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Original papers

Reviews

Anti-cancer effect of engineered recombinant interleukin 18

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Abstract

Background. Interleukin 18 (IL-18) is an inflammatory cytokine belonging to the interleukin 1 (IL-1) superfamily, and is known for its role in anti-cancer activity by promoting type 1 immune response, and thus may be applied to cancer immunotherapy. Our previous report has showed 16 times higher activity of engineered E6K+T63A IL-18 than of native IL-18 in vitro. However, no data has been acquired for its anti-cancer effect in animal model.

Objectives. To investigate the anti-cancer effect of engineered E6K+T63A IL-18 as an immune stimulant in vivo.

Material and methods. Tumor-bearing mice were treated with native IL-18 or E6K or E6K+T63A IL-18 once a day for 10 days after the tumor reached the volume of 100 mm^3. Tumor volume and the number of certain immune cell type in the tumor microenvironment were investigated in this study.

Results. The results showed that tumor progression in mice treated with E6K+T63A was slower than in mice treated with E6K and native IL-18. The volume of the tumor was also smaller and the lifespan longer in the E6K+T63A IL-18-treated mice. The proportions of type 1 helper T cell (Th1) and cytotoxic T lymphocyte (CTL) were significantly higher in mice treated with E6K+T63A IL-18.

Conclusions. These results suggest that our engineered IL-18 conferred strong anti-tumor immunity in the animal model.

Key words: Th1, in vivo, interleukin-18, CTL, anti-cancer immunity

Introduction

Interleukin 18 (IL-18) is an inflammatory cytokine belonging to the interleukin 1 (IL-1) superfamily. Formerly, interleukin-18 was known as interferon γ (IFN-γ) inducing factor due to its function in IFN-γ enhancement.[1] Interleukin 18 drives the polarization of $CD4^+$ T cells toward the helper T cell type 1 (Th1) phenotype when co-stimulated with IL-12 or IL-15, which is identified through IFN-γ production.[2–4] Interleukin 18 can also polarize human natural killer (NK) cells to develop a distinct helper differentiation phenotype ($CD83^+CCR7^+CD25^+$), which effectively produces IFN-γ.[5,6] Moreover, IL-18 can activate M1 macrophage and inhibit angiogenesis.[7] Interleukin 18 has been studied as a tumor suppressor protein in animal models, and has been administered safely to patients in clinical trials.[8–11] Various studies have shown that IL-18 can act as a tumor suppressor when the cytokine is administered through an intraperitoneal,[12–14] intravenous,[12] intratumoral,[15] or peritumoral[16] route.

Many studies have tried to combine the anti-tumor function of IL-18 with other tumor treatment methods, especially immunotherapy. Two studies trialed IL-18 in combination with adoptive dendritic cell (DC) transfer, which resulted in the enhancement of the cytotoxic efficacy of tumor specific cytotoxic T lymphocytes (CTLs) in vitro and recruited the mobilized DCs into the tumor bed in vivo.[17,18] Moreover, IL-18-secreting chimeric antigen receptor T (CAR-T) cells have also demonstrated the augmentation of anti-tumor immunity in solid tumors.[19,20] Using IL-18 as an adjuvant combination with other cytokines, such as interferon α (IFN-α), IL-15, IL-12, and IL-2, can also promote productive dendritic cell T (DC-T) cell interactions, and promote the activation and proliferation of T cells in colorectal cancer patients.[21] A human IL18-IL2 fusion protein has also been developed, and the resulting study[8] showed that it could provoke the IFN-γ production in peripheral blood mononuclear cells (PBMCs), and increase the production of IFN-γ and the cytotoxicity of NK cells to a higher degree than when stimulated with only IL-18 or IL-2 or a combination of both.[8]

In a previous study, we developed a type of engineered IL-18 with higher activity than the native protein.[22] The modification using site-directed mutagenesis based on its receptor interaction and other previous reports.[23–25] We named the modified protein E6K+T63A IL-18, and our study showed that E6K+T63A IL-18 exhibited higher IFN-γ inducing activity from NK-92MI cells by a factor of 16 compared with native IL-18.[22] Molecular dynamic (MD) simulation also indicated that the activity change of this engineered IL-18 came together with some kind of conformational changes at the binding sites of IL-18.

In this article, we demonstrate that in addition to the previous in vitro testing which found the higher IFN-γ induction activity of E6K+T63A IL-18 from cell culture, it also promoted anti-tumor immunity in vivo through increasing the recruitment of Th1 and CTL cells in the tumor bed. This finding indicates the potential of engineered IL-18 as either a sole candidate or with other regimens for cancer immunotherapy in the future.

Material and methods

Cloning and mutagenesis

pPICZα-IL18WT, pPICZα-IL18E6K and pPICZα-IL18E6K+T63A constructions have been described extensively in our previous study.[22] In brief, the IL-18 open reading frame (ORF) was cloned from an RNA sample. We first introduced this ORF to the pTZ57R/T cloning vector (Thermo Fisher Scientific, Waltham, USA) and then subcloned the ORF into the yeast expression vector pPICZαA (Invitrogen, Carlsbad, USA) at the *EcoR*I and *Xba*I sites, resulting in the recombinant plasmid pPICZα-IL18WT. The mutagenesis, using pPICZα-IL18WT as a template, was carried out to generate pPICZα-IL18E6K for the E6K+T63A IL-18. Platinum Pfx DNA polymerase (Invitrogen) was used to perform all mutagenesis processes. The purified PCR fragments were digested with *Dpn*I (Thermo Fisher Scientific) to remove the plasmid template, and transformed into *Escherichia coli* DH5α.

Protein production and purification

Our protein production method was also described in a previous report.[22] Plasmid pPICZα-IL18WT and other mutagenized plasmids were linearized with *Sac*I (Thermo Fisher Scientific) enzyme, electroporated to *Pichia pastoris*, and then selected on yeast extract peptone dextrose (YPD) medium containing 100 μg/mL of Zeocin (Invitrogen). The positive clones were cultured on YPD, buffered glycerol complex medium (BMGY) and buffered methanol-complex medium (BMMY) for inoculum preparation, yeast mass production and protein expression, respectively. For protein expression, 2.0% methanol was used as an inducer. The cell culture was cultivated at 30°C, 250 rpm, for 48 h. The volume of the culture was maintained at 10–30% of the total flask volume to ensure sufficient aeration. Methanol was added every 24 h to maintain the induction at 2% methanol concentration. The collected supernatant confirmed recombinant IL-18 existence using SDS-PAGE and western blotting.

The secreted IL-18 protein was then purified using His-Trap HP column (GE Healthcare, Chicago, USA) according to the manufacturer's protocol. The pH 7.4 binding buffer contained 20 mM sodium phosphate, 0.5 M NaCl and 20 mM imidazole. The elution buffer followed the binding buffer formula but 400 mM imidazole was used instead. The recombinant protein was then concentrated using an Amicon Ultra4 centrifugal filter unit (Merck Millipore, Burlington, USA) and diluted in phosphate-buffered saline

(PBS). The protein concentration was measured using Bradford assay and bovine serum albumin (BSA) was used as a standard. Finally, the purified protein was submitted to Proteomics International Pty Ltd. (Perth, Australia) for LC-MS/MS analysis.

Cell line, animal model and assessment of treatment effect

Mouse CT26-WT colon cancer cells (American Type Culture Collection (ATCC); CRL-2638) were obtained from ATCC (Manassas, USA). CT26-WT is an N-nitro-N-methylurethane-induced BALB/c murine colon carcinoma cell line. The cell line in our study was grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO_2.

Ninty-four male seven- to nine-week-old BALB/c mice (27–30 g body weight) were obtained from the National Laboratory Animal Center (Nakhon Pathom, Thailand) and maintained in the Southern Laboratory Animal Facility, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. The maintenance conditions were a temperature of 22 ±3°C, relative humidity of 55 ±10%, free access to clean food and water, and 12-hour lighting. All animal procedures were performed following guidelines and regulations approved by the Ethics Committee of Prince of Songkla University (animal experimentation application Ref. 15-2017). During the logarithmic growth phase, CT26-WT cells were harvested and screened with trypan blue to allow the preparation of single-cell suspensions with 90% viability before inoculation. Each group of mice was injected subcutaneously with 2×10^6 CT26-WT cancer cells/100 µL of PBS in the left hind flank. Survival times and tumor volumes were monitored after inoculation. The CT26 mice were randomized into 4 groups (9 mice/group) as follows: group A (vehicle group); group B (IL-18WT group); group C (IL-18E6K group); and group D (IL-18E6K+T63A).

The 1st day of treatment was defined as day 0. The mice were continuously observed for 100 days before sacrifice. Kaplan–Meier survival analysis was performed using death as the definition of failure in censoring. Group A mice received PBS solution injection to serve as vehicle control. Groups B, C and D mice received 50 µg/kg of IL-18WT, IL-18E6K and IL-18 E6K+T63A, respectively. The intratumoral injection was performed once a day for 10 consecutive days. Tumor volumes were measured with caliper every day. The tumor volume of 2000 mm^3 or tumor abnormal status (ulcerated, infected and/or >30% body weight loss) indicated the sacrifice notification. No sign of these endpoints in the animal was detected until the end of the experiment. After 10 days of treatment, the mice were sacrificed. The tumor was collected for flow cytometry and weight and size analysis.

Another set of mice has been used for a survival study. Tumor-bearing mice were divided into 4 groups (12 mice/group). The mice were treated with different forms of IL-18 or PBS for 10 days. The animal survival rate was evaluated for 100 days. The animal health and behavior were monitored 4 times/week.

Leukocyte isolation

Inhalation of 4% isoflurane was applied for deep anesthetization and blood samples were extracted. Later, the tumors were removed and kept in complete RPMI media during transportation from the animal facility to the laboratory. The mice were intracardially perfused with saline buffer, and euthanasia was performed under anesthesia by exsanguination. The euthanasia status was confirmed with cervical dislocation.

The tumors were dissected into small fragments and incubated in completed RPMI media at 37°C for 45 min with agitation. RPMI media contained 1 mg/mL type IV collagenase (Sigma-Aldrich, St. Louis, USA), 0.1 mg/mL hyaluronidase (Sigma-Aldrich) and 20 U/mL DNase I (Sigma-Aldrich). The cell suspension was then filtered through a 70 µm cell strainer on a 50 mL tube, and followed by rinsing with 5 mL RPMI medium. The single cell suspension was centrifuged at 300 × g for 10 min to get the cell pellet. The cell pellet was washed using complete RPMI medium and incubated with 5 mL RBC lysis buffer for 5 min. The cells were then washed again and resuspended in 15 mL of 40% Percoll (GE Healthcare) in RPMI. The suspension was under-laid on 15 mL 80% Percoll and centrifuged for 25 min at 400 × g with the lowest descending and ascending rates. The leukocytes were collected from the interphase between the 2 concentrations.

Flow cytometry

The isolated leukocytes were counted using a hemacytometer, adjusted at 2×10^6 cells/mL and incubated in complete RPMI medium containing a cell activation cocktail (BioLegend, San Diego, USA). The cocktail included 20 ng/mL of phorbol 12-myristate-13-acetate (PMA), 1 µg/mL of ionomycin and 5 µg/mL of brefeldin A for 4 h at 37°C in a CO_2 incubator. The cells were washed with a cell-staining buffer (BioLegend) before adjusting to 2×10^7 cells/mL. The cells were then incubated with 10 ng/µL TruStain fcX™ (BioLegend) for 10 min on ice. Cell surface antibodies (anti-mouse CD4 and anti-mouse CD8a) were added, as shown in Table 1, and the solution was incubated on ice for 20 min in a dark environment.

The cells were washed and fixed with ×5 volume of fixation buffer (BioLegend) for 20 min at room temperature in a dark environment. The cells were then washed with cell staining buffer and twice in permeabilization wash buffer (BioLegend) before being stained with fluorescence-conjugated antibodies against intracellular cytokines (anti-mouse IFN-γ and anti-mouse IL-4; Table 1) for 20 min at room temperature in a dark environment.

Table 1. Antibodies used in this study

Antibody	Provider	Clone	Volume used [µL]
Cell surface markers			
FITC Rat anti-mouse CD4	BioLegend	GK1.5	0.5
FITC Rat IgG2b, κ isotype	BioLegend	RTK4530	0.5
FITC Rat anti-mouse CD8a	BioLegend	53-6.7	2
FITC Rat IgG2a, κ isotype	BioLegend	RTK2758	2
Intracellular cytokine markers			
PE Rat anti-mouse IL-4	BioLegend	11B11	1.25
PE Rat IgG1, κ isotype	BioLegend	RTK2071	1.25
APC Rat anti-mouse IFN-γ	BioLegend	XMG1.2	4
APC Rat IgG1, κ isotype	BioLegend	RTK2071	4
Fc block antibody			
Anti-mouse CD16/32	BioLegend	93	2

The double-stained cells were then washed twice with permeabilization wash buffer and resuspended in cell staining buffer before flow cytometry analysis using the BD FACSCalibur™ platform (BD Biosciences, San Jose, USA) and analyzed using Kaluza analysis flow cytometry software (Beckman Coulter, Brea, USA).

Statistical analysis

All data are presented as mean ± standard deviation (SD). Kaplan–Meier method was applied to analyze survival data using the logrank test. Multiple conditions were compared using a parametric one-way analysis of variance (ANOVA), followed by Tukey's post hoc tests. All analyses were performed using SPSS v. 24.0 (IBM Corp., Armonk, USA). The p-value <0.05 justified statistical significance.

Results

Engineered IL-18 increased the lifespan of tumor-bearing mice

In our previous study, we developed 3 types of engineered IL-18, namely E6K, T63A and E6K+T63A IL-18. All showed higher ability to induce IFN-γ production from NK-92MI cells than a native IL-18 with the rates of 9.3, 3.9 and 16.4 times higher at a concentration of 200 ng/mL.[22] In the current study, we have selected E6K and E6K+T63A IL-18 to evaluate the anti-tumor effects of recombinant IL-18 in an animal model since these are the best 2 forms. BALC/c mice were injected with CT26-WT cell line subcutaneously to create tumor-bearing mice. The mice were separated randomly into 4 different treatment regimens as follows: native IL-18, E6K, E6K+T63A, and vehicle. Therapy was instituted according to the schema presented in Fig. 1A.

When the tumors reached 100 mm^3, the mice were injected with one of the recombinant IL-18 types once a day for

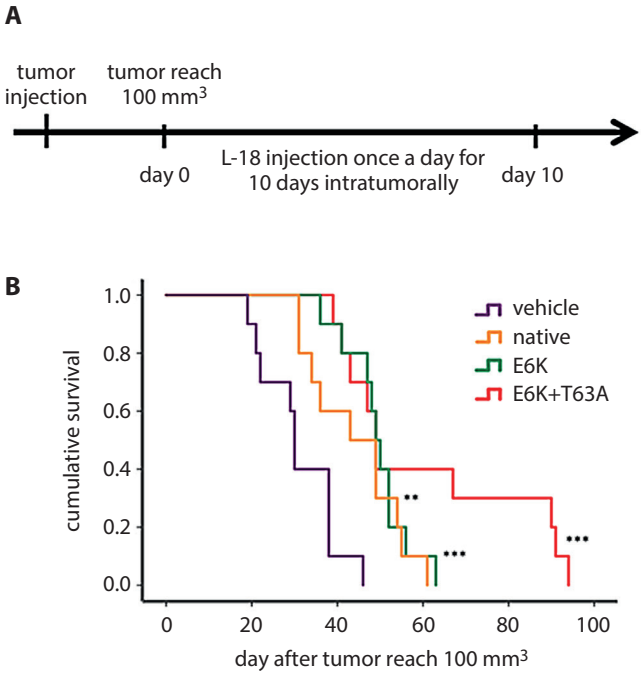

Fig. 1. Mice treated with recombinant IL-18 showed improved survival rates from a colon tumor model challenge. A. The treatment schematic. B. Kaplan–Meier analyses of overall survival in each group of mice (n = 10) (*p < 0.05; **p < 0.01; ***p < 0.001)

10 days intratumorally. After receiving 50 µg/kg of recombinant protein, the average survival times were 31.1 ±2.7 (mean +SD) days in the vehicle group, 44.3 ±3.4 days in the native IL-18-treated group, 49.4 ±2.3 days in the E6K group, and 61.0 ±7.1 days in the double-mutation treatment group (Table 2). This data indicates that the long-term survival of the mice was related to the type of recombinant IL-18 received, since the mice treated with all types of recombinant IL-18 (n = 10) survived significantly longer than the vehicle group (n = 10) (Fig. 1B) with the p-values <0.008 for native IL-18 and <0.0001 for both E6K and E6K+T63A, suggesting the in vivo efficacy of recombinant IL-18. However, the differences between the 3 recombinant IL-18

Table 2. Statistical analysis of survival rate of each group of mice treated with different IL-18 regimens

Intervention	Mean survival time [days]		Pairwise comparisons (Mantel–Cox test)							
	estimate	SE	E6K+T63A		E6K		native		vehicle	
			χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
Vehicle	31.100	2.795	16.195	0.000*	15.312	0.000*	7.002	0.008	–	–
Native	44.300	3.435	3.182	0.074	0.570	0.450	–	–	7.002	0.008
E6K	49.400	2.358	1.510	0.219	–	–	0.570	0.450	15.312	0.000*
E6K+T63A	61.000	7.116	–	–	1.510	0.219	3.182	0.074	16.195	0.000*

* $p < 0.0001$; SE – standard error.

groups were not significant (Table 2), although the mice treated with E6K+T63A showed the longest survival time (61 ±7 days).

Engineered IL-18 slowed tumor progression in vivo

In addition to the survival analysis of each of the regimen groups, we also investigated whether tumor growth was affected by the different types of IL-18. An experimental model was generated as described earlier. The results showed that the double-mutation (E6K+T63A) and E6K groups had significantly lower tumor volumes when compared to the vehicle group ($p < 0.0001$ at day 10 of treatment) and native protein-treated groups ($p < 0.0001$ and $p < 0.05$, respectively) (Fig. 2A,B). Tumor growth in mice receiving intratumoral administration of native IL-18 was also slower than in the vehicle group with the p-value of 0.019 (Fig. 2A,B). Indeed, E6K IL-18 induced about 50% reduction in tumor volume, and the combination of E6K with T63A had a high inhibitory effect (around 70% reduction in tumor volume) (Fig. 2B).

