2	7	0	9	32
B	0	1	1	0	2	7
AB	0	2	0	0	2	7
Total	2	9	14	3	28	100

Table 2. Distribution of blood groups in arterial cryopreserved grafts

Blood group	2011–2013	2014	2015	2016	Total	Rate [%]
0	2	4	6	3	15	54
A	0	2	7	0	9	32
B	0	1	1	0	2	7
AB	0	2	0	0	2	7
Total	2	9	14	3	28	100

It must not be forgotten, however, that not only the freezing protocol itself but also the pre-freezing history of the graft may be responsible for such events. Our previous study in dogs[18] showed that hypothermic storage of vessels in physiological saline for several days lead to considerable edema of the vessel wall. This finding shows the enhanced probability of vessel wall injury caused by crystal formation during freezing. For this reason, we consider it very important to use exclusively organ preservation solutions for intermediate hypothermic storage immediately after vascular graft harvest and to strictly control the timespan between the graft harvest in the PE and start of the cryopreservation procedure in the TE.[17]

Achieving relatively stable ice structures by slow freezing does not, however, guarantee complete avoidance of the devitrification phenomena during thawing, as demonstrated by Pegg et al. in experiments in rabbits.[19] They proved that the formation of microfractures in arteries caused by devitrification during fast thawing was responsible for graft rupture. This finding led us to the implementation of a slow-thawing protocol. In addition, injury caused by recrystallization is prevented in our practice by strict use of a cold chain based on the use of liquid nitrogen temperatures for storage and transport of grafts till thawing before use in the operating room. This is in contrast to the practice of some TEs,[14] which use temperatures of –80°C for transport and even allow intermediate storage of grafts at these temperatures directly in cooperating surgical departments if the graft is not used immediately. In our practice, the graft is always sent back to the TE in the transport cryocontainer if the surgical intervention in the patient must be postponed for unexpected reasons.

Long-term storage of cardiovascular grafts in cryobanks was introduced before 2000.[14,15,20] Development of the storage of vascular tissue in these banks was stimulated in 2000–2010 by the issue of EU Directives and national harmonized norms such as Act No. 296/2008 Coll in the Czech Republic[1,4] or the Tissue Act in Germany,[2] aimed at the assurance of high quality and safety of cells and tissues used for transplantation in humans. This law caused radical changes in the standard procedures used in the tissue banks, including the recovery and processing of vascular tissue.[1,2,4,13] In contrast to Germany, where the use of fresh grafts was practically stopped, Czech law allows the use of fresh grafts in the regimen of organ transplantation regulated by Act No. 285/2002 Coll. within 48 h after harvest.

There is also a difference between the required purity of the environment in the graft processing areas. While Czech law permits the use of grade A environments (according to the EU GMP classification) with a grade C

background, German law requires the same environmental conditions as in manufacturing of sterile medicinal products, i.e., a grade A processing area with a grade B background. As the TE of the UHHK has been retrofitted in compliance with the standards of the International Society for Pharmaceutical Engineering,[21] it is able to assure this high level of quality of the environment that is regarded as standard in other Western European countries.[11] National law may also set some restrictions of the use of grafts. While in some countries, the free sale of grafts to surgical departments is possible, in Germany the use of cryopreserved vascular grafts is strictly limited to clinical trials only.[13] The situation in the Czech Republic is somewhere between these 2 extremes; the use of both fresh and cryopreserved grafts is limited to accredited transplantation or vascular transplantation centers.

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Observations on surgical reconstructive management following the excision of malignant neoplasms of the eyelid and periocular area

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Abstract

Background. The surgical treatment of malignant neoplasms of the eyelid and the periocular area, due to the complex structure of the eye protective apparatus, remains a difficult surgical problem. The aim is to reconstruct the missing tissue as precisely as possible, both from a functional and esthetic point of view. Postoperative disorders of eyelid function may considerably disturb both the functioning and the quality of life of the patients.

Objectives. The aim of the study was to evaluate 262 patients who had underwent operations related to malignant neoplasms of the eyelid and the periocular area, and to demonstrate which reconstruction methods for post-resection defects were the most advantageous in terms of functions and esthetics.

Material and methods. The study was based on an analysis of the medical records of 262 patients. The analysis included the reconstruction techniques used, the patient's age, the sizes and locations of the primary skin cancer, any healing complications, and the distant esthetic and functional outcomes.

Results. Various types of flap procedures were the most frequently used techniques in the studied group of patients (66.8%). Single flaps were used in 83 patients, multiple flaps in 89 patients and complex flaps with a cartilage graft in 3 patients. Free skin grafts were used in 52 patients and were associated with complications in the form of partial necrosis. Complications during postoperative wound healing were observed in 18.3% of the patients. In 12 patients, the complications were associated with a local infection, in 15 patients with partial, marginal necrosis and in 8 patients partial wound breakdown after the transfer of the flap occurred.

Conclusions. The most frequently used mechanisms for correction and reconstruction of the deficits following the excision of the eyelid skin and periorbital malignancies included various flaps used in a total of 175 patients (66.8%).

Key words: flap, wound healing, periorbital neoplasm

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The treatment of eyelid and periocular area malignancies, due to the complex structure of the eye protective mechanism, requires careful surgical management, as the eyelid constitutes an esthetic facial unit that, apart from the protective function for the eye, plays an important role in the expression of feelings, mood and emotions. Eyelid malignancies and the deficits following their resection often constitute a difficult surgical problem that involves the closest possible reconstruction of the resected tissue in a way that preserves both the functional and esthetic values. Postoperative complications and deformations may disturb eyelid function as a protective apparatus and can significantly impair the patient's functioning and quality of life.[1,2]

Reconstruction of the anatomical eyelid structures lost as the result of surgery poses a challenge to the surgeon. It requires not only an understanding of the anatomy and physiology of this part of the face, but also the skills and experience to ensure effective plastic surgery, as well as a sense of esthetics.[3,4] The primary purpose of reconstructive and corrective procedures is to reduce the tissue deficits, to preserve the eyelid function and its protective mechanism for the eye via proper adhesion of the eyelid to the eye, and to maintain tear duct patency. The esthetic effect is equally important as it has a significant impact on the patient's quality of life.[4–6]

Objectives

The aim of the study was to present the methods used for eyelid and periocular area reconstruction following the resection of skin malignancies in patients operated on at the Department of Maxillofacial Surgery of the Frederic Chopin Clinical Voivodeship Hospital in Rzeszów, Poland, in the years 2006–2015, to demonstrate which of the applied methods of post-resective tissue reconstruction were the most frequently used, and to investigate if there were any correlations between the surgical site and the method of reconstruction.

Material and methods

The study was based on a review of the medical records of 262 patients hospitalized at the Department of Maxillofacial Surgery of the Frederic Chopin Clinical Voivodeship Hospital in Rzeszów due to malignancies of the eyelid and the periocular area. This group of patients comprised 139 females and 123 males, aged 23–94 years old. Most surgeries were performed under general anesthesia and involved the excision of the primary tumor with a standard oncological margin, followed by the reconstruction of the tissue deficits during the same procedure. In clinically advanced cases with a risk of cancer recurrence, the intra-operative radical nature of the procedure and the surgical margins were assessed.

The statistical analysis of the material was conducted using STATISTICA v. 10.0 software (StatSoft Inc., Tulsa, USA). The Pearson's χ^2 test was used to assess the relationships between tumor location and the treatment procedures. The 1-tailed significance test for structure indicators was used to verify the hypotheses of proportion values for the given answers. The statistical significance level assumed was $p < 0.05$.

Results

Various techniques were used to reconstruct the post-resective tissue deficits in the periocular area.[4,5,7] Figure 1 presents the methods of reconstruction of the tissue deficits following resection of the primary tumors.

The most frequently used method for eyelid and periocular area reconstruction involved the use various dermo-fascial flaps in a total of 175 patients (66.8%) as single, multiple or complex flaps. Single flaps were used in 83 patients (31.7%) and multiple flaps in 89 patients (33.9%) according to the individual reconstructive requirements.

Complex flaps with a cartilage graft were used in 3 patients (1.2%). No statistically significant differences were found in the frequency of single or complex flap procedures. Single-flap procedures were not statistically more frequent than the next most common method, free skin grafts, although the relationship was approaching the limit of statistical significance. The multiple-flap procedure was performed statistically significantly more often than the free skin graft procedure ($p = 0.0371$).

Table 1 presents the surgical management methods according to the primary tumor site. Following the lead of the authors from Yale University, the division of the eyelid area into 5 zones was adopted: upper eyelid – zone I, lower eyelid – zone II, medial canthus – zone III, lateral canthus – zone IV, and the suborbital and lateral-frontal area – zone V.[7] The data presented in Table 1 confirms the relationship between the tumor site and the reconstruction management method ($p < 0.001$).

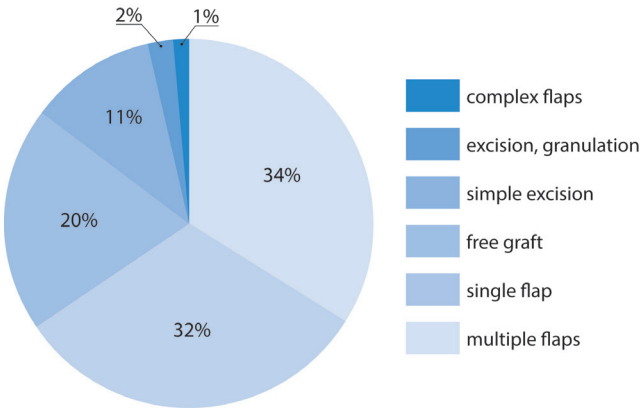

Fig. 1. Methods of tissue reconstruction following the excision of malignant eyelid neoplasms

Zone I (upper eyelid) deficits were treated in a total of 26 patients. No statistical significance was observed regarding the most frequently used methods of reconstructive management. Island flaps, which were used independently in 4 patients (15.4%), and in combination with a free skin graft in 9 patients (34.9%), were applied if the deficit, often full-thickness, exceeded 50% of the eyelid area.

In 7 cases, a dermo-fascial flap from the lower eyelid was used. A total of 6 patients with smaller deficits (25–50% of the eyelid volume) were treated with a simple suture of the postoperative wound.

Zone II (lower eyelid) deficits were reconstructed the most frequently: in 40 patients (48.2%) with single flaps using the Mustardé method, and in 19 patients (23.0%) with a Mustardé flap strengthened by a subcutaneous island pedicle flap. These were full-thickness eyelid tissue deficits, and the relationships were statistically significant ($p = 0.047$), compared to the other methods presented in Table 2. In 3 patients (3.6%), a complex auricular perichondrium-cartilage graft was used. Partial-thickness tissue deficits in this location of less than 1 cm in 7 patients (8.4%) were closed by simple suturing of the operative wound using dermo-fascial flaps from the adjacent tissue or a free full-thickness skin graft, or were left for granulation.

Zone III (medial canthus) deficits were treated in 124 patients. This is the most difficult periorbital location due to the specific anatomical structure and frequent problems with obtaining a radical resection. In the cases where the medial palpebral ligament was also involved, an intraoperative assessment of the surgical margins was performed.

In this location, subcutaneous island pedicle flaps were used, formed in the area of the glabella, the upper eyelid and the nasolabial fold. Two island flaps formed opposite the deficit and moved towards each other were used in 41 patients (33.1%); single flaps were used in 23 patients (18.5%), while in 12 patients (9.7%) a free full-thickness graft was used in combination with the island flap to cover the deficit. In deficits of less than 1 cm, a simple excision was performed and the wound was left for granulation. The medial canthus island flap and 2 island flaps, compared to the frequency of the next most common method (free skin graft), were statistically insignificant.

Zone IV (lateral canthus) deficits were significantly more frequently reconstructed using a full-thickness free skin graft, as confirmed by the statistical analysis ($p = 0.017$). Zone V (the suborbital and lateral frontal area) was not the subject of the presented study.

The healing of postoperative wounds in 214 patients (81.7%) was uncomplicated, whereas complications occurred in 48 patients (18.3%), which extended the healing period. In 12 patients (4.6%), the complications were associated with a local facial infection. Partial marginal necrosis of the free skin graft was found in 15 patients (5.7%), and partial wound breakdown after the transfer of the island flap occurred in 8 patients (3.1%). The data regarding the early outcomes of the surgical treatment is presented in Table 2.