The tumor weights were also measured after 10 days of treatment. As shown in Fig. 2C, the tumor weights in mice treated with recombinant IL-18 were considerably lower than in the vehicle mice. The tumors were also significantly smaller in the tumor-bearing mice injected with all types of recombinant IL-18 (Fig. 2C,D). The average tumor weight from the E6K+T63A-treated group was 5 and 3.6 times lower than the tumors from the vehicle

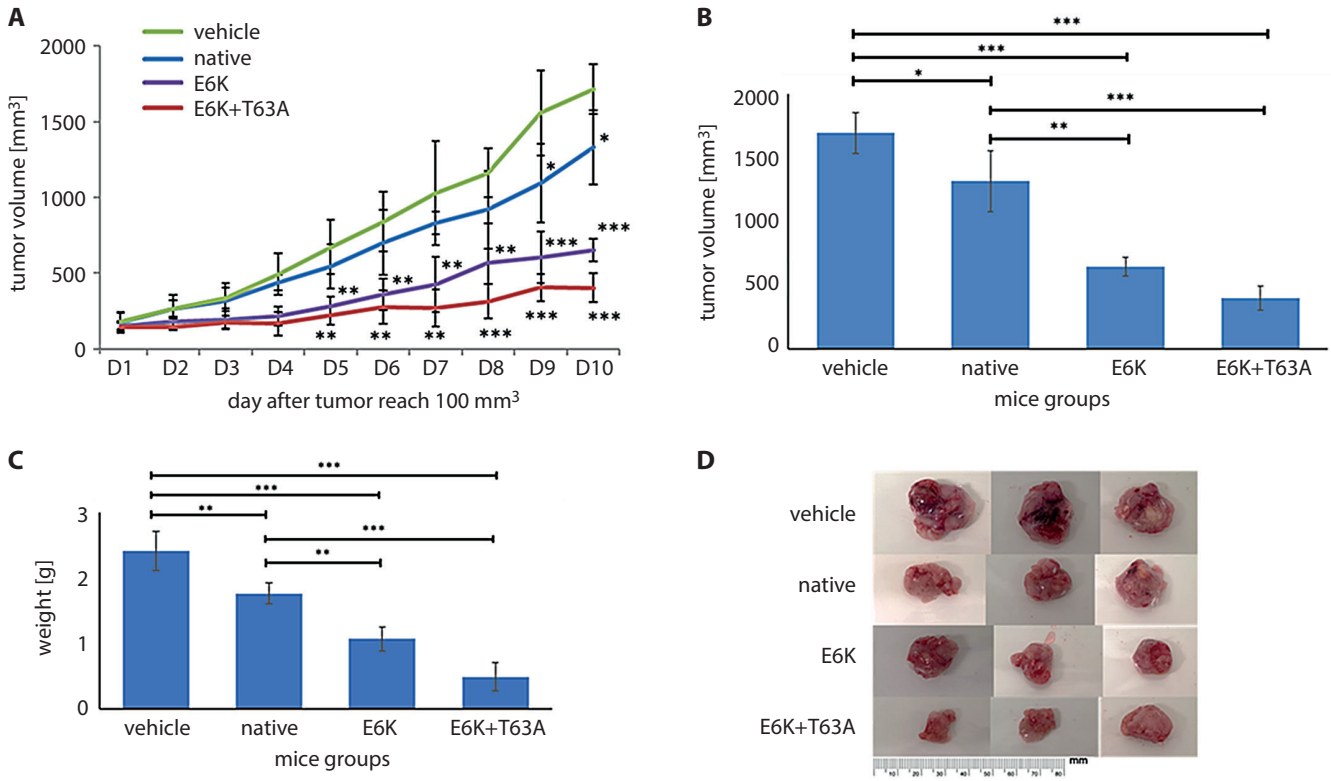

Fig. 2. Intratumoral recombinant IL-18 injection suppressed tumor growth in vivo. A. Groups of 10 tumor-bearing mice were challenged with each type of recombinant IL-8. Tumor size was measured every day starting on day 1 after IL-18 treatment. B–D. The tumor volumes and weights were measured. The image represents tumor growth at 10 days after IL-18 treatment. Results shown as means ±SD of 5 animals/group; values having different signs are significant different (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

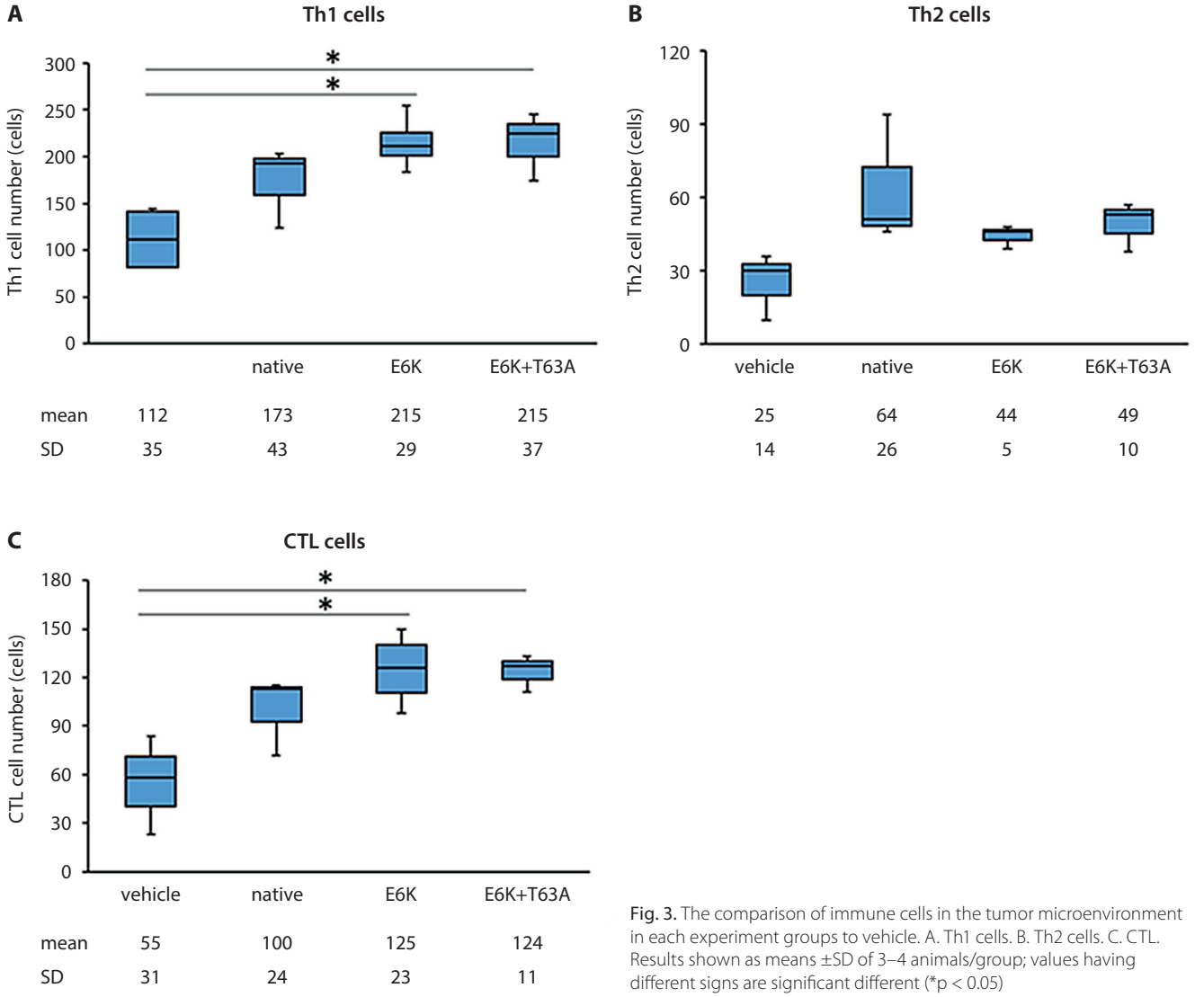

Fig. 3. The comparison of immune cells in the tumor microenvironment in each experiment groups to vehicle. A. Th1 cells. B. Th2 cells. C. CTL. Results shown as means ±SD of 3–4 animals/group; values having different signs are significant different (*p < 0.05)

and native IL-18-treated groups, respectively. This quantification of the tumor size and weight confirmed that E6K+T63A IL-18 significantly reduced the xenograft tumor volume (Fig. 2A,B) and tumor weight (Fig. 2C,D).

Engineered IL-18 increased the number of Th1 and CTL cells in the tumor bed

We investigated the number of different types of T cells to determine whether our recombinant IL-18 indeed induced T cell activity in the context of anti-tumor immunity. The phenotype of lymphocytes in tumors of the CT26-WT-bearing mice after recombinant IL-18 treatment was examined. After treatment with each regimen, the tumor masses were collected and the mononuclear cells were then isolated using Percoll gradient centrifugation. All cells were subjected to flow cytometry after anti-body staining against CD4, CD8, IL-4, and IFN-γ. The Th1, Th2 and CTL cells were identified as $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$ and $CD8^+IFN\text{-}\gamma^+$, respectively, using flow cytometry and gating on viable cells. The results as presented in Fig. 3 showed that the numbers of Th1 and CTL significantly increased in the tumor masses of the E6K- and E6K+T63A IL-18-treated groups compared to the vehicle group ($p < 0.05$) (Fig. 3,4). However, there was no difference in the numbers of Th2 cells from the mice treated with all types of IL-18 as compared to the vehicle group (Fig. 3B,4B). These data demonstrate directly that E6K+T63A IL-18 induced the strongest functional Th1 and CTL response, correlating with the in vivo tumor therapy data.

Discussion

Interleukin 18 was widely reported for its potential as an anti-cancer agent. Application of IL-18 is thought to enhance the stimulation of NK cells and cytotoxic T lymphocytes, the cancer cell growth and spread inhibition, and the expression of Fas-ligand in immune cells.[26–30] Moreover, these anti-tumor functions of IL-18 are mainly

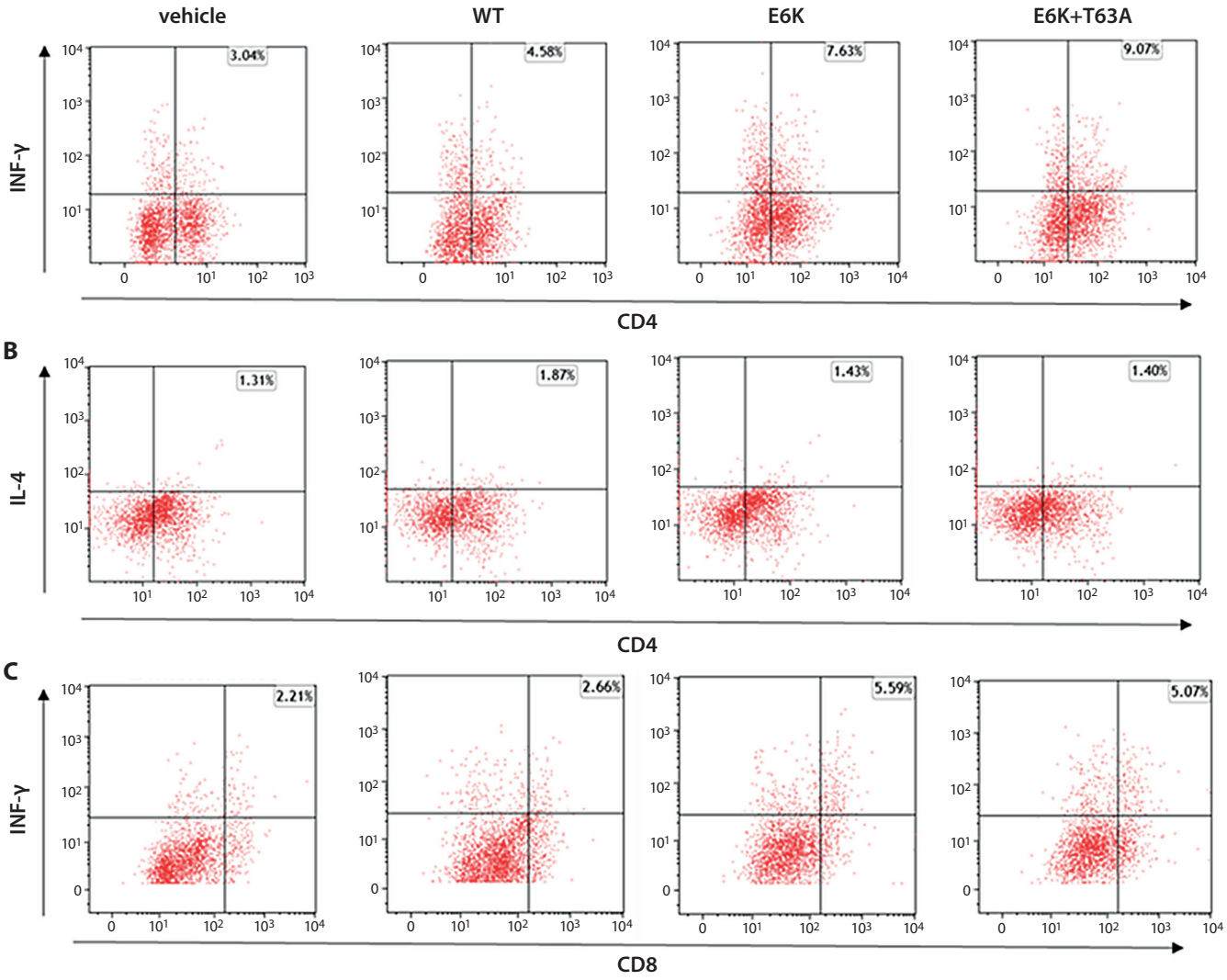

Fig. 4. Representative flow cytometry gating plots showing the percentage of tumor infiltrating. Th1 (A), Th2 (B) and CTL (C) cells from mice treated with different types of recombinant IL-18 or vehicle

mediated through IFN-γ,[31] the downstream molecule after IL-18 activation.[1] In our previous experiment, we demonstrated that altering amino acids as E6K+T63A improved IL-18 activity.[22] The proteins were also produced by the yeast system that gave us a low cost and no need for the refolding steps presented in the *E. coli* system. Moreover, some earlier studies have suggested that the route of administration of IL-18 also plays an important role in the therapeutic effects of a cytokine, since 1 study found that IL-18 only slowed tumor progression when administered directly into the tumor mass or if the cytokine was secreted by tumor cells, indicating that the tumor microenvironment can be an effective target for tumor treatment.[31] This was also confirmed by several other studies in which an intratumoral injection of immunomodulatory compounds was used as the delivery approach for such agents as IL-12,[32] or viral vectors encoding cytokine genes IFN-γ[33] or IL-12.[34] Therefore, to improve the efficacy of IL-18, we chose the intratumoral injection as a delivery method.

In the current study, the syngeneic CT-26WT colorectal cancer model was used to evaluate the anti-tumor effect of engineered IL-18 produced in our laboratory in vivo. Although many studies have described the anti-tumor effect of IL-18 as mentioned above, our study found only a minor ability of native IL-18 to inhibit tumor growth in our animal model after 10 days of treatment with recombinant proteins. This may have been due to the quality of the protein produced in our laboratory, which may lead to the variation in IL-18 quality and activity. However, the engineered IL-18, E6K+T63A, showed a significant anti-tumor effect as demonstrated in Fig. 1 and Fig. 2. The tumors in the mice treated with E6K+T63A grew slower than those in the mice treated with native protein or E6K IL-18. The mean tumor volume and weight of the E6K and double mutation groups in the CT26-WT colon cancer model were also obviously smaller than in the control group. Compared to the native protein, E6K and E6K+T63A exhibited better effects on tumor regression. These results suggest the combination of E6K and T63A could have a synergetic

effect on tumors in immunocompetent mice. In addition to tumor progression, this protein also significantly increased the lifespan of this group of mice (Fig. 1B), indicating the strong anti-tumor capability of our engineered IL-18 in an animal model.

Interleukin 18 is a potent inducer of Th1 immune response.[1] The number of Th1, Th2 and CTL cells in the tumor masses were thus investigated to evaluate the anti-tumor effect of each type of recombinant IL-18, and these cells could be identified as $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$ and $CD8^+IFN\text{-}\gamma^+$, respectively, in accordance with the role of IFN-γ induction. In tumor-bearing mice models, although there was no significant difference in Th2 numbers, the presence of E6K+T63A IL-18 induced significantly greater infiltrations of $CD4^+IFN\text{-}\gamma^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ cells in the tumor masses (Fig. 3,4) compared to the vehicle group supporting a role of our recombinant engineered IL-18 on anti-tumor immunity. This scenario is not surprising, given that IL-18 can recruit T cells to the site,[35] promote the polarization of Th1 cells, induce the proliferation, enhance the cytotoxicity of T cells,[1] and induce the maturation of dendritic cells.[36]

Conclusions

In summary, the present study confirmed that our engineered IL-18 exhibits anti-tumor capabilities by promoting anti-tumor immunity. Although the native IL-18 produced from our laboratory did not show significant anti-tumor effects according to the tumor volume and survival rate, our engineered IL-18, E6K+T63A, significantly slowed tumor progression and increased the lifespans of tumor-bearing mice. These findings may have therapeutic implications, since higher proportions of Th1 and CTL cells were found in the tumor microenvironment of E6K+T63A IL-18-treated mice compared to E6K and native IL-18. Before this technology can be moved into clinical trials, further investigation is needed to elucidate the toxicity and biology of the novel IL-18 type.

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Factors influencing the choice of graft type in ACL reconstruction: Allograft vs autograft

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Abstract

Background. Anterior cruciate ligament (ACL) reconstruction is the prevailing procedure in cases of ACL rupture.

Objectives. To analyze ACL reconstruction based on time and costs of the surgery, postoperative pain, postoperative complications, time it takes to return to work or other such physical activity, and cosmetic effects.

Material and methods. The retrospective analysis involved 62 patients who had undergone ACL reconstruction with the same results in clinical and functional assessment, which were then divided into 2 groups. In one group, an allograft was utilized, while in the other group – an autograft. The time it takes to perform the surgery, the cost, pain expected to be experienced, the possibility of postoperative complications, scarring, and the time needed for return to work were all considered and analyzed.

Results. The surgery time was 40.64 ±4.23 min in group I in comparison to 52.48 ±4.92 min in group II ($p < 0.05$). The cost of surgery was 32% higher in group I. Visual analogue scale (VAS) pain score in group I was from 36.45 ±8.39 mm on the 3rd day to 15.16 ±5.70 mm on the 28th day. In group II, it ranged from 60.67 ±10.15 mm on the 3rd day ($p < 0.05$) to 18.67 ±6.81 mm on the 28th day. The time of return to office work in group I was 6.96 ±1.9 weeks and 9.27±1.57 weeks in group II ($p < 0.05$). The time of return to physical work in group I was 19.85 ±2.79 weeks, and 20 ±3 weeks in group II. Postoperative scar and local complications were statistically less pronounced in group I.

Conclusions. Allografts achieve less postoperative pain, smaller local complications, shorter time necessary to return to work, and better cosmetic effect. However, an allograft is more expensive to perform.

Key words: anterior cruciate ligament, allograft, autograft, ACL reconstruction, knee arthroscopy

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Introduction

The reconstruction of the anterior cruciate ligament (ACL) of the knee joint is the standard procedure used in the treatment of complete ACL rupture for individuals desiring to return to high-level and vigorous physical activities,[1] and is advocated in order to prevent instability of the knee, further intra-articular disease and recurrent injury.[2–4] The most frequently used grafts for reconstruction of ruptured ACL are: autologous grafts (semitendinosus (ST), gracilis tendon (GR), and combined semitendinosos and gracilis (STGR)), or allograft.[5,6] Despite both grafts showing good results after ACL reconstruction, some authors suggest that graft selection should be based on an individual evaluation of patient demand. This suggestion is given because of potential advantages, such as less donor site morbidity or greater overall graft strength, particularly in allografts, due to the fact that the ST and STGR grafts have recently become more popular.[7–10] Nevertheless, ST and STGR grafts, in contrast to allografts, are soft tissue grafts, which have a higher likelihood of slippage and loss of stability caused by slower healing and greater stress at the site of fixation.[11]

Material and methods

The study was conducted in strict accordance with ethics guidelines and principles of the Declaration of Helsinki. Written informed consent forms were signed by all of the participants.

The retrospective analysis involved 61 patients which had undergone primary single-bundle ACL reconstruction with the same results in clinical and functional assessment, organized into 2 groups: one group utilizing allografts, the other group utilizing autografts. Sixty-two patients made up the initial sample. One patient stopped reporting for follow-up examinations. These patients had undergone ACL reconstruction, were operated on by the same 2 surgeons and the operation had been performed in 2014 and 2016. The inclusion criteria were as follows: primary unilateral intra-articular ACL reconstruction with the use of autologous ipsilateral STGR graft or allograft, and no additional injuries of the involved knee joint between the surgery and the 2nd measurement.

Surgical technique

The same surgical team operated on all the patients, using the same surgical technique, and either an allograft or autograft were used. It was fixed using an Endobutton (Smith–Nephew, Warsaw, USA) on the femur and an interference screw, ComposiTCP30 (Biomet, Warsaw, USA), on the tibia. After assessing the intra-articular structures (Fig. 1), the team started preparing the tibial canal. The tibial canal was prepared using the "outside-in" technique. The aimer device was placed under the arthroscopic control, enabling the introduction of K-wire in the center of the tibial ACL attachment (Fig. 2) located in the intercondylar notch, 15 mm frontally to PCL. After the K-wire was introduced using a cannulated drill of 4.5 mm diameter, the tibial canal was drilled, and the team worked to leave the stump in the tibial attachment. Next, the K-wire was introduced into the tibial canal and, under arthroscopic control, was placed in the center of ACL femoral attachment on the inner area of the lateral femoral condyle using the "classic clock face" technique. After obtaining satisfactory placement for the procedure to continue, the K-wire was introduced into the femur through the cortical bone and the soft tissues over the skin of the thigh. Next, the canal was drilled with a drill 4.5 cm in diameter, allowing the introduction of the Endobutton. A measurement of the length of the femoral canal was taken using a scaled device (Fig. 3). Prepared this way into the canals, the wire was introduced with a loop made of a strong thread, through which the threads were pulled. The threads led the graft suspended on the Endobutton loop. After introducing the graft to both canals (Fig. 4), the assistant, while pulling the threads protruding beyond the canal, inspected graft stability in the femoral canal. Under arthroscopic control and with the extended graft, the knee joint was flexed by 90° to make certain the graft tape had settled properly.

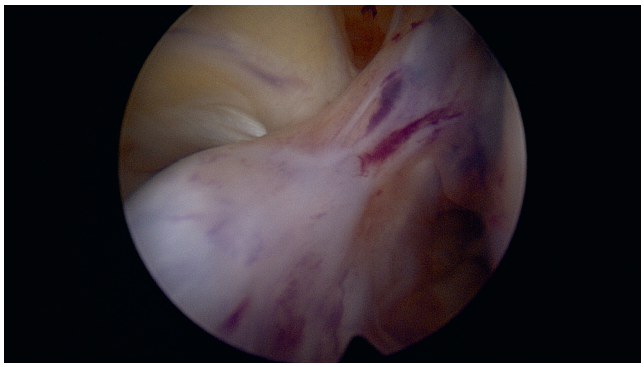

Fig. 1. Arthroscopic image. Left knee joint. A ruptured right ACL

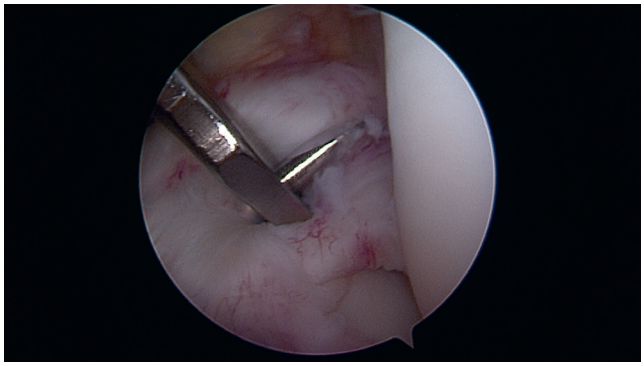

Fig. 2. Left knee joint. The K-wire coming out of the center of the stump, which is an evidence of a proper introduction of the wire into the tibial footprint

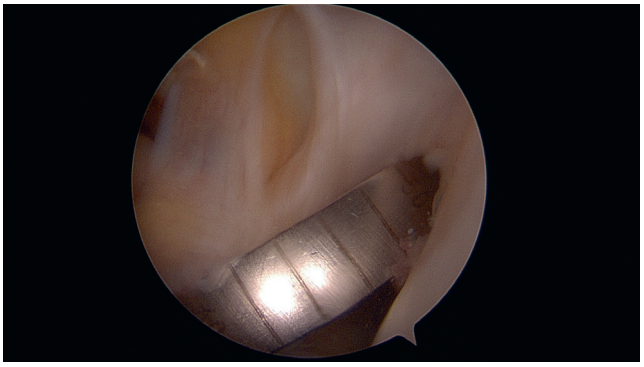

Fig. 3. Arthroscopic image. Left knee joint. Measuring the femoral canal

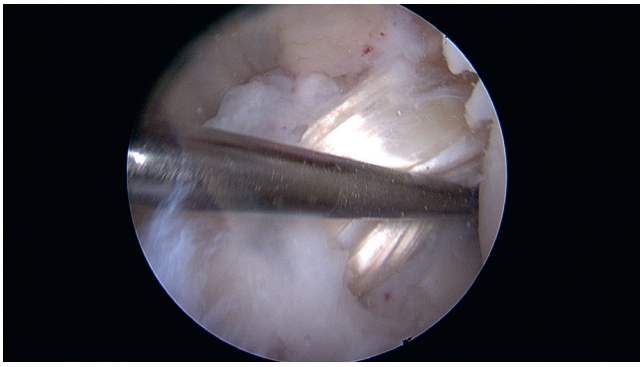

Fig. 4. Arthroscopic image. Left knee joint. The introduced graft

Clinical and functional assessment was made using Lysholm scale and International Knee Documentation Committee (IKDC) 2000 scale. Next, parameters analyzed in the postoperative period were as follows:

– Visual Analogue Scale (VAS) was applied on days 3, 7, 14, and 28 (VAS is a scaled ruler from 0 to 100 mm with an accuracy of 1 mm); complications were noted (hematomas at the collection site and intra-articular hematomas, skin sensation disturbances, pain in and around the back of the thigh).

– Check-up at 3 and 6 months after surgery: complications noted (skin sensation disturbance, pain in the back of the thigh).

– Control examination 18 months after surgery: complications reported (skin sensation disturbances, pain in and around the back of the thigh); measurement of the extent from one end to the other end of the postoperative scar on the tibia.

Statistical analysis

IBM SPSS Statistics v. 20 software (IBM Corp., Armonk, USA) was used in order to perform the statistical analysis. The arithmetic mean (x) and standard deviation (SD) of the patients' age, the timescale between the surgery and measurements for the study groups were calculated. Data distributions for the pain values were evaluated for normality using the Shapiro–Wilk test.[12] The Wilcoxon test was employed for the intra-group to compare pain values between the operated and non-operated knees, and between the preoperative and postoperative values. Differences in values were considered significant if $p < 0.05$.

Results

No statistical differences between groups were found in clinical and functional assessment on Lysholm scale ($p = 0.119$) and IKDC 2000 scale (0.992).

Results of pain assessment

The daily pain felt in the operated limb in group I can be interpreted as a mild pain, being that they were at maximum on the 3rd postoperative day x = 36.45 ±8.39 mm. A comparative inspection of the results of the assessment of the extremity of daily pain of the operated limb in group I exhibited statistically significant differences ($p \leq 0.001$) between the results acquired successively on 3, 7, 14, and 28 postoperative days (Fig. 5).

The intensity of daily pain felt from the operated limb in group I was statistically significantly smaller ($p \leq 0.001$) on postoperative day 7 (x = 25.16 ±6.77 mm) than on postoperative day 3 (x = 36.45 ±8.39 mm). Pain also statistically significantly decreased ($p \leq 0.001$) on the 14th postoperative day (x = 17.74 ±6.17 mm) compared to the 7th postoperative day. In turn, the intensity of pain sensations of the operated limb on the 28th postoperative day (x = 15.16 ±5.70 mm) was comparable to the pain felt on the 14th day after surgery ($p = 0.448$). The relationships between individual results are noted in Table 1.

The pain felt in the operated limb in group II on the 3rd postoperative day was of moderate nature (x = 60.67 ±10.15 mm). In the following postoperative days, the values did not exceed x = 43.67 mm, so they can be considered as mild pain. Comparative analysis of the results of the assessment of the intensity of daily

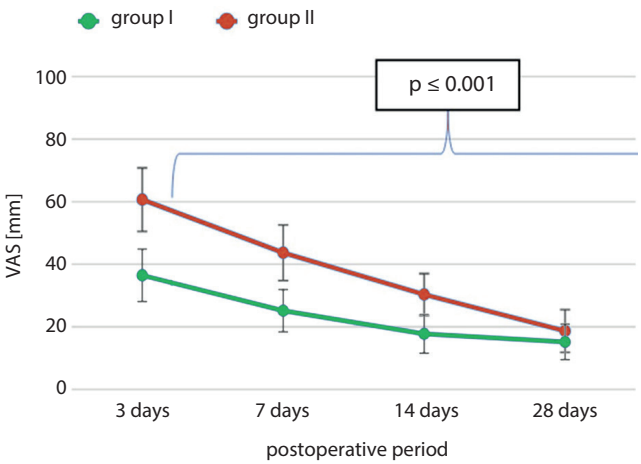

Fig. 5. Results of the assessment of the intensity of daily pain experienced in the operated limb in groups I and II on subsequent postoperative days

Table 1. Comparative analysis of results obtained in assessing the intensity of daily pain using VAS in the operated limb in group I and II between individual postoperative days

VAS								
Postoperative day	day 3		day 7		day 14		day 28	
	group I	group II	group I	group II	group I	group II	group I	group II
Day 3	–	–	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001
Day 7	≤0.001	≤0.001	–	–	≤0.001	≤0.001	≤0.001	≤0.001
Day 14	≤0.001	≤0.001	≤0.001	≤0.001	–	–	0.448	≤0.001
Day 28	≤0.001	≤0.001	≤0.001	≤0.001	0.448	≤0.001	–	–

Values expressed as p (level of statistical significance); VAS – visual analogue scale.

pain of the operated limb in group II, similarly to group I, showed statistically significant differences ($p \leq 0.001$) between the results obtained successively on 3, 7, 14, and 28 postoperative days (Fig. 5).

The intensity of daily pain of the operated limb in group II statistically significantly ($p \leq 0.001$) decreased on the 7th day after the operation (x = 43.67 ±8.90 mm) in comparison to the 3rd day after surgery (x = 60.67 ±10.15 mm). Between the 7th and 14th days following the operation (x = 30.33 ±6.69 mm), the intensity of pain also decreased statistically significantly ($p \leq 0.001$). Pain felt in the operated limb on the 28th day post-operation (x = 18.67 ±6.81 mm) was significantly ($p \leq 0.001$) less intense than on the 14th day after surgery. The results are shown in Table 2.

Comparison of the results of the assessment of the intensity of daily pain experienced in the operated limb showed that in group I, the level of said pain was statistically significantly lower than in group II (from $p \leq 0.001$ to $p = 0.033$). A comparative analysis of the results obtained in both examined groups is presented in Fig. 6.

Results of the return to work evaluation

The patients from group I statistically significantly ($p \leq 0.001$) returned to office work faster (x = 7.00 ±1.93 weeks) than to physical work (x = 19.86 ±2.79 weeks). In group II, patients also statistically significantly ($p \leq 0.001$) returned to office work faster (x = 9.24 ±1.61 weeks) than to physical work (x = 20.00 ±3.00 weeks) (Fig. 7).

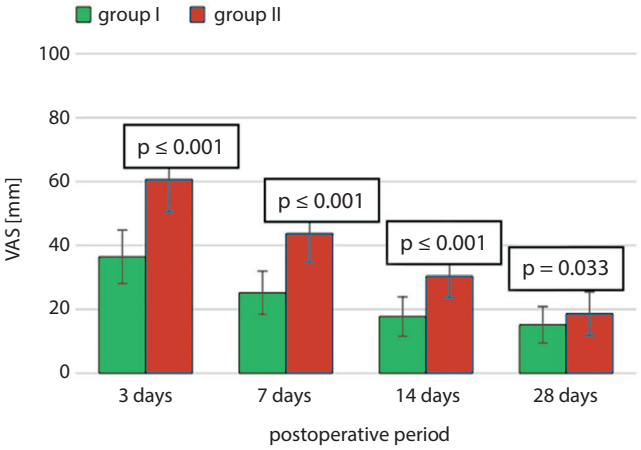

Fig. 6. Comparison between the research groups for pain intensity results in the operated limb on individual days post-operation

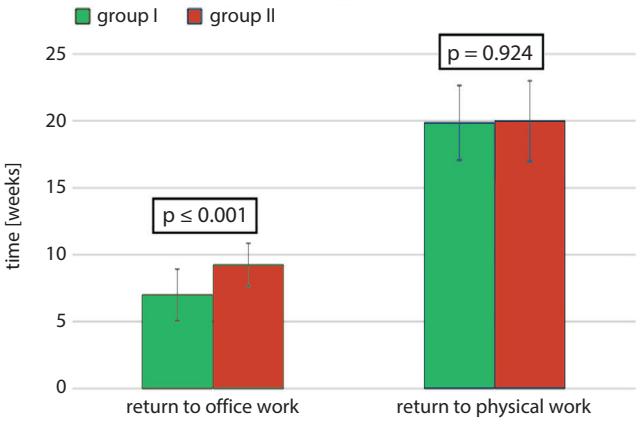

Fig. 7. Comparison of time taken to return to office work and to physical work in both groups

Patients from group I statistically significantly ($p \leq 0.001$) returned to office work faster than patients from group II. The time taken to return to physical work was comparable in both groups ($p = 0.924$) (Fig. 7).

Local postoperative complications

Skin hypoesthesia did not occur in any of the patients in group I (Table 2). In contrast, it occurred in 17 patients in group II, constituting 57% of patients in group II. Symptoms of skin hypoesthesia resolved in less than 3 months after reconstruction of the ACL in 59% of the patients (Table 2). Symptoms of skin hypoesthesia resolved within 3–6 months of surgery in 29% of the patients. In the remaining 12% of the patients with symptoms of skin hypoesthesia, the symptoms resolved more than 6 months after the reconstruction of the ACL.

None of the patients in group I complained of pain in the posterior thigh of the operated limb (Table 2). However, 21 patients from group II reported this symptom, which constituted 70% of the patients from group II. Thigh pain subsided in less than 3 months of reconstruction of the ACL in 81% of the patients (Table 2). This symptom

Table 2. Analysis of the occurrence of skin hypoesthesia symptoms and analysis of pain in the posterior thigh of the operated limb and its remission in group I and group II

Group	Occurrence of symptoms	Remission of symptoms		
		<3 months after surgery	3–6 months after surgery	>6 months after surgery
Skin hypoesthesia in the operated limb, n				
Group I	0	0	0	0
Group II	17	10	5	2
Pain in the posterior thigh of operated limb, n				
Group I	0	0	0	0
Group II	21	17	3	1

Values expressed as n (number of patients in test group).

resolved within 3–6 months of surgery in 14% of the patients. In the remaining 5% of patients, pain in the posterior thigh of the operated limb persisted for more than 6 months after surgery.

Four patients from group II had hematoma at the graft site, which made up 13% of group II. Three out of 4 patients with hematoma received conservative treatment, and 1 patient out of 4 hematoma required surgical intervention.

Discussion

Numerous reports show differing opinions on the choice of an acceptable term of surgical treatment, option of graft and graft fixation approach, bone canal preparation method, or the selection of postoperative approach to rehabilitation[8–12]; however, there are only a few scientific papers regarding cost and patient comfort. Some of them can be compiled as duration and costs of surgery, postoperative pain, postoperative complications, time taken to return to work, and cosmetic effects.

The analysis of the cost of surgery shows that the allograft procedure generates a 32% higher cost compared to the autograft procedure. This, of course, is related to the cost of the graft, because the costs of the implants are the same, and the difference in the time of surgery does not cause financial savings that can offset this expense. This coincides with the observations of other authors.[13,14] However, the difference in the cost of the procedure is greater in this work than in the works of other authors. In the work of Cole et al., this difference was 18%,[15] and according to Nagda et al., the use of the allograft increased the cost of the procedure by only 11%.[16] This is associated with the large number of tissue banks, especially in the USA, implying greater availability and lower transplant prices. This is a field for a broader discussion regarding transplantology in Poland.