Discussion

Skin malignancies, including eyelid neoplasms, constitute an important problem in dermatological, ophthalmological and, for several decades now, maxillofacial surgical practice. From a clinical and histological perspective,

Table 1. Methods of surgical reconstructive management in eyelid malignancies according to their location

Location	Excision granulation	Simple excision	Free skin graft	Island flap			Mustardé flap		Dermal-fat flap	Complex flap + cartilage	Total
				single	2 flaps	+ free graft	single	+ island flap			
Upper eyelid	0 (0.0%)	6 (23.1%)	0 (0.0%)	4 (15.4%)	0 (0.0%)	9 (34.6%)	0 (0.0%)	0 (0.0%)	7 (26.9%)	0 (0.0%)	26 (100.0%)
Lower eyelid	1 (1.2%)	7 (8.4%)	5 (6.0%)	3 (3.6%)	0 (0.0%)	0 (0.0%)	40 (48.2%)	19 (23.0%)	5 (6.0%)	3 (3.6%)	83 (100.0%)
Medial canthus	11 (8.9%)	5 (4.0%)	27 (21.8%)	23 (18.5%)	41 (33.1%)	14 (11.3%)	0 (0.0%)	0 (0.0%)	3 (2.4%)	0 (0.0%)	124 (100.0%)
Lateral canthus	0 (0.0%)	5 (17.2%)	20 (69.0%)	2 (6.9%)	0 (0.0%)	2 (6.9%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	29 (100.0%)
Total	12 (4.6%)	23 (8.8%)	52 (19.8%)	32 (12.2%)	41 (15.6%)	25 (9.5%)	40 (15.3%)	19 (7.3%)	15 (5.7%)	3 (1.2%)	262 (100.0%)

Table 2. Occurrence of healing complications

Number of patients	Normal healing	Local infection, purulent wound	Foreign body sensation, irritation by the suture	Partial necrosis of the graft or flap	Partial wound breakdown	Hematoma in the surgical wound
262	214	12	4	15	8	9
100.0%	81.7%	4.6%	1.5%	5.7%	3.1%	3.4%

it is a very diverse group of neoplasms, which, depending on their periorbital location, poses a considerable challenge for surgeons. Basal cell carcinoma (BCC) is the most common type of neoplasm, which is also confirmed by our own data. According to the literature, malignant neoplasms are mostly located in the lower eyelid, whereas in our own material the most frequent location was in the medial canthus (124 patients). The authors agree that eyelid malignancies require surgical resection with control of the surgical margins, followed by a reconstruction of the post-resective tissue deficit. The majority of researchers, including the authors of this article, believe that the reconstructive procedure should be performed during the same surgery as the primary tumor excision.[1–3] Some authors suggest that the method of choice depends on numerous factors: size of tissue deficit, clinical advancement, location of the lesion in the eyelid area, and experience of the surgeon. The 5 zones in the eyelid area defined by researchers from Yale University have significant use in facilitating the surgical planning process. Reconstructive procedures should provide good protection and covering for the eye, prevent drying and meet the esthetic criteria.[3–5]

Numerous methods for the reconstruction of tissue deficits following the removal of eyelid or periorbital malignancies are available in the specialist literature.[2,4,6] According to most authors, single-stage techniques are preferable, as they provide better functional and esthetic outcomes and are less burdensome for the patient.[1,2,7] The analysis of the present material demonstrates that while the choice of eyelid reconstruction method depends on the location, size and shape of the deficit following the excision of the neoplasm, other important factors are the age, sex and health status of the patient. In elderly patients, which included the majority of the patients, the loss of skin elasticity and natural wrinkles facilitate the closure of post-resective tissue defects.[1,2,6]

In the literature, many authors emphasize the classification of the tissue deficits into partial eyelid thickness deficits, which cover the skin and orbicularis oculi muscle without any damage to the tarsus, and full-thickness eyelid defects, which require the reconstruction of all the lost eyelid layers.[3,8] The tissue loss percentage is sometimes also determined. Therefore, the reconstruction plan should consider the reconstruction of all layers of the eyelid, i.e., the anterior lamella, including the skin and muscle, and the posterior lamella, including the mucosal membrane and tarsus. Many authors also emphasize the need to reconstruct the tarsus, which stabilizes the free margin of the eyelid and prevents eyelid protrusion and entropion, as well as the need of protecting against lacrimation and corneal injury with a cornified epidermis.[9–12]

The retrospective analysis of the records for 262 patients indicates that the most advantageous reconstruction method was individualized management, involving the individual choice of reconstruction technique, considering the size and location of the lesion, as well as the patient's age, sex and health status. In the study group, a total of 83 patients (31.7%) received the single-flap procedure, resulting in a good functional and esthetic effect in various periorbital sites. A total of 89 patients (33.9%) received individualized management involving a combination of 2 island flaps, island flaps with a free skin graft, and the Mustardé procedure with a subcutaneous island pedicle flap. A complex auricular perichondrium-cartilage flap was used in 3 patients, with a good functional and esthetic result following the reconstruction of a full-thickness lower eyelid deficit. The combination of several surgical techniques and complex flaps enabled the reconstruction of all the layers of multi-layer deficits, which helped to restore the protective function of the eye, while the distant esthetic outcomes were mostly accepted by the patients and did not reduce their psychosocial levels of comfort.[3,7,10,13]

Glabella was usually used to form different types of flaps, which were then moved, mostly using the lateral pedicle, to the defect location in the medial canthus, upper eyelid or lower eyelid. Post-resective deficits were often reconstructed using subcutaneous island pedicle flaps, usually to reconstruct the tissue deficits in the medial canthus area. The subcutaneous island pedicle flaps used for the reconstruction of the periorbital deficits were formed in the area of the glabella, nasal bridge or nasolabial fold. A relatively long pedicle for the subcutaneous island pedicle flap enabled the island to be moved to a distant site, due to the axial blood supply to the flap, which conditions normal healing. Subcutaneous island pedicle flaps demonstrate an important characteristic, emphasized by most of their supporters, namely the ability to rotate or double them, which allows the reconstruction of multi-layer deficits.[2,4] However, these flaps also have their shortcomings, as indicated by numerous authors.[4,6] The unesthetic protrusion of the central part of the flap due to the shrinking of a round scar may be considered a disadvantage. This effect was observed in a total of 7 patients in our study.

In patients with systemic conditions, partial thickness and full-thickness free skin grafts were used relatively often. Skin grafts were used in a total of 52 patients (19.8%), being applied primarily in those cases where the primary tumor site was considered to preclude the use of a different surgical technique. Full-thickness and partial-thickness skin grafts are simple, with little burden for the patient. The method is used primarily in extensive, superficial deficits of the skin. The graft thickness is adjusted individually, depending on the thickness of the tissue lost due to the tumor resection.

Free skin grafts usually heal properly, maintaining the original shape and dimensions. Healing complications are sometimes observed in free skin grafts due to partial or complete graft necrosis as a result of the disturbed blood supply at the donor site. This complication is observed most frequently in elderly patients with heart failure or atherosclerosis and diabetic angiopathy. A disadvantage of free skin grafts is their unsatisfactory esthetic effect due to differences in skin color and consistency between

the donor and recipient sites. Disturbed healing of the skin grafts in the periorbital was found in 11 of our patients, which was 21.1% of the grafts used. This was due to a partial loss of the skin graft as the result of a partial necrosis. In our group of patients, there were no cases of complete graft loss.

Conclusions

On the basis of this assessment of patients having had operations due to eyelid and periorbital malignancies, the following conclusions were established.

1. The most frequently used mechanisms for correction and reconstruction of the deficits following the excision of the eyelid skin and periorbital malignancies included various flaps, used in a total of 175 patients (66.8%).
2. The planning and choice of the reconstructive procedure was individualized for each patient, considering the type, stage and location of the neoplasm, as well as the age and general health status of the patient.
3. Individualization of the surgical technique enabled the use of various surgical methods in 1 patient (multiple flaps).
4. Lower eyelid deficits in 82.6% of patients were reconstructed using the Mustardé flap method.
5. Single or multiple subcutaneous island pedicle flaps from the glabella area were used to reconstruct the tissue in the medial canthus.
6. In 15 patients (5.7%), the use of free skin grafts was associated with complications in the form of partial loss of the graft.
7. In 214 patients (81.7%) the healing process was uncomplicated, whereas in 85 patients (32.3%) the complications included partial wound breakdown, local infection at the surgical site and partial breakdown of the surgical wound with a unesthetic scar.

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Withholding and withdrawing life-sustaining treatment: Experiences in limiting futile therapy from three Polish intensive care departments

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Abstract

Background. In intensive care units (ICUs), a patient's vital functions may be maintained, regardless of the patient's chances of survival. A key issue is how to precisely determine the moment in which life-support treatment should be withheld. In many countries, the decision-making process is regulated by the guidelines of scientific societies. However, heuristic errors may influence this process.

Objectives. The objective of this study was to assess factors involved in decisions to implement or withhold treatment in general ICUs in Poland.

Material and methods. The medical records of patients treated in 3 clinical ICUs of general, cardiosurgical and neurosurgical profile were retrospectively analyzed. Patients with a diagnosis of brain death were finally excluded from the study.

Results. The records of 1,449 patients hospitalized between January 1, 2014 and December 31, 2014 were analyzed. Of these, 226 patient cases were evaluated. There were no correlations between the placement of restrictions on resuscitation in specific cases, use of noradrenaline, frequency of blood gas testing, and patients' age. There was a relationship between these factors and the duration of hospitalization in the ICU. There was a direct relation between a "do not resuscitate" (DNR) order in a patient's record and the frequency of both resuscitation procedures and withholding catecholamine treatment in the hours preceding a patient's death.

Conclusions. Treatment was withheld in about 20% of cases involving dying patients in analyzed ICUs, regardless of age. Placing a limit on treatment consisted of either withholding new procedures or withdrawing existing therapy. The length of stay in the ICU affected the decisions to limit treatment.

Key words: critical care, withholding life-sustaining treatment, withdrawing life-sustaining treatment

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Introduction

The treatment of patients in the end of life (EOL) period is now an integral component of critical care, with positive results of life-sustaining treatments leading to the widespread implementation of new methods in clinical practice in intensive care units (ICUs). It is now technologically possible to support the functions of individual organs and systems, without having to justify a patient's chances of survival. A key issue facing intensive care physicians is how to precisely determine the moment in which life-support treatment should be withheld.

Many studies have been published to date on this topic, using different methods and materials. These include studies conducted in France, Spain, the US, the UK, Sweden, Norway, and Denmark.[1–7] One of the largest studies in Europe was based on 127,484 patients admitted to 127 ICUs in England, Wales and Northern Ireland, and concluded that the EOL issues are common in clinical practice.[8] In many countries, withholding treatment is formally regulated by the guidelines of national scientific societies.[5] Many of these guidelines recommend noting the decision to withhold treatment in the patient's medical records, sometimes using specially designed "do not resuscitate" (DNR) order forms.[5]

In some cases, the selection of treatment and subsequent escalation of the treatment in the face of imminent death can be out of proportion and unnecessary in view of a patient's clinical status. Heuristic errors in medical judgment regarding the withdrawal or withholding of treatment may stem from subjective decisions by the physician, the physician's experience and underestimation of important factors that can influence the physician's judgment, such as clinical status of the patient, accompanying diseases and prognosis for outcome.[9] Such errors can also be the result of the physician basing the prognosis on other cases in the literature or clinical practice that bear only a partial similarity to the clinical condition of the patient.[9] These factors can influence decision-making regarding treatment.[10]

The aim of this study was to assess factors involved in decision-making regarding withholding or withdrawing treatment in Polish ICUs.

For the purpose of this study "withholding" was defined as a situation when therapeutic team refrains from administering new forms of treatment. The term "withdrawing" was used to describe the situation when some medical procedures or drugs were terminated despite having been administrated earlier.

Methods

After obtaining the approval of the bioethics committee of Wroclaw Medical University (No. KB-470/2013), the medical records of 1,449 patients treated in 3 clinical ICUs of general, cardiosurgical and neurosurgical profile between January 1, 2014 and December 31, 2014 were retrospectively analyzed. A detailed analysis was performed regarding the last 48 h of treatment for all 226 patients who died in the ICU. There were patients who underwent surgical procedures amongst the analyzed subjects. Patients with a diagnosis of brain death were finally excluded from the study.

The treatments administered in the last 48 h prior to a patient's death were analyzed. When the treatment period was shorter than 48 h, the first measurement was designated as the first measurement taken at the fixed time for recording the data. Data on the patients' characteristics and existence of DNR orders was recorded. Data on each patient's mean arterial blood pressure (MAP), catecholamine dose, serum concentrations of sodium and potassium, resuscitation, and arterial blood gas (ABG) testing at 6-hour intervals was also recorded. Analysis was performed depending on the time spent in the ICU: (1) more than 48 h, (2) 24 h or less, and (3) less than 12 h.

Catecholamine treatment

A noradrenaline, administered intravenously, was most commonly used to maintain perfusion. To evaluate trends, the dose administered was expressed in μg/kg body weight/h. The collected data in the analyzed time points was compared as median of catecholamine doses and MAP. The mutual relations between such values gave the possibility to create trends of MAP and catecholamine dose, which were classified as positive, negative, zero, or zero constant. Table 1 shows examples of trends at 48 h. Values of MAP were calculated using the following formula:

$$MAP = DP + 1/3 * (SP - DP),$$

MAP – mean arterial pressure,
SP – systolic pressure,
DP – diastolic pressure.

Withholding was defined as no administration of catecholamine, accompanied by a negative trend of MAP value. Withdrawal was defined as a decreasing trend

Table 1. Examples of trends in noradrenaline dosage in 48-hour period of observation

Day 2				Day 1				Day of death				Trend
6 a.m.	12 p.m.	6 p.m.	12 a.m.	6 a.m.	12 p.m.	6 p.m.	12 a.m.	6 a.m.	12 p.m.	6 p.m.	12 a.m.	
1.44	1.28	1.28	0.96	0.8	0.8	0.8	0.8	0.8	0.56	death	death	negative
1.12	1.12	1.12	1.12	1.12	1.12	1.12	1.12	1.12	death	death	death	zero
0.32	0.32	0.32	0.32	0.8	0.64	1.92	5.6	5.76	death	death	death	positive
0	0	0	0	0	0	0	0	0	0	death	death	zero constant

The doses of noradrenaline are shown in mg/h.

in catecholamine doses, accompanied by a falling trend in MAP prior to the death of the patient.

Resuscitation

A DNR record in a patient's file was taken as a proof of not performing resuscitation (i.e., withholding therapy). The latter was classified as not performing external chest compression and defibrillation after cardiac arrest, as other advanced life support elements remained in place.

Testing frequency of arterial blood gas and electrolyte concentrations

The frequency of ABG testing provides indirect evidence of the therapeutic effort undertaken by physicians. The frequency of ABG tests in the last 5 days of therapy prior to the patient's death was recorded. When the hospitalization time was shorter than 5 days, the number of tests performed daily was recorded, and relevant trends were identified. The serum concentrations of sodium and potassium ions were recorded for at least 48 h prior to the death of the patient. Concentrations exceeding normal ranges (135–145 mmol/L for sodium and 3.5–5.5 mmol/L for potassium) were recorded.

Statistical analysis

Statistical analysis was performed using STATISTICA software package (StatSoft Inc., Tulsa, USA). In the statistical method, because of the nature of the analyzed data, frequency analysis was used. The relationships between the variables were tested using the χ^2 test (Pearson). Regarding the trend analysis, changes in the hemodynamic and laboratory parameters of the patients over time were evaluated. To prevent a subjective assessment of changes in these parameters, the slope of the regression line was used as an indicator of whether a change in a trend was "positive," "negative" or "zero." This method made it possible to develop indicators of changing trends in these parameters.

Sample size calculation

Because of the insufficient number of published Polish papers in this field, the sample size of the study was not determined.

Results

In the analyzed period, 1,449 patients were hospitalized in the ICUs. Among them 226 died. Twelve cases were excluded from the study due to the diagnosis of brain death. Finally, the records of 214 patients were analyzed. There were 95 (44.40%) women and 119 (55.60%) men. The youngest patient was 19 year old and the oldest was 94. The characteristics of the analyzed patients regarding clinical diagnosis are shown in Table 2. One hundred twenty-seven out of 214 (59.35%) patients were treated surgically, and 87 (40.65%) patients were not.

Table 2. Characteristics of the study group

Diagnosis	Number of patients	%
CNS damage	43	20.09
Multiple organ failure	14	6.54
Severe sepsis	93	43.46

CNS – central nervous system.

In 142/214 cases (66.35%), the length of the stay in the ICU did not exceed 7 days. In 14/214 (6.5%) cases, the hospitalization length exceeded 30 days. Two patients spent 104 and 120 days, respectively, in the ICU. The medical records of 34/214 (15.88%) patients contained a DNR order in the case of cardiac arrest.

The statistical analysis did not reveal any correlation between the centers where the treatments were administered and the frequency of withholding treatment ($p = 0.784$). It also revealed no association between the procedures for placing restrictions on resuscitation and the patient's age ($p = 0.2584$). There was a significant statistical relationship between the presence of a DNR order and the duration of stay in the ICU ($p = 0.0000$). In patients with duration of less than 8 days, a DNR order was present in 10 (7.04%) out of 142 cases. In those with hospitalization durations longer than 30 days, 9 (64.23%) out of 14 patients had a DNR order. The presence of a DNR order in the medical records showed a statistically significant relationship with the frequency of resuscitation procedures performed prior to the patient's death ($p = 0.00$). There was also a significant relationship between the presence of a DNR order and withholding noradrenaline treatment in the hours preceding a patient's death ($p = 0.00$). Detailed data regarding the correlations between withholding treatment and different factors was presented in Tables 3–5.

Table 3. Withholding noradrenaline treatment

Limits placed on noradrenaline treatment	Number of patients	%
Withdrawal	22	10.28
Withholding	31	15
Total	53	25
Correlations between limiting noradrenaline treatment and other factors		**p-value**
Sex		0.144
Medical center		0.784
DNR		0.0000
CNS damage		0.0003
Serum level of sodium above standard (exceeded 145 mmol/L)		0.003
Serum level of potassium above standard (exceeded 5.5 mmol/L)		0.002

DNR – "do not resuscitate"; CNS – central nervous system.

Table 4. Association between withholding treatment and the length of ICU stay

Variables	Duration of ICU stay				
	<7 days (%)	8–14 days (%)	15–30 days (%)	>30 days (%)	p-value
Treatment duration	142/214 (66.36)	29/214 (13.55)	29/214 (13.55)	14/214 (6.54)	–
Limitation of catecholamine treatment	20/53 (37.74)	12/53 (22.64)	12/53 (22.64)	9/53 (16.98)	0.0002
DNR order	10/34 (29.41)	5/34 (14.71)	10/34 (29.41)	9/34 (26.47)	0.0001
Withholding ABG testing	18/55 (32.73)	13/55 (23.64)	17/55 (30.91)	7/55 (12.73)	0.009

ICU – intensive care unit; DNR – "do not resuscitate"; ABG – arterial blood gas.

Table 5. Association between withholding treatment and the patients' age

Variables	Age				
	<65 years (%)	66–75 years (%)	76–85 years (%)	>85 years (%)	p-value
Number of patients	92/214 (43.99)	56/214 (26.17)	46/214 (21.50)	18/214 (8.41)	–
Withholding catecholamine treatment	20/53 (37.74)	16/53 (30.19)	10/53 (18.87)	7/53 (13.21)	0.147
DNR order	12/34 (35.29)	7/34 (20.59)	12/34 (35.29)	3/34 (8.82)	0.292
Withholding ABG testing	22/55 (40.0)	13/55 (23.64)	14/55 (25.45)	6/55 (10.91)	0.314

DNR – "do not resuscitate"; ABG – arterial blood gas.

In 5/214 (2.34%) patients, the time of their hospitalization in the ICU was shorter than 12 h, so in such cases it was impossible to complete the analysis of trends in catecholamine treatment. In 209/214 (97.66%) cases included in the statistical analysis, the noradrenaline treatment doses were administered from the time of admission to the ICU to at least 2 days before death were recorded. They were recorded during a 12-hour period in 187/214 (87.38%) patients and in 137/214 (64.01%) patients during a 24-hour period. The withdrawal of noradrenaline because of a decline in MAP immediately before death was recorded in 22/187 patients who had been in the ICU for at least 24 h prior to death. The withholding of noradrenaline was recorded in 31/187 cases.

In patients with a 24-hour period of hospitalization before death, the physicians decided to cease catecholamine treatment in 24/214 (11.21%) cases due to a decline in MAP immediately preceding death, despite the previous use of noradrenaline. Furthermore, in 67/214 cases (31.3%), 1 day before the patient's death, cardiovascular support was limited to noradrenaline doses smaller than those that had been previously used due to increased hypotension. Central nervous system (CNS) damage was observed in 20/53 (37.73%) cases when catecholamine treatment had been withdrawn or withheld 24 h prior to death ($p = 0.0002$). This relationship between CNS damage and catecholamine limitation remained statistically significant for each available observation time ($p = 0.0026$ for 12 h and $p = 0.0067$ for 48 h). There was no correlation between the patients' ages and the withholding of catecholamine treatment ($p = 0.14$). There was a clear statistical relationship between the frequency of withholding noradrenaline treatment and the duration of the patient's stay in the ICU ($p = 0.0013$). Regardless of the length of time in the ICU, this relationship remained unchanged ($p = 0.00$). Among patients who were treated in the ICU for 7 days or less, noradrenaline treatment was withheld in 20/115 (17%) cases. Noradrenaline treatment was withheld in 9/14 (64%) cases where the patient remained in the ICU for more than 30 days. Catecholamine treatment was withheld from all 2/2 (100%) patients treated for over 90 days. There was no correlation between the treatment center ($p = 0.78$) and sex ($p = 0.14$) of the patient and the withholding of noradrenaline treatment.

Among the patients who remained in the ICU for 24 h prior to death, the concentration of sodium ions increased in 79/214 (36.91%) cases. In 45/214 (21.02%) cases, sodium concentrations surpassed the upper limit of 145 mmol/L. In 113/214 (52.80%) patients, an increased serum level of potassium was noted. In 46/214 (21.49%) cases, the serum level of potassium exceeded 5.5 mmol/L.

A decreasing frequency of ABG testing was recorded in 55/214 (25.70%) patients. Among these patients, noradrenaline treatment was withheld in 23 (10.74%) cases. There was no correlation between a decrease in the number of ABG tests and the age of the patient who died ($p = 0.6$). However, there was a relationship between a lower frequency of ABG tests and the length of stay in the ICU ($p = 0.0089$). A lower frequency of testing was found in half of the cases where hospitalization exceeded 30 days.

Discussion

More than 50% of all deaths in the ICU are preceded by some sort of limit placed on treatment.[4,11,12] A survey of the attitudes of physicians in Poland to withholding treatment showed that these attitudes were reflected in actions taken in ICUs, although documentation on withholding treatment in medical records is very limited.[13] According to the survey, during the course of their professional careers, 93% of Polish anesthesiologists admitted not having administered new medications to a patient in the

EOL stage. Withdrawal was less common but mentioned by 75% of anesthesiologists. Similar results were obtained in a European study, in which 93% and 77% of ICU doctors mentioned withholding and withdrawing treatment, respectively.[13] In cases of patients who died in the ICU, the prevalence of withholding catecholamine treatment was 24.76% and the prevalence of withholding renal replacement therapy was 21.49%.[14] In the UK, the prevalence of withholding treatment was reported to be 31.8%,[8] whereas it was 41% in Sweden.[15] In Southern European countries, the reported prevalence of withholding treatment was much lower (20%). In Northern European countries, different types of limits on treatment were as high as 79% in Norway and above 80% in Denmark of all deaths in the ICU.[6,7]

The Polish guidelines regarding the ineffective maintenance of organ functions were published in 2014.[16] The results of the present study indicated that withholding treatment was a common practice in Polish ICUs, although much less common than that observed in Scandinavian ICUs. The prevalence of withholding treatment in Poland was similar to that recorded in others countries, such as Italy, Greece and Portugal.[14] As indicated by medical records and reported in physician surveys, withholding treatment is less common in these countries than in Northern Europe.[14] Kübler et al. found that only 10% of physicians in Polish ICUs admitted to placing DNR orders in patients' medical records.[13] In contrast, the decision to withhold treatment in Norway was not recorded in only 12% of cases.[6] Vincent found that, similarly to Polish physicians, only 8% of Italian doctors admitted to placing formal DNR orders in a patient's medical records.[14] This is significantly less than in other analyzed European countries, where 58% of physicians admitted to placing DNR orders.[14]

Differences in documenting the withdrawal/withholding of treatment may be based on legal and psychological factors. On the one hand, the absence of legal regulations and medical guidelines on mandatory documentation encourages the omission of such documentation in daily medical practice. On the other hand, the psychological burden of making such decisions and the fear of possible moral and legal recriminations may cause physicians to avoid documentation. In Nordic countries, recording the withholding of treatment is a common practice. With the aim of developing a formal procedure, clinicians in France proposed a 4-step, transparent protocol for qualifying and evaluating patients daily which would be based on 4 groups, categorized by the scope of care most appropriate for the clinical condition of the patient.[17]

According to the analysis of Prendergast and Luce, many patients in whom treatment was withheld had already experienced prior CNS damage, and it confirmed that CNS damage had an important impact on a patient's prognosis.[18] In addition, several reports indicated that older patients gained less therapeutic benefit from critical care than younger patients and that both patients and staff had a low tolerance for aggressive treatments.[19–21] Some studies reported that advanced age was associated with a higher incidence of withholding treatment.[22–25] Age was also reported to affect the quality of life.[26–28] Some reports suggested that the perceptions of treatments for older people by ICU and emergency room physicians differed from their perceptions of treatment indications for younger people.[29,30] Other studies documented some ethically questionable suggestions to reduce the opportunities for admission to the ICU for patients over 85.[31,32] The present study found no association ($p = 0.1384$) between withholding catecholamine treatment and age. However, there was a clear correlation between the patient's age and withholding cardiovascular support. There was no link between age and the incidence of DNR orders ($p = 0.292$) and no link between age and a lower frequency of ABG testing at the EOL stage ($p = 0.3149$). The frequency of withholding treatment in Poland was not associated with the age of the patient, in contrast to France, the USA and other countries where a smaller range of treatment options may be available for older patients.[33]

This study found a correlation between withholding treatment and the duration of time spent in the ICU. The frequency of withholding cardiovascular support increased with the length of the hospital stay. Treatment was withheld in 16% of patients who were hospitalized for less than 7 days and in 64% of patients who were hospitalized for more than 30 days. For hospital stays over 90 days, treatment was withheld in 100% of cases. A similar relationship was observed regarding the placement of DNR orders. For patients hospitalized for less than 7 days, a DNR order was in place in 7% of cases. For patients treated for over 30 days, a DNR order was in place in 64% of cases. The frequency of ABG testing was also related to the duration of the hospital stay. Physicians may perceive the length of stay in the ICU as a prognostic factor when considering making changes to therapeutic targets and indications for treatment.

This study revealed that withholding treatment was more common than withdrawing treatment. In the case of a decline in MAP preceding death, noradrenaline treatment was withheld in 31 (14.5%) cases, whereas the dose was reduced in 22 (10%) cases, despite a decline in MAP. In Norway, an analysis of data on written protocols governing limiting treatment showed that withholding treatment was almost twice as common as withdrawing treatment.[6] A similar situation was reported in studies of physicians' conduct in Denmark and Sweden.[5]

Conclusions

Treatment was withheld in about 20% of dying patients, regardless of age, in Polish ICUs, suggesting that it is a relatively common practice. Withholding new treatment was much more common than withdrawing treatment. The length of stay in the ICU affected decisions to withdraw or withhold treatment.

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Systemic sclerosis and its oral health implications

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Abstract

Systemic sclerosis (SSc) is a chronic, generalized disorder of the connective tissue. It is characterized by immune disorders, abnormalities of morphology and functions of small blood vessels, and the presence of inflammatory process. The pathogenesis of this disorder has not yet been fully understood. The classification criteria were established by The American College of Rheumatology (ACR). A number of clinical types are distinguished due to the diversity of the clinical picture. These types are characterized by a different course, presence of organ complications and prognosis. Connective tissue disorders are interdisciplinary conditions and, therefore, the subject of interest of different medical specialties, including dentistry. The oral cavity may be the place of pathological manifestations within soft and hard tissues. Such manifestations are the results or the primary symptom of systemic diseases. The relationship between the health of the oral cavity and systemic diseases has been frequently reported in the literature. Lesions in the oral cavity in patients with SSc are discussed in detail in the present paper. Management includes the administration of drugs that prevent tissue ischemia and post-ischemic consequences as well as drugs that inhibit inflammatory-immune processes and excessive collagen production.