The type of transplant did not affect postoperative management. In both groups, walking on crutches and using the orthosis device for 3 weeks was recommended. In medical literature, differences have not been encountered in postoperative management and rehabilitation due to the use of the allograft.[17–19]

On days 3, 7, 14, and 28, postoperative pain intensity in patients was monitored in both groups using VAS. Comparing both groups, it is clear that in the material, statistically significantly smaller pain occurred in patients operated on using the allograft. This is in agreement with the studies of other authors who consider significantly less postoperative pain as an advantage of using allografts.[20,21]

In all patients in both groups, there were no inflammatory septic complications or arthrofibrosis. This confirms the safety of using allografts in cooperation with certified tissue banks.[22] Other researchers also point out the lack of difference in the incidence of inflammatory complications using allo- and autographs in the primary reconstruction of ACL.[23]

The most frequently observed local complications concerning the operated limb among patients were skin hypoesthesia, posterior thigh pain and hematomas at the attachment site. Skin hypoesthesia is associated with damage to the skin branch of the femoral nerve. Among patients in the allograft group, skin hypoesthesia was rare and resolved in the first 3 months after surgery. Much more often, the symptom of skin hypoesthesia was reported by patients after primary ACL reconstruction using an autograft. The time until cessation of symptoms in these patients was much longer. This problem is related to the incision performed for graft harvesting and is also indicated by other researchers.[24] It should be noted that this symptom did not affect the function of the knee of the examined patients after reconstruction of ACL; it was only a discomfort for them. Other authors also confirm the frequent occurrence of this symptom without affecting knee joint function after ACL reconstruction.[25] The incidence of this discomfort among patients in the autograft group was an unwelcome surprise. This confirmed the opinions of other researchers that the incidence of damage to the skin branch of the femoral nerve when performing vertical cutting for hamstring is high; it decreases when ST is taken and increases when ST and GR are taken.[24] The risk of damage is also reduced by an oblique incision when accessing hamstrings.[26]

Posterior thigh pain in the early postoperative period occurred in the autograft group in most patients. Due

to the fact that no such symptom was observed in any patient in the allograft group, it should be assumed that this pain is associated with hamstring tendon collection. There have been no instances in literature of any other researchers assessing the occurrence of this symptom in the early postoperative period. In most patients, this symptom resolved within 3 months after surgery, which may confirm the hamstring "regeneration" phenomenon described by other authors.[27] However, other researchers noted the fat degeneration of hamstrings, improper regeneration and weakening of flexor strength in the operated limb.[28] These phenomena may explain the occurrence of pain in the back of the thigh of the operated limb, persisting over 3 months after surgery in 4 patients operated on using an autograft.

Lower limb hematomas only occurred in patients from the autograft group and were associated with bleeding from the hamstring site. In 1 patient, the hematoma required surgical intervention and re-hospitalization. No studies in medical literature have been found analyzing the occurrence of hematomas at the site of taking hamstring tendon harvest.

According to the analysis of our material, local complications related to the operated limb such as skin hypoesthesia, posterior thigh pain and hematomas at the donor site concerned only patients operated on using autografts. Their absence in the allograft group is one of the undoubted advantages of using allografts. These observations are consistent with the published analyses of other authors.[20,29]

There are interesting results concerning time taken to return to work assessed for operated patients. In the group of patients with allografts, the time taken to return to office work was shorter than in the group of patients with autografts. This is because there is less pain in the postoperative period and no local complications in the form of hematomas and pain in the back of the thigh in the group of allografts. In the case of manual labor, the time necessary to return to work was comparable in both groups. No literature was found that compared the time needed to return to work in relation to the graft used. The time taken for patients from the autograft group to return to work coincided with the observations of Groot et al.[30]

Regarding the length of the postoperative scar in the lower leg, the scar was significantly shorter in patients from the allograft group. In our opinion, this has both a better cosmetic effect, which is especially important for women, and is associated with fewer local complications. This coincides with the observations of other researchers.[20,29]

The main limitation is the short-term follow-up. In the future, studies involving long-term follow-up with patients that have undergone fully supervised physiotherapeutic procedures and a comprehensive clinical and functional evaluation should be considered.

At this moment, there are numerous studies being conducted focusing on highlighting genetic predisposition to cruciate ligament injuries. Research on the application of stem cells, plasma rich in platelets and xenografts are also breaking new ground. These present trends in the evolution of ACL surgery will pave the way for a far more individualized method of surgical treatment.

Conclusions

The choice of the graft impacts duration and costs of surgery, postoperative pain, local complications, time necessary to return to work, and cosmetic effect. An allograft reduces the duration of lower surgery, postoperative discomfort and pain, local complications and the time required to return to work, as well as increases cosmetic effect. However, the cost is higher.

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USP26 deubiquitinates androgen receptor (AR) in the maintenance of sperm maturation and spermatogenesis through the androgen receptor signaling pathway

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Conflict of interest
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Abstract

Background. The post-translational modifications of proteins control various physiological and pathological events in cells.

Objectives. In this study, we investigated the influences of the X-linked deubiquitination enzyme USP26 in mediating androgen receptor (AR) deubiquitination in the physiological events of sperm maturation and spermatogenesis through the AR signaling pathway.

Material and methods. The cell cycle results detected with flow cytometry (FCM) showed that both of the proteins, USP26 and AR, could facilitate the transition of G1–G2 phase in the Leydig cells (TM3). This effect also promoted the proliferation of the Leydig cells.

Results. The cell cycle studies performed using FCM detected that the 2 proteins, USP26 and AR, could facilitate the transition of G1–G2 phase in the Leydig cells (TM3). This effect also promoted the proliferation of the Leydig cells. Moreover, the results from co-immunoprecipitation (CO-IP), immunofluorescence and western blot assays showed that the physiological process due to USP26 interacted with AR and influenced AR deubiquitination, thus upregulating the proteins CCND1 and SPATA46 – which are associated with cell cycle progression and spermatogenesis – as well as decreasing the expression of TP73. Thus, these processes took place through the AR signaling pathway. Furthermore, the USP26 mimic plasmid transfection enhanced these activities, while, conversely, USP26 and AR inhibitor plasmid transfection suppressed the physiological events.

Conclusions. Taken together, the effects of AR deubiquitinated by USP26 could modulate sperm maturation and spermatogenesis through the androgen receptor signaling pathway.

Key words: deubiquitination, sperm maturation, USP26

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Introduction

The ubiquitination and deubiquitination of protein post-translational modifications play important roles in numerous physiological processes, such as DNA damage sensing and repair, signal transduction, cell proliferation, protein functions, etc.[1,2] The process of sperm maturation and spermatogenesis requires successive processes of cell mitosis, meiosis and post-meiosis, cell proliferation, gene transcription, gene translation, and protein modification, among others.[3] Thus, the ubiquitin system is identified as being engaged in the process of spermatid metamorphosis and is present in the human sperm centrosome.[4]

The ubiquitin system contains 3 kinds of enzymes: ubiquitin-activating enzyme (E1), ubiquitin carrier proteins (E2) and ubiquitin protein ligases (E3); additionally, there are 2 structural types of the ubiquitin system: polyubiquitination and monoubiquitination.[5] The deubiquitinating enzymes consist of the ubiquitin C-terminal hydrolases (UCH) and the ubiquitin-specific processing proteases.[6] The deubiquitinated enzymes keep the ubiquitin system in balance in physiological events.[7]

The polyubiquitin enzyme Ubi-p63E[8] and the enzyme USP9X[9] were proved to be involved in male meiotic cell cycle progression and germ cell differentiation. While the ubiquitin-specific protease 26 (USP26) belongs to the family of deubiquitinating enzymes, it also exhibits deubiquitinating activity when the ubiquitin carboxy-terminal hydrolase (UCH) motif is present and the protein sequence is homology.[10] Komander et al.[11] showed that USP26 was responsible for maintaining ubiquitin homeostasis of the cells; it has also been found to be highly expressed in type A and type B spermatogonia, spermatocytes and Leydig cells, etc.[10,12] Moreover, a study by Christensen et al.[13] and Ribarski et al.[14] demonstrated that the ubiquitin enzyme USP26 was essential in the biological activity of spermatogenesis, such as sperm cell mitotic division and maturation, etc.

The process of spermatogenesis begins in puberty and the main androgenic hormone, testosterone, is crucial for the initiation and maintenance of spermatogenesis,[15] which is mainly through binding to the androgen receptor (AR). Studies have also shown that AR was essential for spermatogenesis and testis development, which is highly expressed in the Sertoli cells and peritubular myoid cells within the testicular tubules.[16,17] Importantly, the AR signaling pathway is mainly responsible for germ cell mitosis and meiosis, and the maintenance of spermatogenesis.[17] Dirac et al.[18] reported that USP26 was a regulator of AR and that it reversed the ubiquitination of AR in the AR signaling pathway. Therefore, in this study, we intended to study the mechanism of sperm maturation and spermatogenesis in the USP26-mediated AR signaling pathway.

Material and methods

In vitro cell culture and plasmid transfection

Leydig cells (TM3) and 293T cell line cells were plated in a cell culture flask and cultured in a cell incubator in Dulbecco's modified Eagle's medium (DMEM; Hyclone; Logan, USA) with 5% fetal bovine serum (FBS; Gibco, Waltham, USA), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Sigma-Aldrich, St. Louis, USA) with 5% CO_2 at 37°C. In addition, an overexpressed USP26 plasmid and a small interfering RNA plasmid constructed by Gemma Bio-technology Co. Ltd. (Shanghai, China) were used. The methods of plasmid transfection were as follows: firstly, the Lipofectamine 2000 and the constructed DNA plasmid with DMEM were separately dissolved without FBS at a ratio of 1:0.05; then, they were mixed together. Once the cells had grown, they were washed twice in phosphate-buffered saline (PBS). Then, the constructed ov-USP26 plasmid, si-USP26, si-LacZ, si-AR, and si-con plasmids were separately transfected into the cells for 12 h.

Flow cytometry analysis

Leydig cells (TM3) were cultured and the ov-USP26 plasmid, si-USP26, si-AR, and si-con plasmids were transfected as above, and 1 group of TM3 cells was used as a normal control. Then, the cells were collected into tubes once the cell density had reached over 60%, and they were washed in PBS 3 times; 5 mL of cold ethyl alcohol (70–80% concentration) was added for 4 h to stain the cells; later, the cells were washed in PBS twice. Afterwards, the cells were resuspended with 0.5 mL of mixed PI/RNase staining solution (Beyotime, Beijing, China; c1052), and they were incubated at 4°C for 2 h; the cell cycles in G0/G1, S and G2/M phases were analyzed with a Caliber FACS system (Becton Dickinson Biosciences, Franklin Lakes, USA).

Cell proliferation with EdU assay

Leydig cells (TM3) were plated in a 96-well plate (5,000/well). Then, the constructed ov-USP26, si-USP26, si-AR, and si-con plasmids were separately transfected into the cell models for 12 h, while 1 group of TM3 cells was used as a normal control. Later, 100 µL of EdU (Invitrogen, Carlsbad, USA) was added to the cells and they were incubated for 2 h at 37°C; then, they were washed in PBS and fixed in 4% formaldehyde for 30 min, after which they were removed from the medium. At this point, 50 µL of glycine was added to each well in the plate, and the cell plates were shaken in a table concentrator for 5 min. The plates were then removed from the medium and washed in PBS and 0.5% Triton X-100 at room temperature for 20 min. Then, the cells were washed with BSA buffer and incubated with DAPI solution (1:1000; Sigma-Aldrich) at room temperature in the dark for 30 min.

After incubation, the medium was removed and the cells were washed in PBS; the cell graphs were then observed with a fluorescent microscope (Leica, Wetzlar, Germany). Additionally, the ratio of EdU-positive cells (red) to total DAPI-positive cells (blue) was analyzed with Image-Pro Plus (Media Cybernetics, Rockville, USA).

Co-immunoprecipitation

Leydig cells (TM3) were cultured as above and the constructed ov-USP26, si-USP26 and si-con plasmids were separately transfected into the cells for 12 h, until the cell density reached over 70%. Then, the total proteins were collected with RIPA buffer containing phenylmethylsulfonyl fluoride (PMSF). Protein A/G sepharose beads were pre-incubated with anti-USP26 or anti-AR antibody for 1–1.5 h with balanced wheel rotation. Then, the beads were suspended in the protein lysate medium with balanced wheel rotation overnight at 4°C. Afterwards, the beads were washed 3 times and centrifuged at 3,000 g to obtain the immunoprecipitates, which were then analyzed using the western blot method.

Immunofluorescence

Leydig cells (TM3) were plated in a 96-well plate (5,000/well). Then, the constructed ov-USP26, si-USP26 and si-con plasmids were separately transfected into the cell models for 12 h, while 1 group of TM3 cells was used as a normal control. Then, a cell slide was made for each group and they were fixed with 4% polyformaldehyde for 30 min. Afterwards, the cells were permeabilized with 0.1% TritonX-100 for 5 min and blocked with 10% bovine serum albumin (BSA) for 60 min at 37°C. Finally, the cells were incubated with USP26 (Abcam, Cambridge, UK; 101650) and SPATA46 (Thermo Fisher Scientific, Waltham, USA; OTI_2A_9) antibodies for 12 h at 4°C. Then, the cells were washed in PBS and incubated with Alexa Fluor 488- (green) and 594- (red) conjugated anti-mouse IgG (Invitrogen) at room temperature for 1 h. The cell nuclei were dyed with DAPI (blue), and the cell slide was mounted with an anti-fade mounting medium. Then, the images were visualized with a confocal microscope (Leica).

Western blot

Leydig cells (TM3) and 293T cell line cells were cultured as above; the 293T cell line was a control cell line and the constructed ov-USP26, si-USP26, si-LacZ, si-AR, and si-con plasmids were separately transfected into cells for 12 h until the cell density reached over 70%. One group of TM3 cells was used as a normal control; the *LacZ* gene was steadily expressed in the cell lines. Then, the total proteins in each group were collected with RIPA buffer containing PMSF protease inhibitors (Beyotime) and the protein concentration was measured. Afterwards, the proteins were denatured with hot water and separated with SDS polyacrylamide gels. Next, the gels were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% fat-free milk; the membranes were incubated with primary antibodies USP26 (Abcam), AR (Abcam), SPATA46 (Thermo Fisher Scientific), CCND1 (Abcam), TP73 (Abcam), and β-actin for 12 h; then, the membranes were immunoblotted with anti-mouse IgG antibody for 1 h; finally, the bands were visualized with the chemiluminescent ECL substrate in bandscan and the gray intensity of the proteins was analyzed with ImageJ Software (National Institutes of Health, Bethesda, USA).

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) in SPSS v. 19.0 statistical software (IBM Corp. Armonk, USA), with a p-value <0.05 representing significant differences. Then, the differences between groups were graphed with GraphPad Prism v. 6.0 software (GraphPad Software Inc., San Diego, USA).

Results

AR deubiquitinated with USP26 could facilitate Leydig cell division and proliferation

To determine the functional role of USP26 in Leydig cells, we individually transfected the ov-USP26, si-USP26 and si-con plasmids into the TM3 cell groups. The cell cycles and cell proliferation characteristics in each group were then assessed, with the results showing that USP26 could promote the transition of G1 phase to G2 (Fig. 1A), that the cell numbers were also increased with ov-USP26 plasmid transfection (Fig. 2A) and that these processes were inhibited with the si-USP26 and si-AR plasmids (Fig. 1A,2A). Additionally, the differences in cell cycle between the si-USP26-transfected group and the si-con and normal control groups were statistically significant; besides, the differences between the ov-USP26-transfected group and other groups were statistically significant ($p < 0.05$; Fig. 1B), while the differences in cell proliferation between the ov-USP26-transfected group and the si-USP26 and normal control groups were also significant ($p < 0.05$; Fig. 2B). Furthermore, we also transfected the TM3 cells with the constructed si-AR plasmid, and the results suggested that AR mediated the cell cycle transition and cell proliferation, and that the cell cycles and cell proliferation features were inhibited by si-AR plasmid transfection (Fig. 1C,2C). There were significant differences between the si-AR group and the si-con and normal control groups ($p < 0.05$; Fig. 1D,2D).

Moreover, the co-immunoprecipitation (CO-IP) found that USP26 co-mediated AR in the process (Fig. 3A,B).