Key words: connective tissue diseases, oral manifestations, systemic scleroderma

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Introduction

Systemic sclerosis (SSc) is a chronic systemic connective tissue disease. It is characterized by immune disorders, disorders of morphology and functions of small blood vessels, and the presence of the inflammatory process. The predominant symptom of SSc is progressive fibrosis of the skin, organs and systems. The disease usually involves kidneys, lungs, gastrointestinal tract, as well as osteoarticular, cardiovascular and nervous system. Disease activity, the degree and the extent of internal organ involvement, and the prognosis differ among patients.[1]

The pathogenesis of SSc has not been fully understood. The factors considered include the genetic background, environmental factors, blood vessel malfunctions, loss of immune tolerance, impaired biosynthesis, degradation of the extracellular matrix of the connective tissue, and hormonal factors.[1] It seems that in individuals with a certain genetic predisposition, some (toxic) environmental factors may result in blood vessel damage and immune cell activation, which leads to non-specific activation of fibroblasts and other connective tissue cells producing the extracellular matrix components.[2]

The incidence of SSc is higher in women as compared to men (F:M of 3:1). The risk of developing the disease in the general population ranges from 0.009% to 0.026%. A positive family history is the most significant factor predisposing to the development of SSc, in which case the incidence risk is from 10 to 158 times higher compared to the general population. The peak incidence occurs between the 2nd and the 5th decade of life.[3]

The classification criteria of SSc were developed by the American College of Rheumatology (ACR). The presence of 1 large or 2 small criteria allows for the diagnosis of SSc. Large criteria are as follows: hardening of the skin covering the areas located proximally to the metacarpophalangeal joints and metatarsophalangeal joints. Small criteria are the following: sclerodactylia (hardening of the skin) distally to metacarpophalangeal joints, fingertip tissue loss and parabasal pulmonary fibrosis.[4]

In order to facilitate the early detection of the disease when clinical symptoms are not that developed or distinctive yet, a group of symptoms was distinguished that make up a high risk group for the development of SSc, known as early scleroderma. The most important symptom is Raynaud's phenomenon (paroxysmal pallor of fingers, feet, nose, or earlobes due to the contraction of small arteries and arterioles in response to cold, vibration or emotions) and the occurrence of microangiopathy (diagnosed based on nailfold capillaroscopy where the assessment includes the appearance and number of vessels, their morphology, and the presence of extravasation and edema) or the presence of specific autoantibodies in the serum – anti-centromere antibodies or anti-topoisomerase 1 antibodies.[5]

The diversity of the clinical picture of the disease allowed us to distinguish a number of clinical types that differ in terms of the disease course, the presence of organ complications and the prognosis. The distinguished forms are the following:

– systemic sclerosis with generalized hardening of the skin (diffuse systemic sclerosis (dSSc));
– scleroderma with limited hardening of the skin (limited systemic sclerosis (lSSc));
– transitional forms: dSSc/lSSc;
– systemic sclerosis without scleroderma;
– early SSc with limited scleroderma;
– scleroderma overlap syndromes;
– scleroderma in the course of cancer.[6]

Generalized systemic sclerosis

Generalized SSc is characterized by the rapid progression of symmetric hardening of the skin of proximal and distal parts of the extremities, face and trunk. Raynaud's phenomenon appears quickly (time between the occurrence of this symptom and a noticeable hardening of the skin is less than 1 year) and early involvement of internal organs is observed. This form of the disease has a very rapid course, especially in the initial period. The rapid progression of skin lesions and the involvement of internal organs (lungs, heart, kidneys) in the early stage of the disease indicate a particularly bad prognosis. In most cases, anti-topoisomerase 1 Scl-70 antibodies are detected. Capillaroscopy demonstrates characteristic vascularization.

Limited systemic sclerosis

Limited SSc is characterized by hardening of the skin in the areas distal to the elbows and knees. Typical changes involve facial skin. The majority of patients present with Raynaud's phenomenon, which precedes the onset of scleroderma. This form is characterized by a chronic, very slow, but steadily progressive course. Previously, it was described as the CREST syndrome, including soft tissue calcifications (calcinosis), Raynaud's phenomenon, esophageal dysfunction, sclerodactylia, and telangiectasia in the skin (hence the acronym). About 70–80% of patients with this form of the disease have anti-centromere antibodies, and a capillaroscopy shows vessel widening (mega capillaries).

Systemic sclerosis without skin hardening

Systemic sclerosis without skin hardening is also known as "scleroderma without scleroderma". In this disease entity, the involvement of organs and internal systems is observed, Raynaud's phenomenon is not always present and skin changes in the form of hardening do not occur. The results of serological tests are characteristic.

Scleroderma overlap syndromes

The literature describes the cases of the coexistence of scleroderma and other connective tissue diseases, most often rheumatoid arthritis and systemic lupus erythematosus.

The major symptoms of SSc include the so-called tough skin and the presence of atrophic changes such as erosions, ulcers and foci of skin necrosis. Hardening of the skin is due to the accumulation of collagen fibers. The skin becomes smooth and thickened. The changes progress from the distal extremities. Limited systemic sclerosis refers to a disease where tightness primarily affects fingers, hands and distal forearms, feet and legs (hardening confined to skin usually from elbows distally and from knees distally); dSSc also includes hardening of the skin of proximal extremities and the trunk. In the initial stage of the disease, the skin is stretched, smooth and thinned ("too tight"). Muscle contractures with the claw-like hand stiffness are observed, and tendon fibrosis and restricted mobility occur as a consequence.[7] As the disease progresses, clinical signs of internal organ involvement are reported. Gastrointestinal tract symptoms occurring in most patients with SSc are mainly related to motor impairment (impaired peristalsis of the oesophagus and colon, reduced sphincter tonus) and secretion impairment.[5] Involvement of the respiratory system, affecting as many as 2/3 of patients, is one of the most severe complications of scleroderma. In 16–22% of patients, it results in death. Interstitial lung disease (alveolitis and interstitial inflammation with secondary fibrosis) usually develops in the process of fibrosis. Primary and secondary pulmonary hypertension is usually detected in patients.

Cardiac involvement is noted in the majority of patients with generalized scleroderma and about 10% develop cardiomyopathy, resulting from cardiac fibrosis. Conduction disturbances are a common symptom of cardiac involvement. Typical symptoms also include pericarditis. Renal involvement is characterized by a scleroderma renal crisis, which is a newly developed renal failure or malignant hypertension in patients with SSc. Progressive renal failure sometimes forces dialysis.

Typical symptoms of the osteoarticular system are as follows: arthralgia of considerable intensity, edema and stiffness of fingers and knees. Symptoms may occasionally be similar to rheumatoid arthritis. Patients develop chronic arthritis with deposition of fibrin and joint contractures. Subcutaneous nodules may be formed around the joint area, especially in the hands, which results from the deposition of calcium compounds in the connective tissue. The reason of muscle weakness is due to muscle wasting, which is frequent in patients with extensive skin hardening. Muscular changes may be non-inflammatory, fibrosing myopathy with the dominance of myalgia, atrophy or typical cellulitis.[2]

Treatment of SSc is possible only in the active phase of the disease, which is a result of progressive inflammation. Fibrotic-type changes are irreversible. The inhibition of pathologic changes is possible only at the early stage of the disease. Therapeutic treatment with the already present fibrosis is limited to the alleviation of symptoms and treatment of complications. Treatment of scleroderma is multi-directional and focused on the inhibition of the biosynthesis and the accumulation of fibrous proteins, the inhibition of dysfunction within the blood vessels and the reduction of inflammation. As the cause of the disease remains unknown, no causal treatment exists. Therefore, only symptomatic medications can be administered. There are no drugs effectively inhibiting or delaying the progression of the disease. The current trend in the treatment is known as organ-specific therapy, the aim of which is to protect an organ with possibly early treatment of pathologies and potential remodeling of the existing changes.

The following groups of drugs are suggested in the treatment of scleroderma[8]:

– drugs preventing tissue ischemia and its effects (calcium channel blockers);

– drugs inhibiting inflammatory-immune processes (immunosuppressive drugs – cyclophosphamide, methotrexate, mycophenolate mofetil, cyclosporine A);

– drugs inhibiting excessive collagen production (D-penicillamine, colchicine).

The orofacial region remains the most affected by SSc in about 80% of cases.[9] Oral abnormalities are frequently reported in SSc. They may, however, be overshadowed by other systemic symptoms of oral problems, and dental (decayed, missing teeth), periodontal and orofacial anomalies (e.g., xerostomia, mandibular bone resorption or microstomia).[10]

Skin involvement is noted in the majority of patients with SSc. Abnormal accumulation of connective tissue components in the skin results in the loss of dermal elasticity with subsequent dermal thickening and hardening.

The disease is characterized by the occurrence of 3 phases of skin thickening related to the facial area: edematous phase, indurative phase and atrophic phase. In the 1st phase (mostly painless), (non)pitting edema may occur in the face and it may last terminally. The next phase is related to edema that is replaced by a markedly thickened skin and considerably thinned epidermal layer, which in consequence results in the loss of skin creases and a "choking out" of hair follicles, sweat and sebaceous glands.[11] The skin is frequently pruritic with the observed loss of specialized skin structures, which contributes to changes in perspiration and also to the loss of hair growth.[12]

In the 3rd phase the rapidly progressive skin fibrosis in consequence results in changes in the physical appearance. Deposition of subcutaneous collagen in facial skin leads to a smooth, tight, expressionless face, often termed as the "mask-like" appearance with the simultaneous disappearance of wrinkles and perioral furrows.[9]

Nasal alae may undergo atrophy, which may result in a "mouse-like" face. Other significant orofacial manifestations are connected with lacrimal gland fibrosis.

Of ophthalmological manifestations, dry eyes with keratoconjunctivitis sicca or xerophthalmia are the most common. It is particularly problematic due to the fact that eyelid scarring leads to a chronic widening of palpebral fissures and also to inadequate eyelid closure, which contributes to further drying of the eyes.[13]

Other dermal manifestations include telangiectasia, frequently in the region around the mouth, cheeks, lips, and nose. Telangiectasia manifests as small red spots by edema of small blood vessels beneath the skin. Cosmetic problems are also noted due to facial occurrence of telangiectasia.[14] Also, changes in the color of the skin are reported and hyper- and hypopigmentation may also be observed in the affected areas.[12]

Sclerosis of the lips and the skin around the mouth area causes reduced mouth opening (known as microstomia) and width (microcheilia) with the reduced interincisal distance. Microstomia may be related to problems with speaking or chewing. Additionally, oral hygiene and dental treatment is difficult due to the limited access to the teeth. The maximum mouth opening should be measured during the oral examination. The maximum interincisal opening of 40 mm or less should indicate the need for investigation regarding its etiology.[14]

Mouth-opening limitation and reduced cheek and tongue movement contribute to the deterioration in dental health. Oral and facial manifestations related to sclerodactylia may lead to a more rapid decline. Therefore, regular oral examination is of great importance. Maintenance of the existing dentition is significant due to the fact that microstomia can make the prosthetic replacement difficult.[15]

Mouth-stretching exercises are recommended as they reduce and decrease the risk of mouth-opening limitation.[16] Some approaches and techniques connected with active exercises are recommended, and some authors reported success related to achieving expanded movements of the mandible.[3]

Perioral exercise programs need to be done on a regular basis. Combined treatments, including physical therapy, mouth stretching exercises or massage may contribute to the improvement in mouth opening.

Kinesiotherapy is based on specific passive (tongue depressors placed between the posterior teeth), active (thumb placement in opposite corners of the mouth with outward pulling) or assisted exercises to improve the opening of the mouth by means of stretching and exercises that increase mimic muscles mobility.[17]

Connective tissue massage is indicated to increase local bloodstream and to relax the involved tissue. Treatment of the neck and the clavear regions in the case of facial involvement of SSc should not be neglected. Kabat's method, which is a neurorehabilitation technique using movement patterns with proprioceptive facilitation techniques to strengthen neuromuscular recruitment, offers benefits.

Patients with SSc require a multidisciplinary and multitasking rehabilitation approach, which can be provided by an experienced team, including physicians and therapists.[17] In severe cases, surgical procedures (e.g., bilateral commissurotomy or commissuroplasty) are indicated for the increase in mouth opening.[18]

Dermatological treatments (such as photodynamic therapy, phototherapy with UV long wavelength light or photochemotherapy) demonstrate benefits.[16] Comstedt et al. in their pilot study, treated SSc patients with microstomia using intense pulse light (IPL), which is known for its ability to remodulate the collagen formation in the skin. Those scientists observed that IPL could increase dermal mobility around the mouth area and reported a significant post-treatment improvement in the interincisal distance.[19]

Bennani et al. reported that CO_2 laser treatment of severe limited mouth opening in SSc patients was connected with significant improvement in the mouth opening 3 months after the 1st laser application, with further improvement observed at 1 year.[20]

Onesti et al. in their study, used autologous fat transplantation and autologous adipose-derived stromal cells (ADSCs) injection into the perioral region.[21] Their aim was to increase dermal mobility in that region. Autologous fat transplantations have recently become the first-choice technique to hide skin lesions. This approach, which uses the patient's own body fat as a natural filler to achieve structural modifications, decreases the rates of complications related to the foreign material.

Autologous adipose-derived stromal cells are characterized by the ability to differentiate into different types of cells under specific conditions. Advanced cell-based therapies offer promising therapeutic possibilities to improve both repair and regeneration of the damaged tissue.

Favorable outcome was reported in all patients treated with fat transplantation or ADSCs. Improvement was also reported in subjective wellness of the skin in the perioral areas. Post-treatment increased mouth opening was reported in patients.[21]

The involvement of facial tissues and jaw muscles can also cause pressure and mandibular resorption, which is observed in approx. 10% of SSc patients and is related to pathological fractures.[16] Resorption of the mandible is characterized by a multifactorial etiology.[22] Erosions are the result of pressure exertion on the bone via the atrophic muscles at their attachment site. Other authors indicate that mandibular resorption can also be due to ischemia of the bone secondary to vasculitis. Ramon et al. indicated that the condyle, coronoid and mandibular angles areas that demonstrate resorption are supplied by small arterial branches of the internal maxillary artery rather than the main inferior alveolar artery.[23] Systemic sclerosis may be more likely to affect these smaller arterial brunches, and in consequence leads to bone resorption secondary to diminished vascularity in those areas. Skin tightness that can result in pressure resorption of the bone, can be another factor in the resorption of the mandible.[24]

In maxillofacial regions, resorption is noted in attachment area of masticatory muscles (e.g., temporal, lateral pterygoid and anterior belly of digastric muscle).[22] Mandibular angles are usually involved (37.6%), followed by condyle (20.8%), coronoid process (20.0%) and the posterior border of the ramus (14.4%).[25] Mandibular bone resorption is commonly observed bilaterally. Blunting of mandibular angles can be visualized on orthopantomograph.

Mandibular erosions (beside condyle erosions) are mostly asymptomatic and are not related to any clinical symptoms. However, they could affect jaw appearance.[26] They may develop in patients with the full or partial set of teeth.[3] Extensive mandibular resorption increases the risk of mandibular fracture during tooth extraction. Therefore, each patient needs to be screened radiologically for early detection of mandibular resorption to prevent the occurrence of iatrogenic fracture.[22] Marmary et al. suggested that angle resorption without other obvious cause could be considered pathognomic for SSc.[27] Therefore, diagnostic diligence is required when evaluating patients with mandibular resorption, especially if asymptomatic.[27]

Scleroderma may occasionally be related to idiopathic tooth resorption. There are reports on external root resorption resulting from the erosive process influencing the adjacent mandible.[29] Severe resorption can lead to painful trigeminal neuropathy (TN) caused by inferior alveolar nerve compression.[9]

The most frequent oral finding to precede systemic involvement appears to be TN characterized by short recurrent episodes of intense paroxysms of pain in the regions where 1 or more branches of the trigeminal nerve are distributed; TN may involve either 1 or both sides of the face.[28] Sensory involvement is predominant in TN that rarely affects masticatory muscles.[9] Of note, TN may be the very 1st symptom of connective tissue diseases, and awareness of this possibility may allow for early and more accurate treatment.[30] So far, 73 cases of TN have been documented, being the most frequently reported peripheral nervous system involvement in patients with SSc.[31] Neuropathy is related to collagen deposition in the perineurium and/or reduced vascularity to the trigeminal nerve itself.[32]

Temporomandibular joint (TMJ) disorders are also frequently reported in SSc patients and could be the consequence of mandibular resorption. The prevalence of clinical signs of TMJ dysfunction (e.g., pain, TMJ sounds or impairment of mandibular movements) and typical magnetic resonance imaging (MRI) findings (disc, articular surface and bone changes) is higher in SSc patients in comparison to healthy controls.[9]

In SSc patients, the dysfunction of TMJ is likely due to the increased dermal thickness and decreased dermal elasticity caused by collagen deposition, which contributes to the restriction in jaw movements.[16] Systemic scleroderma is characterized by symmetric, erosive synovitis, which can lead to joint irregularity and disability. Temporomandibular joint arthritis is a disease that is present in up to 80% of patients with rheumatic disease and causes pain, facial dysmorphism and lifetime disability if it is not properly early diagnosed. Matarese et al. assessed the detectable prevalence of TMJ symptoms and signs of dysfunction in SSc patients in comparison to healthy controls.[33] One of the main results of that study was that SSc patients showed more symptoms and signs of TMJ dysfunction as compared to healthy controls. Magnetic resonance imaging in SSc patients revealed the following: disk without displacement, disk displacement with reduction, condyle flattening, erosions and irregularities, temporal eminence flattening, TMJ with degenerative bone changes, as well as osteophytes and condyle resorption.[31]

Temporomandibular joint changes in patients may not be caused only by SSc process. Differentiation between TMJ disorders affected by systemic disease and any other local pathology, including, e.g., osteoarthritis or traumatic arthritis, is often challenging. Magnetic resonance imaging should be applied to allow for a complete examination and aid differential diagnosis.[34] High-resolution ultrasound (US) may be implemented as an alternative imaging technique to monitor disorders of TMJ in the case when MRI is not available.[33]

Another crucial oral finding is related to the involvement of oral mucosa. Submucosus fibrosis causes atrophy and then sensitivity of the oral mucosa, which becomes pale and sclerotic. Patients frequently report the burning mouth syndrome or dysesthesia. The sicca syndrome is noted in about 70% of patients, being secondary to salivary gland fibrosis. In 7–14% of cases, patients may present Sjögren's syndrome.

Sclerosis and atrophy of the lips and perioral tissues can result in perioral streaks. With advanced sclerosis, lip retraction may become severe and patients may not be able to close the lips, hence mouth breathing, the sicca syndrome and chewing problems.

The tongue becomes smooth, shiny and hard. It is characterized by a considerably restricted mobility as a result of a reduced length of the frenulum and increased thickness.[35] Initially, due to edema, it may increase in size. However, it later shrinks and is affected by fibrosis. The tongue frenulum is thickened, which may result in speech difficulty. The Raynaud's phenomenon of the tongue is very rarely observed in SSc patients.[36]

Oral mucosal telangiectasias, particularly in the area of the lateral border of the tongue and buccal mucosa of the cheek, are frequently reported.[37] Gastrointestinal (GI) fibrosis is reported in about 90% of patients with scleroderma. Tightening of the perioral skin leads to decreased nutrient absorption. Destruction of the myenteric neurons causes oropharyngeal dysphagia and the risk of malnutrition. Additionally, associated xerostomia and microstomia may decrease oral intake. Esophageal dysfunction is considered to be the most common GI manifestation. Reduced lower esophageal sphincter tone is detected in up to 90% of the patients and, in consequence, may lead to significant

reflux. Gastric dysmotility is also reported. Gastroparesis causes nausea and vomiting. The progressive nature of the disease and GI tract involvement may contribute to malnutrition.[38] Malnutrition, gastroesophageal reflux disease (GERD), deficiencies in vitamins B9 and B12, or exocrine pancreatic insufficiency can exacerbate mucosal atrophy.[9]

Salivary gland hypofunction (SGH) and xerostomia are other frequently reported complaints. The prevalence of clinical xerostomia ranges from 22% to 70%.[10] Salivary gland fibrosis is detected around capillaries and excretory ducts. Capillary wall sclerosis causes functional abnormalities by reducing vascular permeability and periductal fibrosis impairs excretion of saliva.[9] Salivary gland hypofunction is a condition with significantly reduced salivary flow, and can also lead to changes related to the chemical composition of saliva. Xerostomia is a subjective perception of dry mouth, which in fact may be accompanied by a reduction in salivary flow. The symptoms of the disease mostly include halitosis, oral soreness, altered taste, as well as difficulty in swallowing and talking. Both xerostomia and SGH increase the risk of caries, and contribute to periodontal diseases and oral infections, e.g., candidiasis. A decrease of the salivary pH due to xerostomia and GERD may be responsible for enamel and dentin erosion.[9,39]

Patients need to be checked for Sjögren's syndrome, since studies reported a 17–29% prevalence of this syndrome in scleroderma patients.[14] Systemic sclerosis is defined as an autoimmune epithelitis characterised by lymphocytic infiltration of exocrine glands and epithelia in multiple sites, and it can be seen alone (primary SSc) or in association with other autoimmune rheumatic diseases (secondary SSc).[40]

Xerostomia may be caused by some drugs administered to SSc patients, e.g., cardiovascular, antidepressant or antihistamine drugs, diuretics, iron supplements, narcotic analgesics, daily aspirin, or anticholinergics. Assessment of dry mouth in all SSc patients should be made to prevent complications related to this condition.

Symptomatic relief of the dry mouth symptoms can include the following: regular water intake, sugar-free gums (topical salivary stimulants) or oral lubricants. In the case of patients with SGH artificial saliva substitutes, sialagogues (pilocarpine) or anetholtrithione may be of benefit.[9,40,41]

Pilocarpine-related adverse effects include, e.g., hyperhidrosis, nausea, rhinitis, dizziness, intestinal colic, and pollakiuria. The drug is contraindicated in cardiac patients or individuals with glaucoma, chronic obstructive pulmonary disease and asthma.[42]

In contrast to cholinergic agonist agents whose therapeutic effects last between 3 and 5 h, carboxymethyl cellulose salivary substitutes only provide palliative and short-lasting benefits.[32]

Dry mouth, decreased oral aperture and impairment in manual dexterity are responsible for dental plaque and increased risk of developing oral diseases, e.g., plaque-induced gingivitis.[43] Several case-control studies demonstrated that patients with SSc present with more severe gingivitis compared to controls. Baron et al. indicated that SSc patients have significantly worse dental health (more missing teeth, more periodontal disease) in comparison to controls.[10,44] Wood et al. found that more patients with SSc presented with periodontal pockets compared to healthy controls.[45] The cause of gingivitis may be connected to the defective vascularity and changes in the microcirculation of the gingival tissues, which results in gingival bleeding. Felder et al. and Poole et al. suggested that impaired manual dexterity was connected with poor oral hygiene.[46–48] Baron et al. indicated that decreased saliva, GERD and diminished hand function (influencing the ability to maintain good oral hygiene) are related to missing teeth.[43] Reports confirm that SSc patients have higher periodontal indices (plaque index, gingival index) compared to controls.[9] Leung et al. noticed that SSc patients experience a higher percentage bleeding on probing (BOP) and have deeper probing depth (PD) as compared to healthy subjects.[47] Recently, a positive correlation between SSc and periodontitis has been reported.

Some authors reported a widening of periodontal ligament space (PDL) in patients with SSc, which is one of the most frequent radiological findings and may be considered one of the first radiographic signs of the disease.[24] Further studies are needed to evaluate whether the radiographic image can be used as a potential diagnostic marker. Periodontal ligament space thickening can be noted in each group of teeth, with the most frequent involvement of the posterior areas. Baron et al. reported that PDL widening was connected with the severity of the disease.[26] Periodontal ligament space is a soft connective tissue located between tooth roots and the inner wall of the alveolar socket, and connects the cementum to both gingival and alveolar bone. Its normal width ranges from 0.15 to 0.21 mm. The reasons for PDL widening in SSc patients are still unknown, but considering the high fibroblast and collagen content of the PDL, it is not surprising that, as elsewhere in the body, the PDL fibroblasts may be activated, thus producing excess collagen which, consequently, may lead to ligament widening. The clinical consequences of a wide PDL remain unknown. No association has been reported between a wide PDL and missing teeth.[26]

Prevention of caries and periodontal disease is the prime objective in the treatment plan for SSc patients. Patient education is significant, with particular attention paid to mouth and dental hygiene, including brushing techniques and mouthwash with antiseptic agents. Powered toothbrushes may contribute to the improvement in oral hygiene. Most patients will need a compact head or pediatric toothbrush to gain maximum access. In patients with hand deformities and functional limitations, the extension of the toothbrush handle may facilitate the removal of plaque biofilm.[32] Patients also need to be educated and instructed on the significance of flossing

at least twice a day, with consideration given to adapted flossing (i.e., flossing devices that are hand-held).[43] For those patients who do not floss due to impaired manual dexterity, oral irrigators or non-alcohol mouth rinse may be of benefit.[48]

Regular follow-up visits (every 2–3 months) are recommended to control oral infection.[32] Patients should have radiographic examination before immunosuppressive treatment in order to remove potential sources of odontogenic infections.[9]

Prevention of oral candidiasis is connected with cleaning removable prostheses. Special caution should be taken in the case of patients on anticoagulant therapy if antifungal treatment is to be implemented. Measurement of International Normalized Ratio (INR) is obligatory.

Prevention of caries is connected with fluoridation of the enamel with toothpaste and the application of fluoride preparations every 3 months. Panoramic radiography should be used for the early detection of caries to avoid invasive treatment. The dentist can perform conventional dental treatment in SSc patients quadrant by quadrant so as to avoid long visits.

In patients who undergo extraction, antibiotic should be administered 1 day before and their administering should be continued until complete scarring.

Before the initiation of the treatment with bisphosphonates, any unsalvageable teeth should undergo extraction and all invasive procedures should be completed due to an increased risk of osteonecrosis. Oral care can be given under local anesthesia (3% lidocaine). It should be remembered that adrenaline use is to be avoided as it can worsen the microangiopathy.

A number of drug-related adverse events are reported, which are connected to the mouth and gum mucosa. These are the following:

– calcium-channel blockers may cause gingival hyperplasia;

– corticosteroids may induce mouth infections, e.g., oral candidiasis;

– cyclophosphamide also causes mouth infections or stomatitis when administered in high doses;

– methotrexate may be responsible for mouth ulcers;

– antibiotic administration may cause oral candidiasis;

– anticholinergic antidepressants may contribute to the exacerbation of the dry mouth syndrome;

– bisphosphonates may lead to jaw osteonecrosis;

– anti-vitamin K anticoagulants may provoke (spontaneous or induced) gingival bleeding.[49]

Prosthetic treatment by conventional approaches might be challenging due to microstomia. Due to the fact that patients report problems with insertion and removal of the prosthesis, it is necessary to fabricate complete and partial dentures different from the standard ones.[50] Fractionated imprints must be applied. Sectional, collapsible or sectional-collapsible are the types of prostheses recommended for prosthodontics management in patients in whom limited intraoral access is observed. Two segments of sectional dentures can be fixed by means of various systems (clasp retainers, stud or magnetic or swing-lock attachments or pins, and a telescope system).

The use of soft liner is reported for the management of edentulous patients who complain of chronic pain, soreness or discomfort related to prolonged contact between the rigid denture base and the underlying tissues.[50,51]

In conclusion, patients with SSc are exposed to a number of health problems, among which dental issues deserve special attention. These patients should obtain dental care immediately after the diagnosis is established. Early implementation of preventive and curative actions enables the maintenance of oral health. The knowledge of clinical symptoms and of the dynamics of the disease process will make it easier for dentists to apply appropriate treatment. Attention should be firstly paid to the prevention of oral diseases (caries prevention and prevention of the diseases related to the mucous membrane, gingiva and periodontium). Conservative and surgical treatment, and also prosthetic rehabilitation should be performed by a specialized team of doctors based on interdisciplinary cooperation and the latest scientific knowledge.

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How to assess and predict success or failure of intra-detrusor injections with onabotulinumtoxinA

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Abstract

Intra-detrusor injection therapy with onabotulinumtoxinA is generally accepted as a highly effective, minimally invasive and well-tolerated day procedure for patients with refractory overactive bladder (OAB) and neurogenic detrusor overactivity (NDO). The aim of this study was to summarize currently available methods of assessing treatment efficacy and risk factors that may influence the therapeutic effect of this approach. We found that there are discrepancies in the assessment methods. The evaluation of intra-detrusor injections with onabotulinumtoxinA in clinical trials are not always transposable into day-to-day practice. Moreover, the primary endpoints in clinical trials do not explore the entirety of meaningful patient-centered outcomes. Therefore, in daily clinical practice with patients with overactive bladder syndrome, the therapy should be assessed with objective measures (bladder diaries) and patient-oriented outcomes analyzing the quality of life (questionnaires). In neurogenic individuals, therapeutic efficacy should be additionally evaluated with urodynamic studies. Potential risk factors that may influence the treatment outcomes include urodynamically proven detrusor overactivity, elevated maximum detrusor pressure, greater maximum cystometric capacity, impaired bladder compliance, older age, male gender, a higher baseline bother score, previous anticholinergic treatment, and repeated injections with a subsequent decline in efficacy. The risk of intermittent catheterization following injections seems to depend on the etiology of detrusor overactivity, the injected dose, the injection technique, and the definition of significant post-void residual urine requiring catheterization.