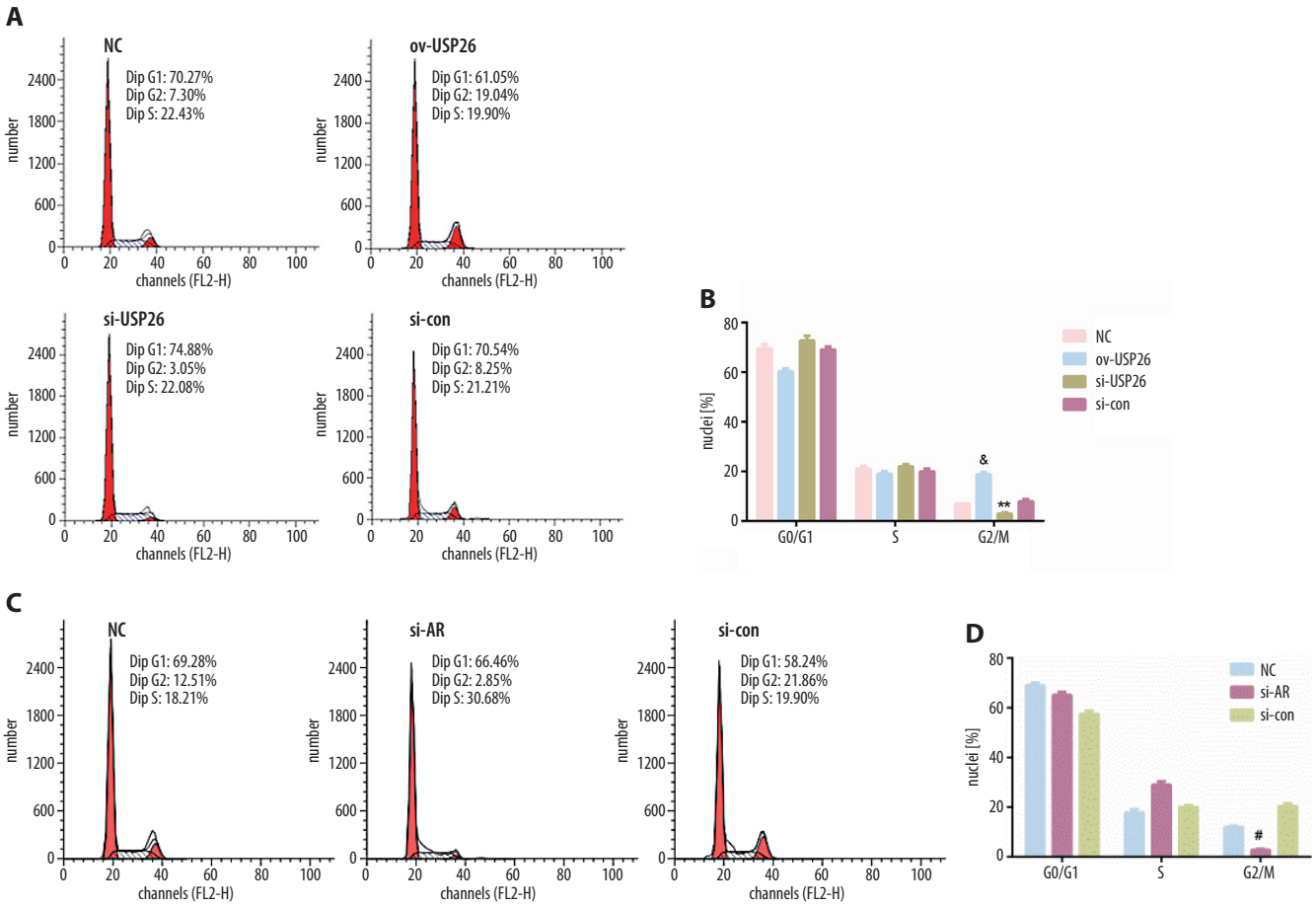

Fig. 1. The proteins USP26 and AR facilitate the cell cycle transition of phase G2 to M. A. Separately transfected into TM3 cells, the ov-USP26, si-USP26 and si-con plasmids varied the cell cycle by group. B. During the G2/M phase, the difference of DNA content in the ov-USP26 plasmid group was higher than in the other groups $^{\&}$ ($p < 0.05$), and in the ov-USP26 group compared with the other groups ($p < 0.001$), while the DNA content was the lowest in the si-USP26 plasmid group when compared with the NC and si-con plasmid groups ** ($p < 0.05$). C. The cell cycle transition of the G2/M phase was inhibited with si-AR plasmid transfection. D. The difference of DNA content in the si-AR plasmid group was lower than in the other 2 groups $^{\#}$ ($p < 0.05$). Mean values and standard deviation (SD) are presented as vertical bars (n = 3)

Therefore, the deubiquitinating USP26 deubiquitinated AR directly in the process of Leydig cell division and proliferation.

USP26 co-regulated with AR to modulate sperm maturation and spermatogenesis through the androgen receptor signaling pathway

Immunofluorescence with confocal microscopy was used to detect the proteins USP26 and SPATA46 with a green- or red-conjugated secondary antibody; both of the proteins were visualized directly. Additionally, both of the proteins were located in the nuclear foci, and the merged image showed that the 2 proteins were co-localized (Fig. 4A). Also, the results showed that si-USP26 plasmid transfection could inhibit the expression of the protein SPATA46. A significant difference was found in the expression of the 2 proteins in the ov-USP26-transfected group compared with the si-USP26, si-con transfected groups and the normal control group (#, & $p < 0.05$, Fig. 4B). Moreover, the proteins associated with the AR signaling pathway, such as USP26, AR, SPATA46, CCND1, and TP73 were detected. The results showed that the constructed plasmid was transfected successfully into the TM3 cells and the 293T cell line cells, and that it significantly regulated the expression of the proteins (Fig. 5A–D). Additionally, the proteins USP26, AR, SPATA46, and CCND1 were noticeably upregulated with ov-USP26 plasmid transfection; conversely, si-USP26 plasmid transfection inhibited this effect while upregulating the expression of TP73 that is associated with cell cycle arrest (Fig. 5A,B). In addition, si-AR plasmid transfection also suppressed the expression of the proteins CCND1 and SPATA46, while upregulating the expression of the protein TP73 (Fig. 5C,D). The difference in the proteins among the groups are presented in Fig. 5E–H. The proteins AR, CCND1, and TP73 are part of the AR signaling pathway, hence USP26 deubiquitinated AR to promote sperm maturation and spermatogenesis through the AR signaling pathway.

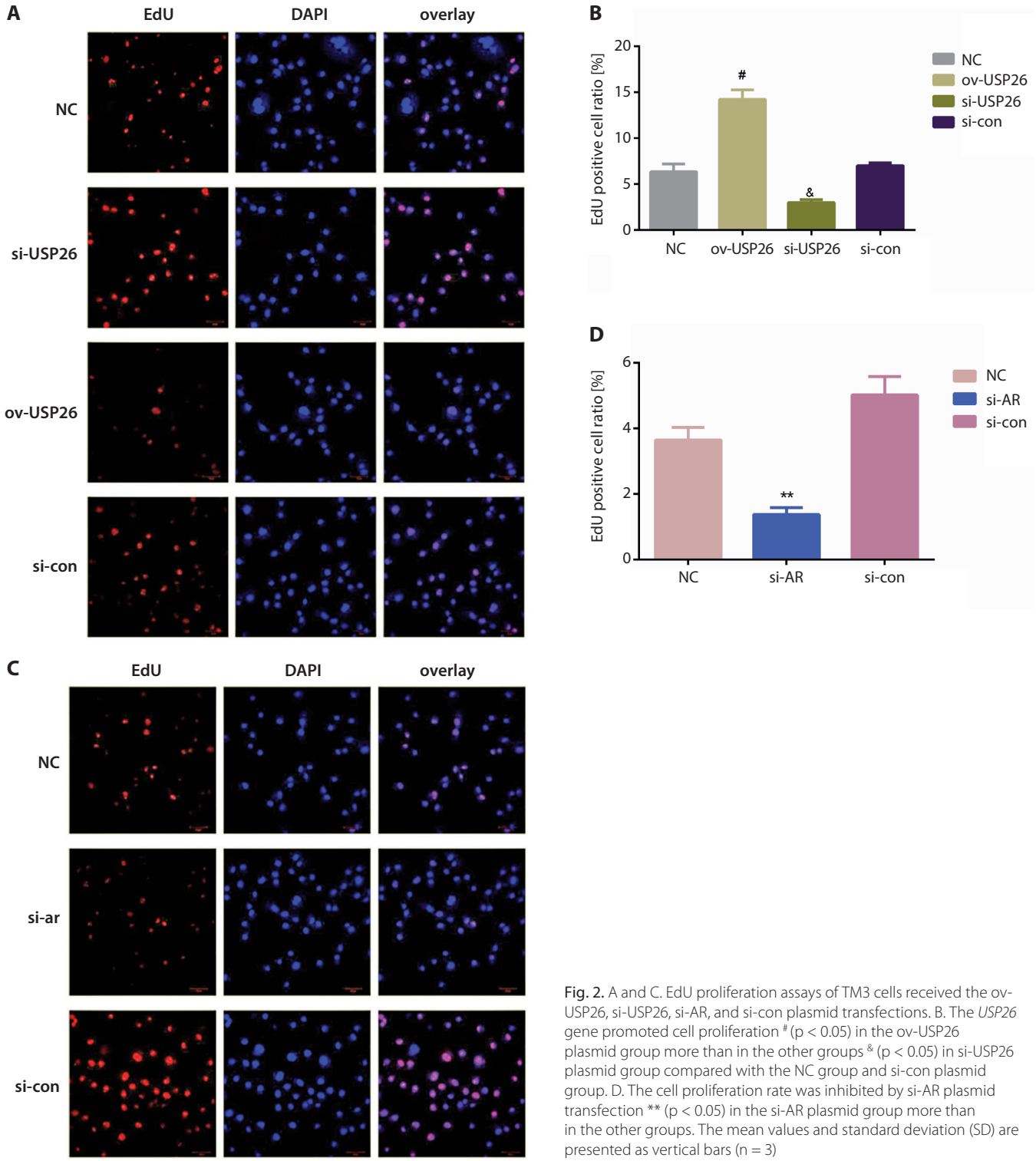

Fig. 2. A and C. EdU proliferation assays of TM3 cells received the ov-USP26, si-USP26, si-AR, and si-con plasmid transfections. B. The *USP26* gene promoted cell proliferation # ($p < 0.05$) in the ov-USP26 plasmid group more than in the other groups & ($p < 0.05$) in si-USP26 plasmid group compared with the NC group and si-con plasmid group. D. The cell proliferation rate was inhibited by si-AR plasmid transfection ** ($p < 0.05$) in the si-AR plasmid group more than in the other groups. The mean values and standard deviation (SD) are presented as vertical bars (n = 3)

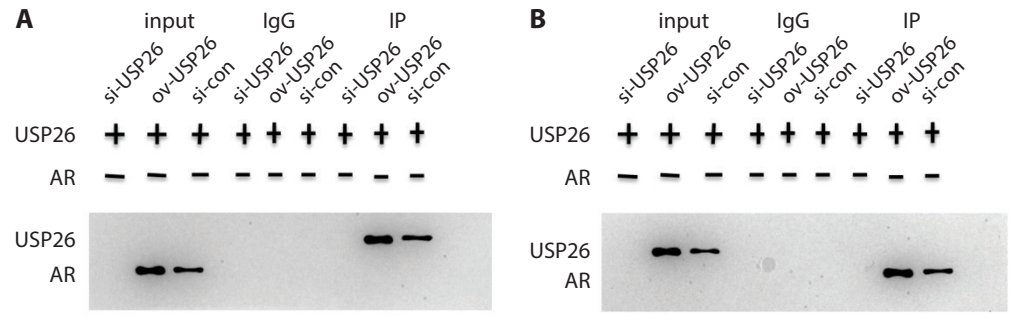

Fig. 3. A and B. The co-immunoprecipitation (CO-IP) was performed in TM3 cells of the groups transfected with ov-USP26, si-USP26 and si-con plasmids. The protein USP26 interacted with AR

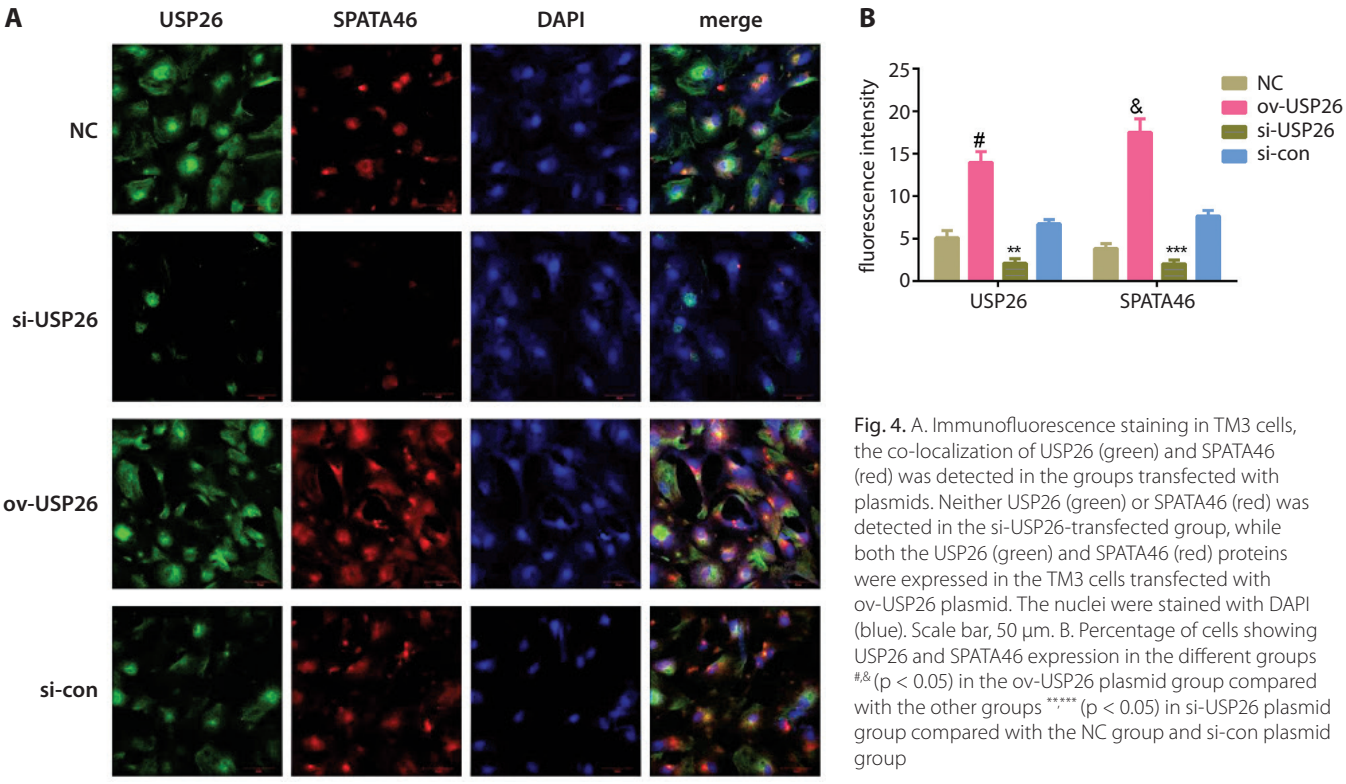

Fig. 4. A. Immunofluorescence staining in TM3 cells, the co-localization of USP26 (green) and SPATA46 (red) was detected in the groups transfected with plasmids. Neither USP26 (green) or SPATA46 (red) was detected in the si-USP26-transfected group, while both the USP26 (green) and SPATA46 (red) proteins were expressed in the TM3 cells transfected with ov-USP26 plasmid. The nuclei were stained with DAPI (blue). Scale bar, 50 μm. B. Percentage of cells showing USP26 and SPATA46 expression in the different groups $^{\#,\&}$ ($p < 0.05$) in the ov-USP26 plasmid group compared with the other groups **,*** ($p < 0.05$) in si-USP26 plasmid group compared with the NC group and si-con plasmid group

Discussion

Sperm maturation and spermatogenesis is a physiological process that is dependent on the regulation and modification at transcriptional and post-transcriptional levels.[19] The ubiquitin system has been suggested as being involved in the different phases of spermatogenesis[4,20]; in this study, we found that the deubiquitinating enzyme USP26 played important roles in the process of sperm maturation and spermatogenesis by facilitating the cell cycle transition of phase G2 to M and cell proliferation in the AR signaling pathway. The *USP26* gene is a novel ubiquitin-specific protease gene, located on the X chromosome and consisting of 835 amino acids, which is a controversial gene in mediating the spermatogenesis and fertility. A study by Felipe-Medina et al.[21] and Zhang et al.[22] clearly demonstrated that USP26 was not essential for spermatogenesis and fertility, though many studies reported that USP26 was essential for testis development and spermatogenesis.[10,15,23] Thus, the deubiquitinating enzyme USP26 is thought to be an important regulator in germ cell development. Importantly, we observed that USP26 monoubiquitinated the AR expressed in Leydig cells in biological events. This finding was consistent with the research Bernards et al.[18] and Dirac et al.[19] A study by Lahav-Baratz et al.[25] found that USP26 binds to Mdm2 through its C-terminal domain to regulate AR in testis development. Therefore, there is a possibility that other E3-ligases were collaboratively engaged in our study.

Although androgens are necessary for male sex development in male secondary sexual characteristics starting from puberty and the AR gene is a key regulator in the process,[26] their mechanism is still not clear. In our study, we found that the proteins USP26, AR, CCND1, TP73, and SPATA46 were expressed in the Leydig cells, and that the siRNA-mediated USP26 and AR knockdown in cultured TM3 cells confirmed the efficiency of the proteins USP26 and AR in promoting sperm maturation and spermatogenesis. Besides, this process was enhanced with overexpression of protein USP26. Moreover, the proteins CCND1 and TP73 were closely associated with the cell cycle transition.[27,28] In our study, the upregulation of CCND1 and the downregulation of TP73 participated in the process of G2/M phase transition and cell proliferation. To our knowledge, the AR signaling pathway in germ cell development mainly controls the cyclin-dependent kinases (Cdks) at the appropriate time during the cell cycles.[17,18,29] The AR has also been identified in the regulation of sex determination and differentiation in zebrafish.[30] Moreover, the protein SPATA46 located on the nuclear membrane was recognized as an important regulator in spermatogenesis in mice.[31] Therefore, the *CCND1*, *TP73* and *SPATA46* genes are possible downstream signals in the AR signaling pathway. However, the direct upstream regulator of SPATA46 still requires further study as part of the AR signaling pathway.

Taken together, USP26 significantly modulated sperm maturation and spermatogenesis by deubiquitinating AR in the AR signaling pathway.

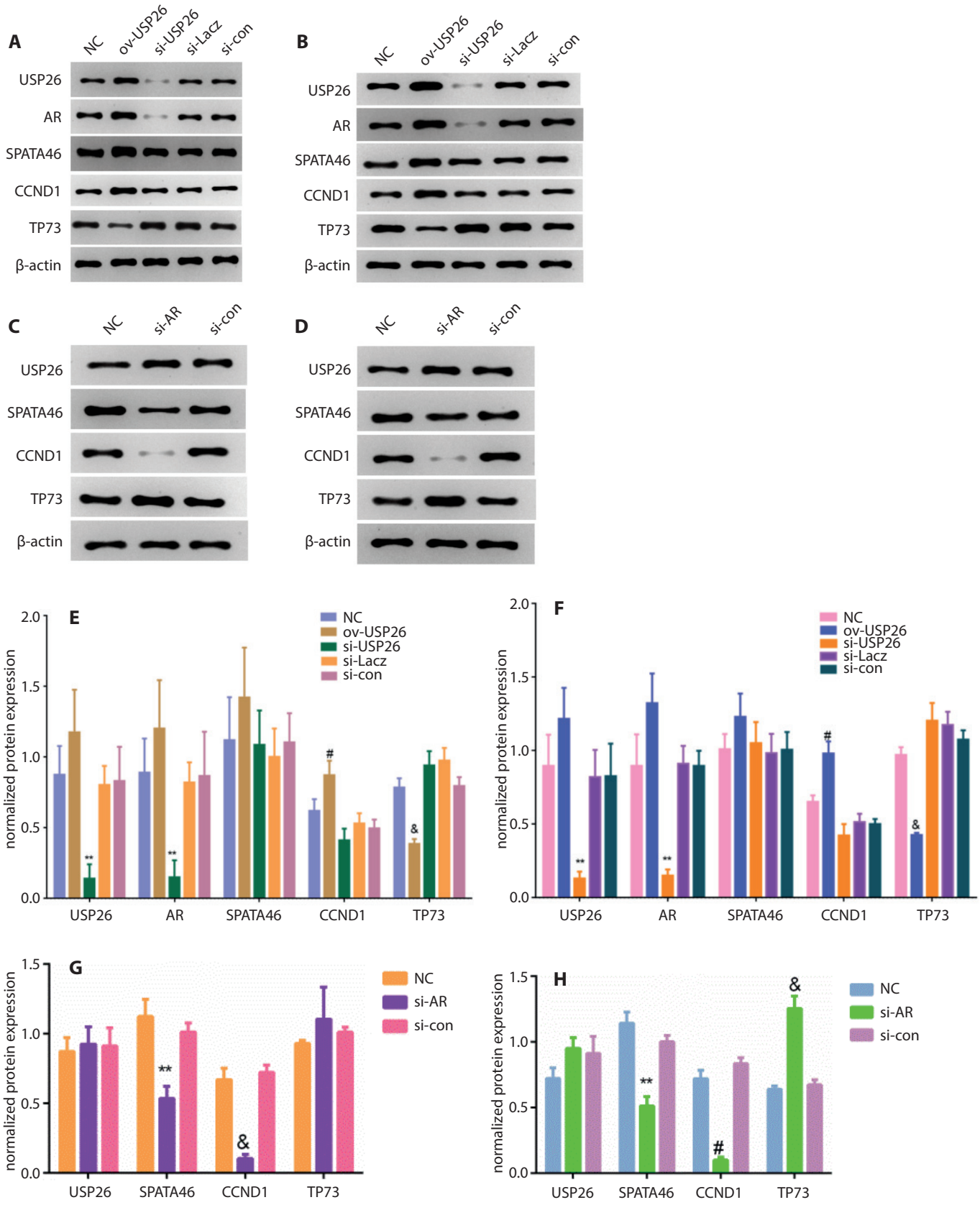

Fig. 5. The USP26, AR, SPATA46, CCND1, and TP73 proteins were detected using the western blot method. A and B. The protein bands represented the protein expression in the TM3 and 293T cell groups transfected with ov-USP26, si-USP26, si-LacZ, and si-con plasmids, along with the normal control group. C and D. The protein bands represent the protein expression in the TM3 and 293T cell groups transfected with si-AR and si-con plasmids, along with the normal control group. E and F. ** $p < 0.05$ of USP26 and AR proteins in the si-USP26 plasmid group compared with the other groups, $^{\#,\&}p < 0.05$ of proteins CCND1 and TP73 in the ov-USP26 plasmid group compared with the other groups. G and H. ** $p < 0.05$ of protein SPATA46 in the si-AR plasmid group compared with the other groups, $^{\#,\&}p < 0.05$ of protein CCND1 and TP73 in the si-AR plasmid group compared with the other groups, $^{\&}p < 0.05$ of protein TP73 in the si-AR plasmid group compared with the other groups

Conclusions

USP26 interacted with AR and monoubiquitinated AR to facilitate sperm maturation and spermatogenesis through the AR signaling pathway.

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Antiviral treatment in congenital HCMV infection: The six-year experience of a single neonatal center in Poland

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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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Abstract

Background. Antiviral treatment is recommended for symptomatic newborns with congenital cytomegalovirus infection (cCMV).

Objectives. To compare 2 treatment methods in neonates with cCMV – ganciclovir-based therapy (intravenous ganciclovir (GCV) or sequential GCV + valganciclovir (VGCV) therapy) with oral VGCV-based therapy – in Polish neonates.

Material and methods. A total of 98 symptomatic infants with cCMV (positive HCMV DNA in urine ≤21st day of life) hospitalized in the neonatal intensive care unit (NICU) between 2012 and 2017 were enrolled. Clinical characteristics, the viral load in blood and urine, hematological and biochemical tests, neuroimaging results, and the length of hospitalization were compared between the study groups at baseline and at the 2nd hospitalization.

Results. In 2012, GCV was used in 57% of the cases, sequential therapy in 33% and VGCV in 10%. In 2017, VGCV monotherapy was used in 83% of the infants treated. Valganciclovir treatment allowed the length of hospitalization to be shortened over 2.5 times during the six-year observation period. Infants treated intravenously had lower birth weights and head circumferences, and more frequently presented splenomegaly, petechiae, thrombocytopenia, and hepatitis. The baseline viral load in the blood and urine were similar in both groups, but at follow-up visits 4–6 weeks later, a viral load about 70 times lower was observed in the blood of the VGCV-based group (1029 viral copies/mL compared to 72,188 viral copies/mL in the GCV-based group; $p = 0.04$). The prevalence of neutropenia was similar in both groups at the follow-up visits.

Conclusions. Valganciclovir became the first line of antiviral therapy in cCMV in the study population. Compared to GCV-based therapy, VGCV monotherapy allowed shorter hospital stays and reduced the viral load in blood due to continuing treatment at home. Valganciclovir monotherapy did not provoke more side effects such as neutropenia. Intravenous GCV is still suitable for patients with severe disseminated disease, born prematurely, with low birth weights, or not tolerating enteral feeding. In those infants, the sequential therapy seems to be optimal.

Key words: congenital, human cytomegalovirus, ganciclovir, valganciclovir, cytomegaly

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Introduction

Human cytomegalovirus (HCMV), which belongs to the *Herpesviridae* (HHV-5), is widespread all over the world. It is estimated that 50–80% of the population is HCMV-seropositive in developed countries, and up to 95–100% in developing countries.[1,2] Congenital cytomegalovirus infection (cCMV) is the most common intrauterine infection.[2,3] In the USA, it affects about 0.5–1% of newborns, which means as many as 40,000 infected newborns per year.[4,5] However, only 10–15% of infected newborns are symptomatic at birth.[6,7] Central nervous system (CNS) abnormalities include microcephaly, abnormal neuroimaging (intracerebral calcifications, intra-/paraventricular cysts, ventriculomegaly), sensorineural hearing loss (SNHL), and chorioretinitis.[8] Hepatobiliary and reticuloendothelial system disorders consist of hepatomegaly, splenomegaly, petechiae, thrombocytopenia, neutropenia, hepatitis, and cholestasis.[9] Among HCMV-seropositive newborns that are initially asymptomatic, up to 15–25% will develop late-onset sequelae. The SNHL and neurodevelopmental retardation, including cognitive delays and motor impairments such as cerebral palsy, are the most common long-term outcomes in cCMV.[3,5,10] Antiviral treatment improves audiological and developmental outcomes.[11] Two of the 5 drugs approved by the Federal Drug Administration (FDA) for treatment of HCMV – ganciclovir (GCV) and its oral pro-drug valganciclovir (VGCV) – were studied in newborns with cCMV. Initially, antiviral treatment for cCMV consisted of 6 weeks of intravenous therapy with GCV.[12] Then, it was documented that an oral solution of VGCV provided comparable plasma concentrations of GCV as intravenous GCV.[13] Finally, randomized clinical trials showed that for infants, 6 months of VGCV therapy was much more beneficial than 6 weeks of GCV, and both treatments were approved by the American Academy of Pediatrics (AAP).[9,11,14]

The aim of the study was to compare 2 treatment methods in neonates with cCMV over time: GCV-based therapy (intravenous GCV or sequential GCV + VGCV therapy) and oral VGCV-based therapy alone.

Material and methods

Caucasian neonates with symptomatic cCMV on antiviral treatment were recruited for the study at the Neonatal Intensive Care Unit (NICU) of the Children's Memorial Health Institute in Warszawa (Poland). The analyzed data was collected retrospectively between 2012 and 2017. Inclusion criteria for the study were positive real-time polymerase chain reaction (RT-PCR) results for HCMV DNA in urine before or on the 21st day of life, symptomatic infection and antiviral treatment. A symptomatic case was defined by 1 or more CNS abnormalities and/or a minimum of 3 hepatobiliary and reticuloendothelial system disorders. The following definitions of laboratory abnormalities were adopted in the study: elevated direct bilirubin >1 mg/dL for cholestasis; elevated levels of aspartate transaminase (ASPAT) >84 U/L and/or elevated levels of alanine aminotransferase (ALTAT) >60 U/L for hepatitis; thrombocytopenia below 100 G/L; neutropenia below 1000 G/L; positive CMV-immunoglobulin G (IgG) ≥6 AU/mL; negative CMV-IgG < 6 AU/mL; positive HCMV-immunoglobulin M (IgM) ≥1 AU/mL; grey-zone HCMV-IgM 0.85–0.99 AU/mL; and negative CMV-IgM < 0.85 AU/mL. Exclusion criteria were negative RT-PCR CMV DNA in urine before or on the 21st day of life; a lack of the PCR results before or on the 21st day of life; asymptomatic infection including isolated symptoms such as prematurity or intrauterine growth restriction (IUGR); and congenital malformations. Depending on the type of treatment, 2 groups were distinguished in the study: group 1 – GCV-based therapy (neonates treated only intravenously with GCV and neonates treated sequentially with GCV followed by oral VGCV); and group 2 – VGCV-based therapy (neonates treated only orally). Ganciclovir was scheduled to be administered intravenously at a dose of 6 mg/kg every 12 h for 6 weeks during baseline hospitalization. Infants treated intravenously were assessed again 4–6 weeks after the cessation of antiviral therapy. Valganciclovir was administered orally at a dose of 16 mg/kg every 12 h during baseline hospitalization, and continued at home and during the follow-up hospitalization. Drug levels were measured during treatment (data not presented). The clinical evaluation, hematological and biochemical tests, serology, qualitative and quantitative RT-PCR for HCMV DNA in blood and urine, and qualitative RT-PCR for HCMV DNA in cerebrospinal fluid (CSF), neuroimaging (ultrasound (US) and magnetic resonance imaging (MRI)), newborn hearing screening tests (otoacoustic emission (OAE)) and ophthalmologic consultations were performed at baseline and in part also at the follow-up visits (about 4–6 weeks after the 1st hospitalization). The HCMV DNA viremia and viruria (viral load) were determined in whole blood and urine samples using RT-PCR and GeneProof Cytomegalovirus PCR Kits (GeneProof, Brno, Czech Republic). The results were expressed as copies per mL (blood or urine). The qualitative detection of HCMV DNA in CSF was done with RT-PCR using the SYBRGreen I format and a primer set specific for the phosphoprotein *pp65*(*UL83*) gene.[15] The sensitivity of the assay was 250 copies/mL. The statistical analysis was performed using STATISTICA v. 7.0 for Windows (StatSoft Inc., Tulsa, USA). All data was presented as mean ± standard deviation (SD) or number (%). The p-values below 0.05 were considered statistically significant.

The study protocol was approved by the ethics committee on human subjects of the Children's Memorial Health Institute (Warszawa, Poland). We declare compliance with ethical practices in the study.

Results

A total of 123 newborns with cCMV were hospitalized in the NICU during the study period. The final analysis was performed in 98 symptomatic newborns offered antiviral therapy. Twenty-five asymptomatic newborns who were not treated were excluded. Among the treated neonates, there were 30 preterm newborns (4 neonates <32 weeks of gestation). Only 2 preterm newborns had extremely low birth weights (ELBW), 1 newborn had a birth weight appropriate for the gestational age (AGA) and 1 had intrauterine growth restriction (IUGR).

In most cases, antiviral therapy was introduced during the 1st month of life. Ganciclovir was used in 60 participants (group 1) – in 13 cases as a monotherapy (12 cases in 2012) and in 47 participants as sequential therapy. Valganciclovir was used as a monotherapy in 38 neonates (group 2). The characteristics of the study population and treatment groups are presented in Table 1.

The mean duration of intravenous therapy was 24 ±10 days in the entire study population. When GCV-based therapy (group 1) was analyzed in detail, GCV was administered for 34 ±9 days in the monotherapy and 22 ±9 days in sequential therapy ($p < 0.0001$). The details of the antiviral treatment were presented for 2012 (the beginning of the study) and for 2017 (the end of the study) of the entire observation period. In 2012, GCV monotherapy, sequential therapy and VGCV monotherapy were used in 12 neonates (57%), 7 neonates (33%) and 2 neonates (10%), respectively. In 2012, the mean duration of intravenous GCV therapy was 31 ±19 days (36 ±7 days for GCV monotherapy, 21 ±10 days for sequential therapy). The mean length of hospitalization depended on the type of antiviral therapy; it was 38 ±8 days for GCV monotherapy, 28 ±9 days for sequential therapy and 6 ±1 days for oral VGCV monotherapy. By contrast in 2017, GCV was not used as monotherapy in any case; 29 neonates (83%) were treated with oral VGCV monotherapy, while 6 neonates (17%) underwent sequential therapy. In 2017, when sequential treatment was applied during the 1st hospitalization, the mean duration of GCV administration was 21 ±9 days. Then, VCGV was used for 9 ±6 days, until the day of discharge from the hospital, and then continued at home. The mean length of hospitalization was associated with the type of antiviral therapy; it was 29 ±13 days for sequential therapy and 9 ±4 day for VGCV monotherapy.

During antiviral treatment, we measured the viral load in blood and urine at every visit. At baseline, before drug administration, viremia and viruria were comparable in both groups ($p > 0.05$) (Table 1). At the follow-up visit, the HCMV DNA concentration in blood was significantly lower in group 2 than in group 1 ($p = 0.04$) (Table 2). Positive results of PCR for HCMV DNA in CSF were observed in 16.8% of the study population (20% in group 1 and 11% in group 2) at baseline (Fig. 1), but in only 3% of the infants in both groups at follow-up (Table 2).

At baseline, the prevalence of neutropenia (<1000 G/L) was 18% in the entire cohort. There were no statistical differences in neutrophil counts between the 2 study groups (Table 1). At the follow-up hospitalization, the prevalence of neutropenia was 24% in the entire cohort. There were no statistically significant differences in neutrophil counts and the prevalence of neutropenia between the study groups during the 2nd hospitalization (Table 2). However, severe neutropenia (grade 4: <500 neutrophils G/L) was observed, mainly in group 1 (5 cases), with the lowest count of 153 neutrophils G/L, while in group 2 neutropenia was noted in 1 patient with the lowest count of 459 neutrophils G/L.

As far as clinical characteristics were concerned, we observed the following abnormalities during the baseline visit: intra/paraventricular cysts (74.4%), vasculopathy

Table 1. Baseline characteristics of symptomatic newborn infants with cCMV and comparisons of the study groups in relation to the treatment regimen

Characteristics	Study population n = 98	Group 1 GCV only or GCV + VGCV n = 60	Group 2 VGCV n = 38	p-value
Birth weight [g]	2535 ±690	2395 ±665	2756 ±680	0.011[a]
Gestational age [weeks]	37 ±3	37 ±3	38 ±2	NS
Head circumference [cm]	33 ±3	32 ±2.9	34 ±2.2	0.002[a]
Age at admission to NICU [days]	15 ±13	13 ±13	17 ±12	NS
Thrombocytes [G/L]	163 ±122	118 ±95	234 ±128	<0.001[a]
Neutrophils [G/L]	1987 ±1398	1871 ±1511	2171 ±1195	NS
ASPAT [U/L]	71 ±117	91 ±145	38 ±13	0.033[a]
ALAT [U/L]	33 ±50	40 ±62	21 ±10	NS
Blood viral load [number of viral copies/mL]	–	339×10^3 $\pm 830 \times 10^3$	104×10^3 $\pm 190 \times 10^3$	NS
Urine viral load [number of viral copies/mL]	–	5830×10^3 $\pm 4517 \times 10^3$	7520×10^3 $\pm 3960 \times 10^3$	NS

Data is presented as mean ±SD; NS – not significant (p-value above 0.05); cCMV – congenital cytomegalovirus infection; GCV – ganciclovir; VGCV – valganciclovir; NICU – neonatal intensive care unit; ASPAT – aspartate transaminase; ALAT – alanine aminotransferase; [a] p-values below 0.05 are statistically significant.

Table 2. Comparison of follow-up characteristics of symptomatic infants with cCMV in relation to the treatment regimen

Characteristics	Group 1 GCV only or GCV+VGCV n = 60, n (%)	Group 2 VGCV n = 38, n (%)	p-value
Age [days]	77 ±26	55 ±17	<0.001[a]
Weight [g]	4519 ±1042	4170 ±1118	NS
Head circumference [cm]	37 ±3	39 ±12	NS
Thrombocytopenia, n (%)	1 (2)	0 (0)	NS
Thrombocytes [G/L]	376 ±137	478 ±134	0.001[a]
Neutropenia [<1000 G/L]	15 (25)	10 (26)	NS
Neutrophils [G/L]	1866 ±1501	1822 ±1098	NS
Splenomegaly, n (%)	8 (13)	1 (3)	NS
Cholestasis, n (%)	5 (8)	0 (0)	NS
ASPAT [U/L]	45 ±38	44 ±31	NS
ALAT [U/L]	36 ±30	36 ±33	NS
Vasculopathy at US, n (%)	16 (27)	12 (32)	NS
Peri/intraventricular cysts at US, n (%)	21 (35)	12 (32)	NS
Positive PCR CMV in CSF, n (%)	2 (3)	1 (3)	NS
Ophtalmologic abnormalities, n (%)	13 (22)	3 (8)	NS
Blood viral load [number of viral copies/mL]	$72 \times 10^3 \pm 432 \times 10^3$	$1 \times 10^3 \pm 2 \times 10^3$	0.044[a]
Urine viral load [number of viral copies/mL]	$132 \times 10^3 \pm 510 \times 10^3$	$680 \times 10^3 \pm 235 \times 10^3$	NS

Data is presented as numbers (%) or mean ±SD; NS – not significant; cCMV – congenital cytomegalovirus infection; GCV – ganciclovir; VGCV – valganciclovir; ASPAT – aspartate transaminase; ALAT – alanine aminotransferase; US – ultrasonography; CSF – cerebrospinal fluid; [a] p-values below 0.05 are statistically significant.