Key words: onabotulinumtoxinA, failure, risk factors

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Intra-detrusor injection therapy with onabotulinumtoxinA (Botox®; Allergan Inc., Irvine, USA) is generally accepted as a highly effective, minimally invasive and well-tolerated day procedure for patients suffering from refractory overactive bladder (OAB) and neurogenic detrusor overactivity (NDO). Its efficacy has been widely investigated and proven in systematic reviews with meta-analyses of randomized, double-blind, placebo-controlled multicenter trials,[1–3] and onabotulinumtoxinA is currently recommended by the leading urological societies for both neurogenic and non-neurogenic voiding dysfunctions.[4] Treatment with onabotulinumtoxinA in patients suffering from OAB mainly improves their quality of life and reduces incontinence, while in those with NDO it additionally helps preserve renal function.

OnabotulinumtoxinA, a specific formulation of botulinum toxin, is a neuromodulator that directly inhibits efferent acetylcholine-mediated detrusor contractions and reduces muscle spasticity.[3] This therapy also has other mechanisms of action, and inhibits the release of different vesicle-mediated neurotransmitters responsible for inappropriate afferent signaling from an overactive detrusor, including effects on afferent sensory neurons via TRPV1 and P2X3 receptors. Therefore, the ability of onabotulinumtoxinA to synergistically target the afferent and efferent neuronal pathways of bladder control may explain the profound effect observed on urgency, frequency and urgency incontinence, as well as the failure of prior anticholinergic therapy. Consequently, onabotulinumtoxinA offers a complex inhibitory effect on multiple targets in the bladder wall that may cause OAB and NDO.

It is of utmost importance to identify patients in whom intra-detrusor Botox® therapy has failed to help or who may not benefit from it. However, appropriate patient enrollment and assessment of treatment success/failure may be challenging for physicians, as these are generally based on subjective outcomes. There is no single study summarizing currently available assessment methods of therapy success/failure and predicting factors for this treatment. Recognition of predictors is a prerequisite for an individualized approach, and adequate knowledge could help clinicians avoid ineffective procedures and potential treatment-related adverse effects. Furthermore, assessment methods of response/non-response to the treatment have important applications in future research. Therefore, the aim of this review is to summarize methods of assessing the success/failure of the therapy and to present a list of risk factors that may influence the outcomes of intra-detrusor injections of onabotulinumtoxinA.

Material and methods

Maximum data were collected according to different methods, including searches with multiple and specific keywords and reference checks. Our searches targeted the terms "neurogenic bladder", "neurogenic lower urinary tract dysfunction", "overactive bladder", "onabotulinumtoxinA", "success", "failure", and "risk factors". We searched PubMed (including MEDLINE and PubMed Central), the Cochrane Library and the Web of Science databases. During full-text screening, references in pertinent papers were checked manually to identify other related papers. In order to present reliable assessment methods of treatment success/failure, only randomized double-blind placebo-controlled trials were included. They were also selected from currently available systematic reviews with meta-analyses of OAB and NDO to avoid omitting important trials.[1–3,5–11] The reports on risk factors that we found (searching through all the available literature) were analyzed with a modified version of the Oxford grading system for recommendations, using levels of evidence (LE) and grades of recommendation (GR).[12]

Results

Assessment methods

There is no consensus among researchers how to assess the success/failure of treatment with onabotulinumtoxinA injections. To make matters even more complex, most of the randomized clinical trials on OAB and NDO used either objective measures from bladder diaries, subjective patient-reported outcomes from questionnaires or urodynamic-related parameters as primary experimental outcomes.

Overactive bladder

Overactive bladder can be debilitating for patients, and may have a profound negative impact on the patient's quality of life. Oral anticholinergics are the mainstay of first-line pharmacologic treatment,[4] but may be associated with unwanted side effects including dry mouth, constipation and blurred vision, leading to poor compliance and a high discontinuation rate in clinical practice. Therefore, minimally invasive treatment with onabotulinumtoxinA offers an effective alternative to other minimally invasive therapies such as neuromodulation, as well as to invasive surgical bladder augmentation. Our study revealed that there is no homogeneity among randomized clinical trials in reporting outcomes. Detailed comparisons between published randomized placebo-controlled trials are hampered by differences in the outcome measures used, whether diary data were compared daily or weekly, and the fact that studies recruited both men and women. Clinicians should also keep in mind that our current knowledge regarding the efficacy of the treatment is limited to patients with OAB wet (i.e., OAB with concomitant urinary incontinence). There is a paucity of reliable data analyzing treatment

with onabotulinumtoxinA in individuals suffering from OAB without urinary incontinence (OAB dry), who comprise approx. 2/3 of all OAB patients.[13]

To date, 9 randomized placebo-controlled clinical trials have been conducted in order to evaluate the efficacy of onabotulinumtoxinA in OAB.[14–25] A summary of the patients included, the doses evaluated and primary and secondary experimental outcomes is presented in Table 1. The most common primary experimental outcome, used in 4 studies, has been defined as a change from baseline in the number of urgency urinary incontinence episodes. Three of these studies used an additional co-primary outcome, defined as the proportion of patients with a positive treatment response on the 4-score Treatment Benefit Scale (2 studies) and symptom scores assessed with a validated questionnaire (1 study). One study used a change from baseline in urinary frequency per 24 h as a primary experimental outcome, 1 study evaluated time to treatment failure (defined as a score of 4 or greater on the Patient Global Impression of Improvement) and 1 used the proportion of patients showing >50% improvement compared to baseline of both urgency and urgency urinary incontinence episodes. Two studies mainly based their analyses on urodynamic parameters and used maximum cystometric capacity as a primary experimental outcome.

A time analysis of the studies included showed that the effects of onabotulinumtoxinA are commonly perceived between 3 days and 2 weeks after injection.[14,17,22,24] Although the mean duration of onabotulinumtoxinA-related improvement is approx. 9–10 months,[14,15] the efficacy of the therapy should be assessed between weeks 4 and 12, as some experimental outcomes have been shown to become insignificant, with decreasing treatment response compared with a placebo after 12 weeks (symptom reduction benefits typically show a slight trend towards decrease after 20–24 weeks).[14,17,18,23,24] In patients with more severe OAB at baseline (i.e., more than 8 episodes of incontinence per day), efficacy should be assessed no sooner than 6 weeks after injection.[16]

Neurogenic detrusor overactivity

Neurogenic detrusor overactivity is one of the most challenging problems in urology. Various disorders or injuries affecting the central nervous system (e.g., stroke, Parkinson disease and multiple sclerosis) may cause this chronic dysfunction, in which the bladder becomes overactive and empties too frequently/quickly. The primary goals of treatment for NDO are to protect the upper urinary tract by decreasing bladder pressure, reducing incontinence and improving quality of life. Initial NDO treatment consists of anticholinergics, often with clean intermittent catheterization (CIC). However, long-term treatment may be suboptimal, with patients stopping medication due to a lack of efficacy and bothersome side effects, similar to those with OAB. Minimally invasive treatment with onabotulinumtoxinA has been of benefit to those patients, as it induces paralysis of the detrusor muscle and consequently results in a decreased risk of vesicoureteral reflux, preventing upper urinary tract deterioration and possible kidney damage.

To date, 6 randomized placebo-controlled clinical trials have evaluated the efficacy of intra-detrusor injections of onabotulinumtoxinA in neurogenic patients.[26–34] These studies mainly included patients with spinal cord injuries or with multiple sclerosis. All of these studies used a change from baseline in the number of urgency urinary incontinence episodes per week as the primary experimental outcome. Five of the studies assessed this primary experimental outcome at week 6.

Improvements usually occur starting from week 2 and are maintained for the duration of 24 weeks of follow-up.[27,30–34] In 3 studies with an open-label phase and longer follow-up, the median duration of the effect – the time lapse before patient-requested retreatment – was 36, 42.1 and 60 weeks, respectively.[28,29,31] These benefits reached their maximum between the 2nd and 6th week.[10] Approximately 60% of the patients may maintain the treatment effect for up to 9 months.[34] However, some of the efficacy parameters tend to become insignificant after 18–24 weeks[26,28] and efficacy evaluation before 6 weeks may be too early for reliable assessment.[34]

Risk factors for treatment failure

Detrusor overactivity

Detrusor overactivity has been defined as the presence of involuntary detrusor contractions during the filling phase of urodynamics. Whereas in neurogenic individuals urodynamically proven detrusor overactivity is necessary to establish a diagnosis of NDO, in those with OAB, although common, it is not present in all patients and not required for an OAB diagnosis.

The impact of detrusor overactivity on the final effect of treatment with onabotulinumtoxinA in OAB patients is still under debate, as the majority of available studies required detrusor overactivity for inclusion. Dowson et al. conducted a randomized controlled trial examining the effects of onabotulinumtoxinA exclusively in patients with OAB without concomitant detrusor overactivity (LE 1, GR B).[21] The trial was halted after the recruitment of 23 patients as a result of poorly perceived patient benefit and no clinical benefit, with no change observed in the symptom score and quality of life for the majority of the participants. Individually, only 2 patients in the treatment arm of the study derived any benefit from the injections, whereas the remainder experienced no change in their symptoms. Of note, the study enrolled only 1/3 of the patients suggested by the power calculation, and the primary endpoint (maximum cystometric capacity) was statistically significant. In a large randomized placebo-controlled clinical trial

Table 1. Currently-available randomized placebo-control clinical trials evaluating the efficacy of onabotulinumtoxinA in OAB. The summary of included patients, evaluated doses, and primary and secondary experimental outcomes

Authors	Year	Single/multi-center study	Included patients (M – men; W – women)	Doses	Follow-up (after injections)	Primary experimental outcome (change in the indicated parameter)	Secondary experimental outcome (change in the indicated parameter)
Sahai et al.[14]	2007	single-center	M: 15; W: 19	200 U	1, 4 and 12 weeks	Maximum cystometric capacity assessed with urodynamics.	Urinary frequency, urgency and episodes of urgency urinary incontinence assessed with a 3-day bladder diary. Quality of life assessment and patient-orientated evaluation of the treatment assessed with validated questionnaires (Incontinence Impact Questionnaire – IIQ-7, Urogenital Distress Inventory – UDI-6). Maximum detrusor pressure, reflex detrusor volume (the volume of saline infused at which the first reflex detrusor contraction occurred during the filling phase regardless of symptoms) and post-void residual assessed with urodynamics.
Brubaker et al.[15]	2008	multi-center	W: 43	200 U	monthly during 12 months	Time to failure (defined as the score from Patient Global Impression of Improvement of 4 or greater).	Episodes of urgency urinary incontinence (assessment method not defined). Quality of life assesment and patient-orientated evaluation of the treatment assessed with validated questionnaires (Pelvic Floor Distress Inventory, Pelvic Floor Impact Questionnaire, Sexual Function Questionnaire, SF-36, Patient Global Impression of Symptom Control).
Flynn et al.[16]	2009	single-center	W: 22	200 U, 300 U	3 days; 3 and 6 weeks	Episodes of urgency urinary incontinence per 24 h assessed with a bladder diary. Symptom scores assessed with validated questionnaires (Incontinence Impact Questionnaire – IIQ-7, Urogenital Distress Inventory – UDI-6).	24-hour pad weights and number of pads used per 24 h assessed with a pad test. 24-hour voiding frequency, diurnal urinary frequency and nocturia assessed with a 3-day bladder diary. Maximal cystometric capacity, volume of first uninhibited detrusor contraction, presence of stress leakage, value of detrusor pressure at peak flow, peak urine flow and post-void residual assessed with urodynamics.
Dmochowski et al.[17] (data from bladder diaries)	2010	multi-center	M: 25; W: 288	50 U, 100 U, 150 U, 200 U, 300 U	7 days; 2, 6, 12, 18, 24, 30, and 36 weeks	Episodes of urgency urinary incontinence per 1 week assessed with an electronic bladder diary at week 12.	Urinary frequency, urgency, nocturia and volume voided per micturition assessed with a 7-day electronic bladder diary.
Rovner et al.[18] (data from urodynamic studies)	2011				12 and 36 weeks		Maximum cystometric capacity, volume at first idiopathic detrusor contraction, peak (amplitude) detrusor pressure during first idiopathic detrusor contraction (maximum detrusor pressure), detrusor compliance measured at maximum cystometric capacity or prior to terminal idiopathic detrusor contraction, whichever occurred first, and post-void residual urine volume via catheterization assessed with a urodynamic study.
Fowler et al.[19] (data from questionnaires)	2012				7 days; 2, 6, 12, 18, 24, 30, and 36 weeks		Quality of life assessment and patient-orientated evaluation of the treatment assessed with validated questionnaires (Incontinence Quality of Life – I-QoL; King's Health Questionnaire – KHQ; Medical Outcomes Study 36-Item Short-Form Health Survey – SF-36).
Brubaker et al.[20] (data from questionnaires)	2012				2, 6, 12, 18, 24, 30, and 36 weeks		Patient satisfaction assessed with questionnaires (the modified version of the Overactive Bladder Patient Satisfaction with Treatment Questionnaire – OAB-PSTQ, Patient Global Assessment – PGA).

Table 1. Currently-available randomized placebo-control clinical trials evaluating the efficacy of onabotulinumtoxinA in OAB. The summary of included patients, evaluated doses, and primary and secondary experimental outcomes – cont.

Authors	Year	Single/multi-center study	Included patients (M – men; W – women)	Doses	Follow-up (after injections)	Primary experimental outcome (change in the indicated parameter)	Secondary experimental outcome (change in the indicated parameter)
Dowson et al.[21]	2011	single-center	M: 6; W: 15	100 U	4 and 12 weeks	Maximum cystometric capacity assessed with urodynamics at week 12.	Urinary frequency, urgency and episodes of urgency urinary incontinence assessed with 3-day voiding diary. Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Impact Questionnaire – IIQ-7, Urogenital Distress Inventory – UDI-6, Patient Perception of Bladder Condition Questionnaire – PPBC).
Tincello et al.[22]	2012	multi-center	W: 240	200 U	6 weeks; 3 and 6 months	Urinary frequency per 24 h assessed with bladder diary at 6 months.	Urinary urgency (moderate or severe on the Indevus Urgency Severity Scale – IUSS) and episodes of urgency urinary incontinence assessed with 3-day bladder diary. Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (International Consultation on Incontinence Questionnaire short form – ICIQ-SF; Incontinence Quality of Life – IQOL). Need for additional treatment during follow-up and time to return of troublesome symptoms.
Denys et al.[23]	2012	multi-center	M: 12; W: 87	50 U, 100 U, 150 U	8 days; 1, 3, 5, and 6 months	The proportion of patients showing >50% improvement compared to baseline of both urgency and urgency urinary incontinence episodes assessed with 3-day bladder diary at month 3.	Urinary frequency, urgency, episodes of urgency urinary incontinence and pads used per day assessed with 3-day bladder diary. Volume at first and at strong contraction, detrusor pressure, maximum detrusor pressure, maximum cystometric capacity and post-void residual assessed with urodynamic studies. Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life – I-QoL; EQ-5D visual analogue scale – VAS).
Nitti et al.[24]	2013	multi-center	M: 60; W: 497	100 U	2, 6 and 12 weeks (if re-treatment was necessary additional follow-up at 18 and 24 weeks)	Episodes of urgency urinary incontinence per 1 day assessed with bladder diary at week 12. The proportion of patients with a positive treatment response on the 4-score Treatment Benefit Scale (condition greatly improved or improved) at week 12.	Urinary frequency, urgency, nocturia and volume voided per micturition assessed with 3-day bladder diary. Quality of life and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life – I-QoL; King's Health Questionnaire – KHQ). The proportion of patients achieving a 50% or greater, or a 100% reduction in episodes of urinary incontinence.
Chapple et al.[25]	2013	multi-center	M: 75; W: 473	100 U	2, 6 and 12 weeks (if re-treatment was necessary additional follow-up at 18 and 24 weeks)	Episodes of urgency urinary incontinence per 1 day assessed with bladder diary at week 12. The proportion of patients with a positive treatment response on the 4-score Treatment Benefit Scale (condition greatly improved or improved) at week 12.	Urinary frequency, urgency, nocturia and volume voided per micturition assessed with 3-day bladder diary. Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life – I-QoL; King's Health Questionnaire – KHQ). The proportion of patients achieving a 50% or greater, or a 100% reduction in episodes of urinary incontinence.

Table 2. Currently available randomized placebo-control clinical trials evaluating the efficacy of onabotulinumtoxinA in neurogenic detrusor overactivity (NDO). The summary of included patients, evaluated doses and primary andsecondary experimental outcomes

Authors	Year	Single/multi-center study	Patients included (M – men; W – women)	Doses	Follow-up (after injections)	Primary experimental outcome (change in the parameter indicated)	Secondary experimental outcome (change in the parameter indicated)
Schurch et al. (data from bladder diaries and urodynamic studies)[26]	2005	multi-center	M: 36; W: 23	200 U, 300 U	2, 6, 12, 18, and 24 weeks	Episodes of urinary incontinence per 1 week prior to each follow-up appointment assessed with a bladder diary.	Maximum cystometric capacity, reflex detrusor volume and maximum detrusor pressure during bladder contraction assessed with an urodynamic study.
Schurch et al. (data from questionnaires)[27]	2007						Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life – I-QoL).
Herschorn et al.[28]	2011	multi-center	M: 34; W: 23	300 U	1, 3, 4, 6, 24, and 36 weeks	Episodes of urinary incontinence per 1 day assessed with a bladder diary at week 6.	Reflex detrusor volume at first contraction, maximum detrusor pressure during filling, volume at maximum detrusor pressure during filling and maximum cystometric capacity assessed with a urodynamic study. Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life – I-QoL; International Consultation on Incontinence Questionnaire – ICIQ).
Cruz et al. (data from bladder diaries and urodynamic studies)[29]	2011	multi-center	M: 120; W: 155	200 U, 300 U	2, 6 and 12 weeks (and then at 6 or 12-week intervals until 52 weeks, if requested retreatment)	Episodes of urinary incontinence per 1 week assessed with a bladder diary at week 6.	Maximum cystometric capacity, maximum detrusor pressure during first involuntary detrusor contraction, first idiopathic detrusor contraction, detrusor compliance and volume per void assessed with urodynamic studies. Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life – I-QoL (only total score)).
Sussman et al. (data from questionnaires)[30]	2012				6 and 12 weeks (and then at 12-week intervals until 52 weeks, if requested retreatment)		Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life –I-QoL; modified Overactive Bladder Patient Satisfaction with Treatment Questionnaire – OAB-PSTQ; Patient Global Assessment – PGA).
Ginsberg et al. (data from bladder diaries and urodynamic studies)[31]	2012	multi-center	M: 171; W: 245	200 U, 300 U	2, 6 and 12 weeks (and then at 6-week intervals until 52 weeks, if requested retreatment)	Episodes of urinary incontinence per 1 week assessed with a bladder diary at week 6.	Maximum cystometric capacity and maximum detrusor pressure during the first involuntary detrusor contraction assessed with a urodynamic study. Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life – I-QoL (only total score)).
Chancellor et al. (data from questionnaires)[32]	2013				6 and 12 weeks (and then at 12-week intervals until 52 weeks, if requested retreatment)		Quality of life assessment and patient-orientated evaluation of the treatment assessed with questionnaires (Incontinence Quality of Life – I-QoL; the modified version of the Overactive Bladder Patient Satisfaction with Treatment Questionnaire – OAB-PSTQ; Patient Global Assessment – PGA).
Rovner et al. (pooled analysis of urodynamic data form studies of Ginsberg and Cruz)[33]	2013	multi-center	M: 291; W: 400	200 U, 300 U	6 weeks		Maximum cystometric capacity, maximum detrusor pressure only at first involuntary detrusor contraction, volume at first involuntary detrusor contraction, and detrusor compliance (change in volume / change in detrusor pressure, where change in volume = maximum cystometric capacity; and change in detrusor pressure = end fill pressure) assessed with urodynamics at week 6. Percentage of patients with no involuntary detrusor contraction on post-treatment urodynamics.
Apostolidis et al.[34]	2012	multi-center	M: 63; W: 10		2, 6 and 12 weeks (and then at 6-week intervals until 78 weeks, if requested retreatment)	Episodes of urinary incontinence per 1 week assessed with a bladder diary at week 6.	Volume per void assessed with a 7-day bladder diary. Maximum cystometric capacity, maximum detrusor pressure during first involuntary detrusor contraction and volume at first involuntary detrusor contraction assessed with a urodynamic study. Duration of effect (time from first treatment to patient request for retreatment).

by Dmochowski et al. (n = 313; LE 1, GR B/C), a subgroup analysis for patients with and without detrusor overactivity showed that the patient-reported outcomes were similar in the 2 groups, although statistical significance in comparison with the placebo was not reached in these much smaller subgroups.[17] Further analysis of the urodynamic data also demonstrated that patients with detrusor overactivity at baseline experienced treatment efficacy compared to those without it.[18] The authors proposed that urodynamic confirmation of detrusor overactivity before treatment may not be predictive in determining treatment success, as patients with OAB with and without detrusor overactivity benefited from the treatment. These results are in line with those presented by Schmid et al. from an open-label study (n = 100; LE 2, GR C).[35] Although the last 2 trials demonstrated some clinical improvement in patients with and without detrusor overactivity, physicians should keep in mind that they were not specifically designed to examine the influence of detrusor overactivity on treatment outcomes and the data presented may be inconsistent.

Maximum detrusor pressure

There have been 2 OAB studies evaluating the influence of elevated maximum detrusor pressure on final treatment outcomes. Sahai et al., in a subanalysis of urodynamic data from their randomized clinical trial (LE 1, GR B/C), reported that very high maximum detrusor pressure (>110 cm H_2O) may predict a poor response to treatment with 200 U of onabotulinumtoxinA (sensitivity 0.86; specificity 1.0), indicating that higher doses may be necessary in these patients.[36] In turn, Rovner et al. demonstrated that elevated maximum detrusor pressure is a poor indicator of treatment response (LE 1, GR B/C).[18] Similarly to Sahai et al., their analysis was based on urodynamic data from a previously published randomized clinical trial. It is noteworthy that in the study by Rovner et al., the mean baseline maximum detrusor pressure ranged from 21.7 cm H_2O (SD ±18.26) to 24.3 cm H_2O (SD ±18.40), whereas in the study by Sahai et al. it was much higher, ranging from 74.4 cm H_2O (SD ±32.6) to 138.0 cm H_2O (SD ±30.7).

In a retrospective analysis of 292 neurogenic patients monitored up to 7 years, high maximum detrusor pressure has been identified as a risk factor of treatment failure. (LE 3, GR C).[37] Nevertheless, the multivariate analysis failed to confirm it as an independent long-term risk factor.

Maximum cystometric capacity

A prospective study by Álvares et al. on individuals who had suffered spinal cord injury (n = 34) showed that patients with greater maximum cystometric capacity responded significantly better than others (LE 2, GR B/C).[38] The authors also demonstrated that cystography parameters, including bladder shape, capacity and the presence of diverticula, were not significantly different between responders and non-responders to the therapy.

Bladder compliance

It has been suggested that pre-existing bladder wall fibrosis may limit therapeutic efficacy. The impact of bladder compliance on treatment results was assessed by Schmid et al. in their prospective non-randomized study.[35] A total of 23 men and 77 women with OAB were consecutively treated with injections of 100 U onabotulinumtoxinA and then followed up with urodynamics at 4, 12 and 36 weeks. Treatment failed in 8 patients, all of whom had low bladder compliance (less than 10 mL/cm H_2O) and maximum cystometric capacity less than 100 mL due to bladder wall fibrosis (LE 2, GR B). These results are in line with those presented by Kim et al. in neurogenic patients.[39] Preoperative bladder compliance was significantly lower in non-responders to onabotulinumtoxinA injections (25.11 ±32.59 vs 8.64 ±6.52; p = 039). Furthermore, a regression analysis revealed that decreased bladder compliance (<10 mL/cm H_2O; odds ratio (OR) = 6.041; 95% confidence interval (CI) = 1.189–30.677; p = 0.030) was an independent predictor of poor response (LE 3, GR C). A long-term study with a 7-year follow-up of neurogenic patients confirmed in a univariate analysis that decreased bladder compliance is a risk factor for therapy failure (LE 3, GR C).[37] Moreover, it has been reported that the efficacy of onabotulinumtoxinA injections in patients with low bladder compliance has a shorter duration (12–24 weeks) than in those with normal compliance.[40]

Age

Komesu et al. compared the efficacy and adverse events of onabotulinumtoxinA injections in women <65 and ≥65 years suffering from refractory urgency urinary incontinence (LE 1, GR B).[41] Even though the authors showed that both older and younger women experienced beneficial reductions in urgency urinary incontinence episodes, similar rates of adverse events and improved quality of life, the study revealed that younger women experienced greater absolute continence, symptom improvement and fewer urinary tract infections. Women <65 years had 3.3-fold greater odds of ≥75% symptom resolution than women ≥65 years (95% CI = 1.56–7.02). Compatible results were presented by Cohen et al., but the relationship was not statistically significant in a multivariate analysis (LE 1, GR B).[42] Lower long-term success rates have also been noted in frail elderly patients (LE 2, GR B).[43]

Sex

Hsiao et al. showed that male gender is an independent factor associated with OAB treatment failure (LE 2, GR B).[44] The study included a total of 89 patients (46 men

and 43 women) and found that female gender (OR = 3.75) was an independent factor associated with treatment success.

Symptoms

It has been shown that low baseline OAB symptom scores and the presence of OAB wet are independent factors associated with therapeutic efficacy (LE 2, GR B).[44] The authors also found that high OAB symptom scores were an independent factor for predicting low response, but the coefficient was low (i.e. −0.12). Thus, symptoms of OAB (other than incontinence) seem to be a poor predictor for assessing efficacy compared to simple evaluation of the presence or absence of incontinence.

Previous anticholinergic treatment

Makovey et al. reported that patients with poor antimuscarinic efficacy experienced less therapeutic effect from onabotulinumtoxinA injections (LE 3, GR C).[45] Success rates were lower in patients reporting a lack of efficacy of antimuscarinic drugs (34 of 57, 68%), compared to those who stopped because of side effects (24 of 28, 86%). In contrast, a pooled analysis of 2 trials of 100 U of onabotulinumtoxinA showed no difference in treatment effect irrespective of the number of antimuscarinic preparations tried, or whether oral medication was stopped due to side effects or a lack of efficacy (LE 1, GR B).[46]

Repeated injections

As the effects of onabotulinumtoxinA therapy are of limited duration, patients usually require further treatments. A recently published study suggested that in OAB patients who respond to the treatment, the duration of the response declines after the 5th injection, suggesting a possible tolerance effect and a subsequent decline in efficacy (LE 3, GR C).[47] The mean time between patients receiving intra-detrusor onabotulinumtoxinA and being added to the surgical waiting list for retreatment varied between 8.5 and 10.4 months for the first 5 cycles of treatment, with the longest time period between the 3rd and 4th cycle. It then decreased to 5.5 and 5.25 months between the 5th and 6th cycle and between the 6th and 7th cycle of treatment, respectively. Further data extrapolation has shown that at the 9th or 10th treatment, the mean duration of response to the treatment would be less than the recommended 4 months. The authors also proposed that treatments with symptomatic benefit of less than 3 months should be regarded as "treatment failure", i.e., patients who undergo further treatments with onabotulinumtoxinA may not get a sufficient therapeutic benefit to justify repeating the procedure. These results are similar to those presented by Tincello et al., who found an increased rate of symptom recurrence following 2nd and 3rd treatments when compared to symptom recurrence after the 1st treatment (LE 3, GR C).[48] Marcelissen et al. showed that almost 70% of all patients abandoned repeated treatments after a mean follow-up of 97 months (LE 3, GR C).[49] Of the patients included (n = 128), 27% experienced insufficient effect and 43% had tolerance issues.