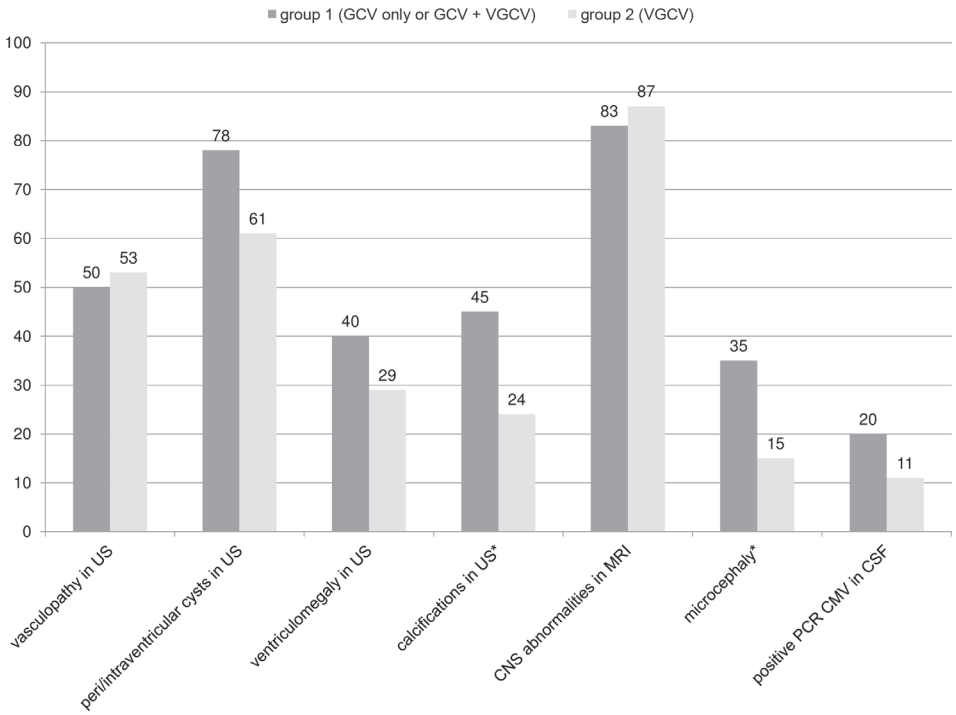

Fig. 1. Comparison of baseline CNS involvement between the GCV group (GCV or GCV + VGCV) and the VGCV group. *For statistically significant differences, p-values <0.05 are shown

(52%), abnormal OAE in at least 1 ear in 45 children among 96 with available results (46.8%), thrombocytopenia (43.9%), ventriculomegaly (37.7%), intracranial calcifications (36.7%), ophthalmologic abnormalities (34.6%), splenomegaly (27.5%), neutropenia (22.4%), cholestasis (22.4%), petechiae (21.4%), and positive PCR HCMV DNA in CSF (16.3%). The neonates in group 1 had a significantly higher prevalence of petechiae, splenomegaly, cholestasis, thrombocytopenia, elevated liver enzymes, intracranial calcifications in US, lower birth weights, and lower head circumferences (Table 1, Fig. 1,2). At the follow-up visit, the results of physical examination, laboratory blood tests

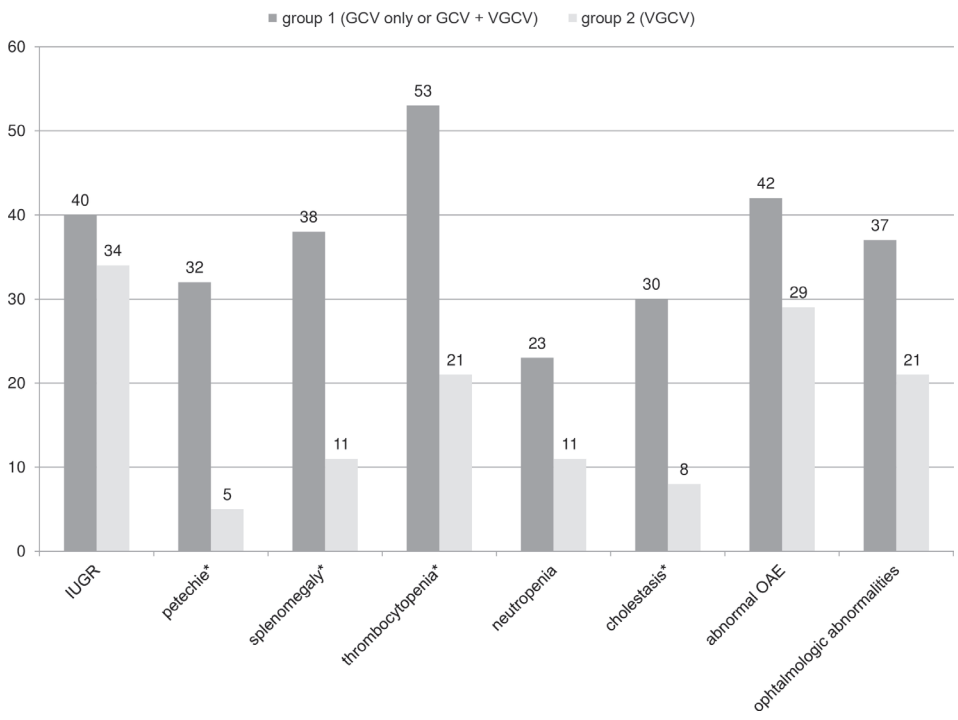

Fig. 2. Comparison of baseline characteristics between the GCV group (GCV or GCV + VGCV) and the VGCV group. *For statistically significant differences, p-values <0.05 are shown

and neuroimaging were not significantly different between the 2 study groups, except for a significantly lower mean platelet count in group 1. However, thrombocytopenia was found in only 1 patient in this group (Table 2).

The baseline serological status showed that the 96 (100%) newborns with results available had positive CMV-IgG (in 2 cases the results were not available). Only 48 of 96 patients (50%) had positive CMV-IgM and 5 of 96 patients (5.2%) had grey-zone CMV-IgM.

Discussion

We compared 2 treatment methods in a cohort of neonates in Poland with symptomatic cCMV: GCV-based therapy (intravenous GCV or sequential GCV + VGCV therapy) compared to oral VGCV monotherapy. Initially, on the basis of international recommendations,[12] 6 weeks of intravenous GCV therapy for cCMV were recommended in Poland. When the oral suspension of VGCV was available in Europe, we introduced oral therapy for cCMV, with a final extension to 6 months, as the first-choice of treatment for cCMV, in accord with international and European recommendations.[9–11,13,14,16,17]

As expected, we noted that between 2012 and 2017, the percentage of neonates with cCMV on oral VGCV increased from 43% to 100%, while those on intravenous GCV as a monotherapy decreased from 57% to 0%. Similarly, the proportion of infants with cCMV in the USA who had VGCV treatment increased significantly between 2009 and 2015.[5]

The average duration of intravenous treatment was about 5 weeks for infants on GCV monotherapy. If sequential therapy was applied, intravenous treatment was reduced to about 3 weeks. In our center, we tended to switch to oral therapy as soon as the clinical status and laboratory results improved and the oral drug could be administered. Our study showed that among infants on VGCV monotherapy, the length of hospitalization was over 6 times shorter than for infants on GCV monotherapy. The growing number of infants with cCMV on oral VGCV treatment led to the time of hospitalization becoming over 2.5 times shorter during the six-year observation period.

Oral treatment methods also entail other benefits, such as decreased risk of nosocomial infections, diminished stress for parents and infants, and lower economic costs.[18] Additionally, oral therapy avoids the complications of intravenous injections. In Poland, neonates have to stay in the hospital for the duration of intravenous therapy through peripheral intravenous catheters or peripherally inserted central catheters (PICCs). In a study by Amir et al., infants received GCV through a central venous catheter (usually a PICC line), which allowed for home treatment and eliminated the risks of prolonged hospitalization. However, 2 patients experienced central line infections.[19] In our study, we did not analyze the frequency of catheter infections.

Antiviral treatment eliminates HCMV from blood, urine and CSF.[13] Although recent research shows that the viral load in blood has no clinically meaningful predictive value for long-term outcomes, it was earlier observed that asymptomatic infants with a reduced viral load in the blood and urine were at a lower risk for hearing loss in the future.[20,21] We showed a 70 times lower viral load in the blood in the VGCV-based group than in the GCV-based group during the 1st follow-up visit. It is widely known that viruria and viremia can recur about

2 weeks after discontinuation of antiviral therapy. Thus, the rebound-effect after completing intravenous treatment might explain the higher viral load in the blood and urine in the GCV-based-group at the follow-up visit. It should be emphasized that HCMV can be excreted in the urine for up to 2–5 years after infection.[13,22,23] We detected HCMV DNA in CSF in only 16.8% of the cases, although over 80% of the infants had abnormal neuroimaging. The detection of HCMV DNA in CSF using PCR was not a sensitive marker of cCMV neuroinfection in the current study. Interestingly, Lisowska-Mikołajków et al. suggested expanding the assessment of other newborn fluids such as blood and CSF if the number of HCMV copies in urine exceed 500 copies/mL. In other situations, high numbers of HCMV copies in blood or CSF are unlikely.[18]

Myelosuppression, especially neutropenia, was the main side effect of GCV/VGCV treatment in our study. It was the main reason for discontinuing intravenous therapy. Temporal cessation of oral therapy was also necessary in cases of severe neutropenia. When grade 4 neutropenia occurred during treatment, we usually stopped antiviral therapy and administered recombinant human granulocyte colony-stimulating factor (rhG-CSF) until the patient's blood tests results improved. Ronchi et al. presented serious consequences of neutropenia for infants, including bacterial sepsis.[24] Fortunately, we did not observe any severe bacterial sepsis or death due to neutropenia during antiviral therapy. In previous studies, grade 3 or 4 neutropenia was reported in 63% of infants with cCMV during six-week GCV treatment, but in only 21% of infants during six-month VGCV-treatment and in 27% of infants in a placebo group.[11,12,25] Thus, the problem was more pronounced in GCV therapy.[5] In our study, however, there were no statistically significant differences between the 2 study groups in terms of percentages of neutropenia and mean levels of neutrophils at the follow-up visits.

Undoubtedly, newborn infants with severe disseminated disease ("septicemic") and those who are unable to absorb medications from the gastrointestinal tract (including preterm infants) benefit from intravenous GCV therapy until a stabilization of their general condition and the safe introduction of oral treatment.[14] In our study, newborns on GCV therapy more often demonstrated petechiae, splenomegaly, cholestasis, thrombocytopenia, calcifications in CNS, as well as lower birth weights and head circumferences, and they were usually born prematurely.

We also investigated baseline serological status of the study population. It is obvious that all cCMV-infected newborns had positive CMV-IgG, due to transplacental transfer, as opposed to CMV-IgM, which was produced by the fetus itself. Positive CMV-IgM was found in only 50% of the entire cohort of symptomatic newborns. Interestingly, Ohyama et al. observed positive CMV-IgM in 25/32 of symptomatic infants with cCMV infection (78%).[26] Undoubtedly, negative CMV-IgM did not exclude cCMV infection and assessment of neonatal serological status is not a reliable tool for a diagnosis of cCMV.[18,26]

Unfortunately, the available data limited our observation period in the current study to 1 follow-up visit about 4–6 weeks after the 1st hospitalization, so we could not analyze long-term outcomes. Kimberlin et al. observed that 6 months of VGCV administration led to improved hearing or protection of normal hearing at 12 months and at 24 months. Similarly, patients with CNS abnormalities also had better outcomes from baseline to 12 months and 24 months.[11] By contrast, Fukushima et al. observed that infants with microcephaly and those who were small for their gestational age (SGA) at birth presented severe sequelae and development quotients <70 at around 18 months of age, despite 6 weeks to 6 months of VGCV treatment.[27] Undoubtedly, further investigations with long observation periods are needed to evaluate neurodevelopmental outcomes among orally treated cCMV-infected patients.

Another limitation of our study was incomplete data of GCV level due to the retrospective character of the study. Moreover, the low number of infants on exclusive intravenous therapy did not allow us to separate these infants and create a 3rd group for comparison. Finally, hearing evaluation was based on OAE, because data on auditory brainstem responses (ABR) were not available for all the participants.

The strength of the study is the relatively large and homogenous population consisting of 98 symptomatic newborns with confirmed cCMV. To the best of our knowledge, our cohort of Polish newborns with symptomatic cCMV is one of the biggest cohorts that has been described to date.

Conclusions

In summary, VGCV became the first line of antiviral therapy in cCMV in the study population. Compared to GCV-based treatment, VGCV monotherapy allowed shorter hospital stays and reduced the viral load in blood due to the treatment being continued at home. Moreover, severe neutropenia was less frequent in VGCV monotherapy than in GCV-based treatment. However, intravenous GCV is still suitable for patients with severe disseminated disease, born prematurely, with low birth weights, or not tolerating enteral feeding. In those infants, sequential GCV + VGCV therapy seems to be optimal.

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Single vs dual rod constructs in early onset scoliosis treated with magnetically controlled growing rods

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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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Abstract

Background. Non-invasive distractions of recently introduced magnetically controlled growing rods (MGCRs) spare multiple operations in the surgical treatment of early onset scoliosis (EOS). Since the costs of the implants are high, concerns have been raised regarding cost-effective, optimal but safe MGCR options: single or dual constructs.

Objectives. To report deformity control, spinal growth and complication incidence in EOS patients treated with MCGR single- or dual-rod constructs.

Material and methods. The study involved 47 patients with MCGRs inserted at Great Ormond Street Hospital, London (UK) in 2013–2014, who were followed up for at least 1 year. In 32 patients, T1–S1 distances, and coronal and sagittal curves were measured on preoperative and postoperative X-rays, and at a one-year follow-up. All complications were recorded. The patients were analyzed in 2 groups: those with single-rod constructs (24 patients) and those with dual-rod constructs (23 patients).

Results. Comparing postoperative with one-year follow-up measurements, T1–S1 length increase was better in the dual-rod group (3.29%) than in the single-rod group (0.34%) ($p = 0.031$). In the whole series, mean scoliosis magnitude dropped by 27.5% at the one-year follow-up. The dual-rod group showed better mean curve correction: 36.5% compared to 15.3% in the single-rod group ($p = 0.0076$). Overall, 34.04% of the patients had complications: 45.8% in the single-rod group and 30.4% in the dual-rod group ($p = 0.0413$). Metalwork failure was observed in 8 patients, lengthening problems in 5 and wound infections in 2; there was also 1 case of proximal junctional kyphosis (PJK). Preoperative hyperkyphosis was associated with more complications (75%, $p = 0.037$), most of which were metalwork failure (41.6%).

Conclusions. The MCGRs are efficient at controlling EOS; however, the complication rate is high, particularly in single-rod constructs. The use of dual-rod constructs allows for better curve control, greater T1–S1 length increase and a lower complication rate.

Key words: early onset scoliosis, magnetically controlled growing rod, dual rods, spinal growth

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Introduction

Patients with progressive spinal deformities which cannot be controlled with conservative measures are usually candidates for surgical treatment. The goal of the treatment of scoliosis in an immature, growing spine – early onset scoliosis (EOS) – is not only to control deformity progression but also to allow the growth of the spine. It is particularly important in children below the age of 5 years, since normal growth of the thoracic spine ensures the necessary development of the chest and lungs.[1]

In order to meet these goals, growth-friendly systems of spinal implants were developed. Three approaches are present in clinical practice: growth guidance, tethering and the most popular: distraction-based systems.[2] Distraction-based systems are efficient at deformity control, concomitantly preserving spinal growth; however, the method has a discouragingly high complication rate, and multiple surgical distractions are needed to follow spinal growth.[3]

The introduction of magnetically controlled growing rods (MCGRs) allowing for noninvasive distractions raised hopes of reducing complications and avoiding multiple operations associated with standard growing rods.[4] The method is fairly new, and the efficiency of the system in terms of deformity control, spinal growth and complication rate has not yet been decisively established. The high cost of the method is another concern. Therefore, questions have been raised about whether the application of a single rod might be a safe, efficient and cost-effective clinical approach.

The aim of this observational study was to report spinal growth, coronal curve control and the complication rate in EOS patients treated with dual or single MCGR constructs.

Material and methods

The study involved 47 patients with diagnosed progressive EOS who underwent MCGR insertion between 2013 and 2014 in the Spinal Unit of Great Ormond Street Hospital, London (UK). All the patients were followed up for at least 1 year (range: 12–30 months). The study group included 18 girls and 14 boys with a mean age of 8.8 years (range: 2.5–11 years) at the time of the surgery. Thirty-two patients had all the measurements conducted on preoperative and follow-up X-rays. The remaining 15 patients missed a follow-up X-ray or the X-ray quality was insufficient for detailed and precise measurements; therefore, those patients were excluded from the analysis. In the series, all types of EOS were included (idiopathic, neuromuscular, congenital, and syndromic). All distractions were performed in the outpatient clinic with a mean distraction frequency of 3 months.

The magnitude of coronal curves and T1–S1 distances were measured on the X-rays obtained in a vertical position, prior to the operation, immediately after the operation (during the hospital stay) and 1 year from the operation. The initial sagittal profile was assessed; thoracic kyphosis (measured between the end plates of T2 and T12) above 50° was considered a kyphotic profile; 20–50° was considered normal; and below 20° was considered lordotic (hypokyphotic).

All complications were recorded and divided into 4 groups: metalwork failure (rod breakage, fixation failure – a screw/hook dislodging or loosening), distraction issues (pin breakage, unknown), infections, and proximal junction kyphosis (PJK).

The patients were analyzed in 2 groups. The single-rod construct group contained 24 patients and the dual-rod construct group included 23 patients. Data and comparisons between the groups were statistically analysed using Student's t-test. In cases of non-homogeneity of variance and/or non-normal distribution ($p > 0.05$), the Mann– Whitney U test was used. The Mann–Whitney U value was used when groups had fewer than 20 samples, while the Z value was used when one of the groups had ≥20 samples. A p-value less than 0.05 was considered statistically significant.

Results

T1–S1 distance

The average T1–S1 distance increased in the whole series of patients. The initial increase in the distance after surgery (preoperative compared to immediately postoperative) was similar in the single-rod group (10.7 ±8.42%) and the dual-rod group (10.44 ±6.51%) ($p = 0.463$). Slightly better growth was observed in the dual-rod group, comparing the mean preoperative T1–S1 distance to 1 year after the operation; the single-rod group showed 11.03 ±8.03%, while the dual-rod group showed 14.03 ±8.04% ($p = 0.316$) growth. Comparing initial postoperative T1–S1 lengths with one-year postoperative lengths, substantially greater increases were obtained in the dual-rod group (3.29%) than in the single-rod group (0.34%) ($p = 0.031$; Fig. 1).

An EOS etiology analysis showed the greatest increase in the T1–S1 distance in syndromic patients (16.06%) ($p = 0.049$) and neuromuscular patients (14.93%) ($p = 0.042$). The lowest growth was observed in idiopathic patients (10.58%) and those with congenital deformities (7.12%) (Fig. 2).