There have been 2 studies investigating the efficacy of repeated injections in neurogenic individuals in terms of risk factors for treatment failure. A recent study by Denys et al. have showed that patients with NDO with a ≥50% reduction of urinary incontinence after their 1st onabotulinumtoxinA treatment continued to experience consistent improvements in urinary incontinence and quality of life with subsequent treatments over a period of 4 years (LE 2, GR B).[50] Initial positive responses were generally maintained to the same extent after repeat treatments. A <50% reduction of urinary incontinence after the 1st treatment did not necessarily predict low response with subsequent treatments, as more than 1/3 of the patients with poor initial response showed better results and increasing response with subsequent injections. The authors concluded that these results underscore the importance of attempting at least a 2nd treatment with onabotulinumtoxinA before deeming neurogenic patients unsuitable for this therapy. Joussain et al. in their retrospective analysis of 292 patients, reported a failure rate of 12.6% (8.6–16.5%) after 3 years, 22.2% (16.6–27.3%) after 5 years and 28.9% (21.9–35.3%) after 7 years of follow-up, whereas the primary failure ratio was 5.1% (n = 15; LE 3, GR C).[37]

Other factors

In neurogenic patients, other possible risk factors that may predict treatment failure include a high number of febrile urinary tract infections (LE 3, GR C) and kidney ultrasound abnormalities (LE 3, GR C).[37]

In OAB patients, smoking status has been found to be a predictive factor for non-response in urgency episodes (with smokers having nearly 3 times greater odds of non-response compared to non-smokers), and higher numbers of baseline leakage episodes are correlated with failure to achieve continence (for every additional increase in the number of baseline leakage episodes, patients had a 17% increase in the odds of failing to achieve continence; LE 1, GR B).[51] Body mass index (BMI) has been shown to have marginal associations with non-response to the treatment. It is worth noting that the risk factors identified in the study were derived from OAB women treated with 200 U of onabotulinumtoxinA and urodynamically proven detrusor overactivity. Furthermore, the authors assessed potential patient factors correlated with non-response only at 6 weeks after treatment. Urinary tract infections in OAB patients were not found to affect the outcomes of the treatment (LE 1, GR B).[51]

Predictive factors for CIC

There are no universally accepted predictive factors for the need for CIC following injections of onabotulinumtoxinA. However, the reported rates of CIC use seem to depend on the etiology of detrusor overactivity, the injected dose along with the injection technique, the definition of significant post-void residual urine, and other factors. It has been shown that the risk of post-treatment retention requiring CIC can be 2-fold higher in neurogenic patients than in those with OAB (LE 2, GR B).[52] The affected neural mechanism for voiding in neurogenic individuals has been proposed as a reason for this phenomenon. The majority of available studies observed a dose-response relationship in terms of initiating CIC following onabotulinumtoxinA injections (LE 1, GR A).[23,26,35] Currently recommended doses (200 U in NDO, 100 U in OAB) represent an appropriate balance between the benefits and safety profile of the drug. A meta-analysis of efficacy and adverse events after trigonal vs extratrigonal injections revealed that trigonal injections were more often associated with acute urinary retention, but this correlation was not statistically significant.[53] Trigonal injections also lead to non-significantly higher values of post-void residual urine. The post-void residual (PVR) volume at which patient should start CIC varies among the available studies. Proposed cut-offs include 100 mL, 150 mL, 200 mL, or even 300 mL.[14,15,35,52,54] To make matters even more complex, the symptoms associated with retention have not always been included in the study definitions of significant retention. There are no strict criteria for CIC cessation, thus the duration of CIC may have been overestimated in some studies. Finally, a recent study has shown that CIC initiated on the basis of an arbitrary PVR volume does not benefit the patient, and CIC use should be based on the individual patient's symptoms (LE 3, GR B/C).[55] Nevertheless, a peak effect and dose-dependent mean increase in PVR volume following onabotulinumtoxinA treatment is usually observed at week 2, with a gradual decrease between weeks 4 and 12 (LE 1, GR A).[17,24] Furthermore, the duration of catheterization (by CIC or indwelling catheter) is typically longer with higher injected doses.[17] The need for CIC also depends on the patient's medical history of previous injections. On the one hand, patients who do not need CIC after their first injection are at a lower risk of needing CIC in later treatment cycles (LE 1, GR A).[56] On the other hand, clinicians should remember that if CIC is necessary after the first injection, it seems to be needed after all subsequent treatments (LE 2, GR B).[54] In patients with a preoperative PVR volume >100 mL, retention may appear in up to 95% of patients (LE 3, GR C).[57] Similar findings have been demonstrated in terms of low voiding efficiency (<90% of the voided volume compared to the pre-void bladder volume) (LE 3, GR C).[58] Furthermore, it has been shown that patients with low maximum flow rates (<15 mL/s), low projected isovolumetric pressure (<50, calculated as the maximum urinary flow rate + detrusor pressure at the maximum urinary flow rate in women) and low bladder contractility index (<120, calculated as 5 times the maximum urinary flow rate + detrusor pressure at the maximum urinary flow rate in men) are at increased risk of needing CIC after onabotulinumtoxinA injections (LE 3, GR C).[59] Clean intermittent catheterization may also become necessary after later injections even if it was not initially required. Other factors that may increase the risk of CIC include older age (>61–76 years) (LE 2, GR B),[43,60] frailty (LE 2, GR B)[43] and higher parity (particularly a higher number of vaginal deliveries; LE 2, GR B).[60] A recent study has shown that BMI and concomitant comorbidities do not significantly influence PVR volumes and the risk of CIC, even though one would expect that these factors could provoke safety issues.[60] Nevertheless, Wang et al. showed that patients with diabetes have a significantly increased incidence of PVR volumes greater than 150 mL (60.4%) compared with nondiabetic patients (33.3%; LE 3, GR C).[61]

Although it may sometimes be necessary to introduce CIC, studies have shown that the need to perform CIC do not negatively impact the outcome of onabotulinumtoxinA therapy, and that improvements in patients' symptoms are similar with and without CIC.[31,57] It has been shown that the risk of new-onset urine retention with a need for CIC usually disappears 2 weeks after the injections.[60]

Discussion

To the best of our knowledge, this is the first study summarizing reported methods of efficacy assessment and factors predicting failure in onabotulinumtoxinA therapy. Currently, onabotulinumtoxinA is the only formulation of botulinum toxin that has been investigated in properly powered, multicenter, multinational randomized controlled trials, and only onabotulinumtoxinA is licensed in the USA and Europe for the management of NDO and OAB.

Our study revealed that most of the randomized controlled trials investigating the efficacy of intra-detrusor injections with onabotulinumtoxinA, both in OAB and NDO patients, commonly used the number of incontinence episodes as the primary experimental outcome. However, this parameter cannot be directly applied in daily clinical practice, as it is not always well correlated with subjective outcomes, including health-related quality of life, treatment satisfaction, subjective assessments of global "improvement", or the safety of the upper urinary tract.[20]

As OAB is a syndrome in the absence of urinary tract infection or other obvious pathology, outcomes from treatment are subjective, highly individual and influenced by patients' lifestyle as well as their expectations from treatment. As the patient's perception of treatment success in OAB is an important component of overall success, clinicians should keep in mind that they underestimate the extent to which patients are affected by their symptoms

in 25–37% of cases.[62] Therefore, reports of medical outcomes after treatment should always include independent, validated questionnaires self-administered by patients to avoid interviewer bias.[63] Furthermore, it has been demonstrated that objective measures from bladder diaries have correlated poorly with patient-related outcome measures, suggesting that objective and subjective assessments measure different aspects of treatment efficacy.[64] Therefore, any evaluation of the efficacy of intra-detrusor injections with onabotulinumtoxinA in day-to-day clinical treatment of OAB patients should be based on objective measures (from bladder diaries) as well as patient-reported outcomes (from questionnaires). The International Consultation on Incontinence has evaluated specific criteria for currently used questionnaires and developed a recommendation grading system. Questionnaires with a Grade A recommendation (highly recommended) should be used in clinical practice. Among them are the Overactive Bladder Questionnaire (OAB-q), the Overactive Bladder Satisfaction Questionnaire (OAB-S), the Overactive Bladder Symptom Scores Questionnaire (OABSS), the Incontinence Impact Questionnaire (II-Q), and the Urogenital Distress Inventory (UDI). Nowadays, single-question questionnaires (e.g., the Patient Global Impression Scale, the Treatment Benefit Scale, the Likert scale, and VAS score[65]) are becoming more popular and are widely used in daily practice. The lack of a positive change in these scales following treatment could be used to define treatment failure. Each questionnaire can be used alone or in combination with other questionnaires to improve assessment or monitoring of treatment outcomes.[4] Some experts suggest that in daily clinical practice, treatment outcomes in OAB patients can be judged by patient communication regarding changes in their symptoms or improvements in their quality of life, without objective documentation. In our opinion, this practice should be avoided. Assessment of treatment efficacy with urodynamics in OAB patients is not currently recommended, as a recently published systematic review with meta-analysis has shown that urodynamic parameters may not differ despite significant improvement in patient-oriented outcomes.[2] Current guidelines advise that follow-up should be patient/symptom directed and a new injection may be considered when the clinical benefit of the previous injection diminishes, but a period of 3 months must elapse between each injection.[66] Also, the manufacturer of onabotulinumtoxinA recommends that retreatment should not be considered within 12 weeks from the previous bladder injection, and the total dose should not exceed 360 U in a 3-month period for all indications.[67]

There are no guidelines or recommendations available for following up neurogenic patients treated with intra-detrusor onabotulinumtoxinA injections. As neurological diseases are usually progressive, videourodynamic study and the use of validated questionnaires with a bladder diary at baseline are recommended. The primary goal of bladder management in neurogenic patients is to achieve low-pressure urine storage and low-pressure emptying of the bladder. Thus, the therapy should be assessed with a urodynamic study, even though available randomized controlled trails used changes in the frequency of urinary incontinence episodes as a primary experimental outcome. In high-risk patients (i.e., those with vesicoureteral reflux, elevated detrusor pressures, decreased bladder compliance, and worsening hydronephrosis or renal function), experts recommend repeating urodynamic studies after injections regardless of the clinical outcome.[68] In the current literature, there is a tendency to define unsuccessful treatment with maximum storage pressure >40 cm H_2O and/or detrusor compliance <20 mL/cm H_2O after injections. Moreover, it has been proposed that the following parameters should be recorded: the presence of involuntary detrusor contractions, the volume of bladder filling at the first involuntary contraction, the maximum detrusor pressure of involuntary contractions of greater intensity, the maximum cystometric capacity, the first voiding desire, bladder compliance, maximum detrusor pressure, maximum flow, detrusor pressure at maximum flow, and post-void residual volume.[69] In low-risk patients (i.e., those with low detrusor storage pressures and appropriate compliance, and when other clinical evaluations also suggest stable lower and upper urinary tracts in a non-progressive neurologic disease), urodynamic studies after injections can be delayed if the patient responds clinically to the treatment.[68]

Our study revealed that possible risk factors that may influence treatment outcomes include urodynamically proven detrusor overactivity (LE 1, GR D), elevated maximum detrusor pressure (LE 1, GR B/C), greater maximum cystometric capacity (LE 2, GR B/C), impaired bladder compliance (LE 2, GR B), older age (LE 1, GR B), male gender (LE 2, GR B), higher bother score from symptoms at baseline (LE 2, GR B), previous anticholinergic treatment (LE 3, GR D), repeated injections with a subsequent decline in efficacy (LE 3, GR C), and other factors (LE 2/3, GR B/C). Patients with these risk factors should be informed that they may experience less efficacy. It might be suggested that the predictors of success identified by our study may vary depending on treatment dose, but it has recently been emphasized that there is no physiological or pharmacological reason why such a difference would exist.[51]

In patients who did not respond to the treatment, other potential underlying disorders for the symptoms reported should be taken into account. It is noteworthy that patients with OAB symptoms may in fact suffer from painful bladder or interstitial cystitis; these individuals appear to respond less favorably to with onabotulinumtoxinA,[70] and if the treatment is effective, it has a shorter duration of action.[71] In patients with decreased bladder compliance, special attention should be given to any possible underlying neurological disorders, as impaired compliance is usually a sequela of neurologic disease, but it may also result from any process that destroys the elastic or viscoelastic properties of the bladder wall. In neurogenic patients, failed

treatment may also result from progression of their underlying neurological disorder. Physicians should remember that short duration of efficacy or treatment failure can be dependent upon procedure-related factors, such as problems with the storage of onabotulinumtoxinA vials, the reconstitution process or the injection technique. Optimization of the injection procedure (the first indication; dose personalization depending on clinical and urodynamic parameters, but also patient expectations; injection localization) could help improve the global efficacy of onabotulinumtoxinA treatment. Moreover, associated urinary tract infections can mask effective treatment. Development of neutralizing antibodies against the neurotoxin is possible, but very unlikely. It has been demonstrated that <1% of patients develop neutralizing antibodies to onabotulinumtoxinA after treatment cycle 6 and their presence has no significant impact on treatment efficacy.[23,72] Another hypothesis to explain treatment failure could be histological modifications induced by injections. However, studies have demonstrated no difference in terms of inflammation, fibrosis or edema before and after 1 or multiple injections.[73] Moreover, no histological differences were found between responders and non-responders. Patients for whom the treatment failed should be questioned with regard to changes in lifestyle or the use of new medications that may have contributed to the worsening of lower urinary tract symptoms.

If the treatment fails and/or a physician predicts that intra-detrusor injections of onabotulinumtoxinA might be ineffective, a switch to a different toxin seems to be more effective than administering another injection of the same toxin. Replacing onabotulinumtoxinA with abobotulinumtoxinA may provide satisfying results.[74] It has been also proposed that in non-responding patients planned for repeated injections, the dose may be increased by an extra 50 U in OAB and an extra 100 U in NDO individuals.[69]

Some studies describe a phenomenon called "secondary failure". This term is used for patients who responded well to the first injection(s), but eventually had very limited benefit after subsequent treatments. Possible causes include an underlying immunological mechanism (antibody formation) or a technical issue with subsequent injections (less adequate delivery). Therefore, some experts have proposed that a repeat injection at least 3 months after the failed injection should be performed in patients with secondary failure.[75]

Conclusions

In conclusion, onabotulinumtoxinA is well established as a second-line treatment for patients with OAB and NDO. When following up OAB patients treated with onabotulinumtoxinA, a validated questionnaire should be used to measure the degree of symptom bother, while a voiding diary should be utilized to assess the response to the treatment. In neurogenic individuals, the therapy should be also evaluated with a urodynamic study. Patients with risk factors for treatment failure should be appropriately informed of the possible lack of efficacy. This article can serve as a reference document for future research assessing the efficacy of onabotulinumtoxinA in specific clinical situations and help urologists select appropriate patients and evaluate their response to the treatment.

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