Deformity control

In the whole series, the initial postoperative mean scoliosis curve magnitude dropped by 32.4%, then in 1 year it increased by 7.2%, resulting in an overall curve reduction of 27.5% at the end of the follow-up. The dual-rod group showed better initial curve correction

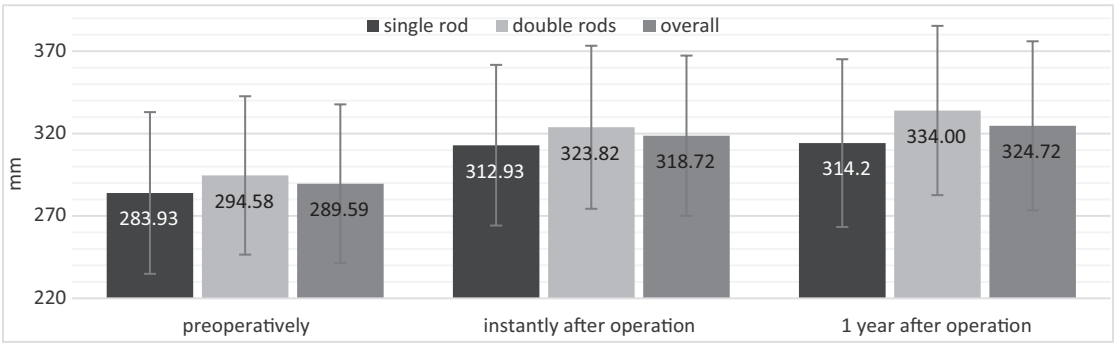

Fig. 1. The mean T1–S1 values [mm] in the single- and double-rod groups

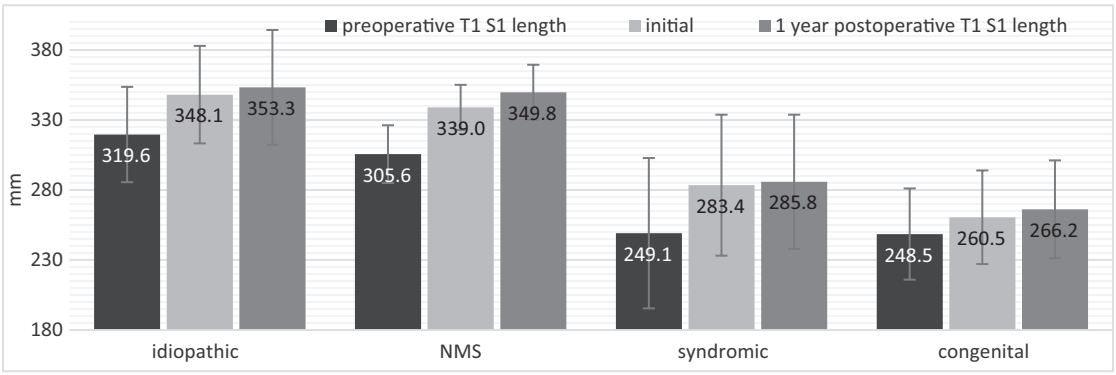

Fig. 2. The mean T1–S1 values [mm] in terms of spinal deformity etiology

(40.08%) than the single-rod group (21.8%) (p = 0.004). At the one-year follow-up, the mean Cobb angle had increased by 5.8% in the dual-rod group and by 8.3% in the single-rod group (p = 0.722). At the end of the follow-up, a 15.3% reduction in the coronal curve was observed in the single-rod group and 36.5% in the dual-rod group (p = 0.0076) (Fig. 3).

Patients with neuromuscular scoliosis had the greatest curve correction, followed by those with idiopathic and syndromic scoliosis, with the weakest effect in patients with congenital scoliosis; however, no statistical significance was observed (Fig. 4).

Complications

As shown in Table 1, in the whole series of 47 patients, 17 complications (36.17%) were observed in 16 patients (34.04%). Metalwork failure was the most frequent complication, observed in 8 patients (17.02%). Two patients (4.25%) had deep wound infections (both revised). Problems with MCGR lengthening were observed in 5 cases (10.63%). Proximal junction kyphosis was observed in 1 child, but no revision was necessary during follow-up. Overall, 13 revisions were performed: the 2 infections, 3 distraction issues and 8 metalwork failures.

Table 1. Complications and patient characteristics

Complications n = 17 (36.17%)		Metalwork failure	Lengthening issues	Infection	PJK	General
Etiology	idiopathic (n = 20)	2 (10%)	3 (15%)	1 (5%)	1 (5%)	7 (35%)
	neuromuscular (n = 10)	1 (10%)	1 (10%)	1 (10%)	0	3 (30%)
	congenital (n = 6)	2 (33.3%)	0	0	0	2 (33.3%)
	syndromic (n = 11)	4 (36.4%)	1 (9.1%)	0	0	5 (45.5%)
Sagittal profile	kyphotic (n = 12)	5 (41.6%)	2 (16.6%)	1 (8.3%)	1 (8.3%)	9 (75%)
	lordotic (n = 15)	1 (6.6%)	3 (20%)	0	0	4 (26.6%)
	normal (n = 20)	3 (15%)	0	1 (5%)	0	4 (20%)
Rod construct	single rod (n = 24)	5 (20.8%)	3 (12.5%)	1 (4.2%)	2 (8.3%)	11 (45.8%)
	dual rod (n = 23)	4 (17.4%)	2 (8.7%)	1 (4.34%)	0	7 (30.4%)
Curve magnitude	<70° (n = 25)	3 (12%)	3 (12%)	1 (4%)	1 (4%)	8 (32%)
	>70° (n = 22)	6 (27.3%)	2 (9%)	1 (4.5%)	0	9 (40.9%)

PJK – proximal junctional kyphosis.

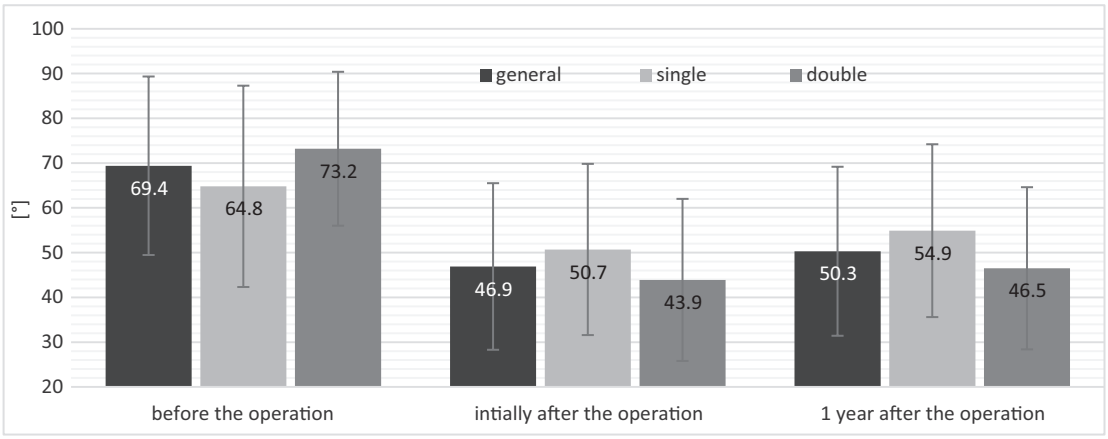

Fig. 3. Average Cobb angle in all groups

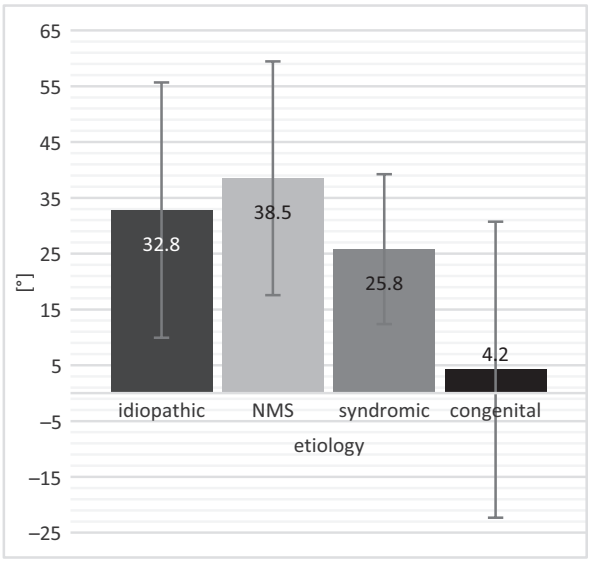

Fig. 4. Curve magnitude change in relation to etiology

The highest rate of complications was observed in the 11 patients with syndromic scoliosis (45.5%), most of which (36.6%) were metalwork issues. Preoperative hyperkyphosis tended to produce a high rate of complications. Among the 12 hyperkyphotic patients, 9 (75%) had complications; most of these were metalwork failures (41.6%) (Fig. 5). These results proved to be statistically significant ($p = 0.037$).

There was a higher overall complication rate in the single-rod (45.8%) than in dual-rod (30.4%) constructs ($p = 0.0413$). Metalwork problems, PJK and rod distraction issues occurred more frequently in single-rod construct patients. The single- and dual-rod groups each had 1 case of infection.

Curve severity was not found to contribute to the complication rate; however, a slightly elevated frequency of metalwork failure was present in patients with scoliosis above 70° ($p = 0.195$).

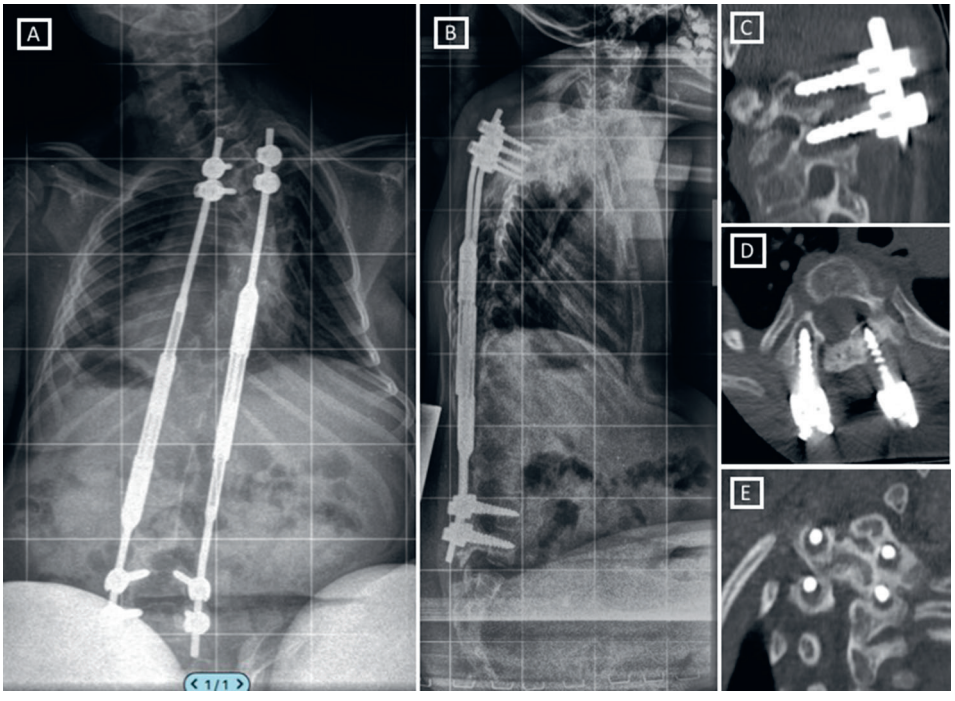

Fig. 5. Loss of proximal fixation of MGCR. Plain whole spine X-rays in postero-anterior (A) and lateral (B) projections. The CT images of loosened screws in sagittal (C), axial (D) and coronal (E) views

Discussion

The popularity of MCGRs is continuously growing, mainly due to the major advantage of avoiding open distractions. Primary reports concluded that MCGRs are a safe and effective option for the treatment of progressive pediatric scoliosis.[4] However, the presented series were rather small in number, and as more observations emerged, more doubts were raised, particularly regarding the incidence of complications, which appeared to be higher than previously considered.[5–8]

In our retrospective analysis of a consecutive series of patients treated with MCGRs, we observed overall good curve control and spinal growth; however, better growth of the T1–S1 distance and better curve control was noticed in the dual-rod group. The incidence of complications was generally high in the whole series (34%), but it was particularly high in patients with initial hyperkyphosis (75%) and those with single-rod constructs (45%), as opposed to dual-rod constructs (30%). The complication rate was comparable in all types of scoliosis, and we did not find any correlation between the initial coronal curve magnitude and the complication rate.

This study has several limitations. It is only a one-year retrospective follow-up of a heterogeneous group of EOS patients treated with MCGRs; however, the series analyzed is rather large in comparison to other reports available in the literature. A comparable cohort to ours was presented only by Dannawi et al.[9]

Several other papers have reported similar MCGR results to ours: overall good coronal curve control and T1–S1 distance growth.[4,10–12] Comparisons of traditional (TGRs) vs magnetic (MCGR) growing rods demonstrated similar ability to control major curve as well as to obtain good T1–S1 distance growth5, but the advantage of MCGRs over TGRs is an avoidance of multiple planned operations for the rod distractions.

The high costs of MCGRs and the lower invasiveness of the operation might have motivated surgeons to insert constructs based on single rods, but the data presented suggests that this solution results in lower spinal growth (11% in single-rod compared to 14% in dual-rod constructs) and inferior scoliosis correction (by 15% and 36.5%, respectively). Akbarnia et al. and Dannawi et al. both reported minor improvement in T1–S1 distance growth in patients with single rods compared to those with dual rods, but in our series, the differences were more pronounced (65–55° compared to 73–46.5°) than in the studies by Akbarnia et al. (68–36° compared to 56–29°) and by Dannawi et al. (67–44° compared to 70–40°).[9,10] It appears that, like TGRs, single rod growing constructs entail more frequent complications, particularly rod breakage and fixation problems.[12,13]

Surgical treatment of EOS is known to have significant complication rates, especially due to infection and metal/work failures. According to recent reports comparing TGRs and MCGRs, there is lower incidence of infection in MCGRs.[7,14] Reports regarding metalwork problems are more inconsistent, showing lower,[13] similar[5] or higher complication rates in MCGR[7] compared to TGRs. The MCGR patients undergo fewer surgical procedures than TGR patients, but the incidence of unplanned surgical revisions has been reported as similar in the 2 groups.[5] The cohorts in the studies by Teoh et al. and Akbarnia et al. included a significant number of single-rod constructs, and the majority of patients had the first generation of MCGRs implanted.[7,10] These 2 facts may contribute to poorer curve control and spinal growth results, and to the higher complication rate.

The background (etiology) of a deformity often influences treatment decisions and may affect treatment outcomes.[15] In the present study, complications most frequently occurred in syndromic scoliosis, mainly metalwork failure. In this group, a significant number of patients consisted of osteogenesis imperfecta (OI) patients with very poor bone quality; 1 patient experienced fixation dislodgment twice.

We observed that the preoperative spinal sagittal profile (unlike the preoperative magnitude of the primary coronal curve) may affect the complication rate. In our results, hyperkyphosis significantly elevates the risk of metalwork failure – nearly half of our hyperkyphotic patients had this complication. These observations are consistent with those made with TGRs.[3,16]

Despite being the most modern surgical solution for progressing pediatric spinal deformities, MCGR treatment has limitations. Some of them are similar to those noted with TGRs, particularly metalwork problems and distraction issues. Since there are many similarities between TGRs and MCGRs, lessons learned from TGRs should be transferred to clinical practice with MCGRs; after all, it is still a distraction-based growing system. Nonetheless, we are far from an ideal solution and there is a strong need for further research with a special focus on reducing complications.

Conclusions

The MCGRs are efficient in controlling deformities in EOS and provide good spinal growth. The complication rate remains high, as in other surgical methods of EOS treatment; however, the use of dual-rod constructs can decrease the complication rate in comparison to single-rod constructs. Particular caution is required in cases of hyperkyphosis, since these patients are more inclined to develop metalwork problems. Dual-rod constructs provide better curve control, greater T1–S1 length growth and lower complication rates than single-rod constructs.

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The role of N-acetylcysteine in preventing hepatic injury associated with systemic oxidative stress after extracorporeal shock wave treatment

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Abstract

Background. Systemic oxidative stress may cause detrimental consequences for the liver, leading to hepatic fibrogenesis.

Objectives. To investigate histopathological changes in liver tissues due to the increased systemic oxidative stress associated with rat extracorporeal shock wave lithotripsy (SWL) model and to document the consequences of N-acetylcysteine (NAC) administration.

Material and methods. In this experimental SWL model, 18 Wistar albino rats were randomly assigned into 3 groups. The control group (group I) had no intervention. Group II underwent SWL treatment with intraperitoneal saline injection. Group III also had SWL with intraperitoneal NAC and was divided into short-term (group III-14 days) and long-term (group III-28 days) subgroup. Hepatectomy was performed for histopathological examinations. Histopathological alterations were evaluated with light microscopy. Immunohistological staining for p53 and myeloperoxidase was also performed.

Results. Blood samples revealed a significant increase in plasma oxidative stress index (OSI) after plasma total antioxidant status (TAS) and total oxidant status (TOS) had been measured. It was shown that this increased systemic oxidative stress adversely affected liver tissues. Predominantly, sinusoidal dilatation was remarkably observed in rats with significantly high OSI values ($p = 0.043$). Similarly, periportal necrosis significantly increased in rats with high OSI values ($p = 0.033$). p53 positivity was also remarkable in rats with systemic oxidative stress ($p = 0.049$). N-acetylcysteine administration provided a significant decrease in OSI. N-acetylcysteine also improved all these alterations, including p53 staining. Particularly, sinusoidal dilatation was significantly protected in the long-term NAC group (group III-28 days).

Conclusions. We demonstrated that SWL-induced systemic oxidative stress causes histological alterations in liver tissues. Increased p53 and myeloperoxidase staining as markers of oxidative damage were also detected. N-acetylcysteine may protect from these histological and ultra-structural alterations related to oxidative stress.

Key words: liver, oxidative stress, N-acetylcysteine, sinusoidal dilatation, p53

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Introduction

Systemic production of oxidative stress may produce detrimental consequences for several organs, particularly the liver. For instance, oxidative stress has long been considered a key driving factor of many obesity-related liver diseases. Non-alcoholic fatty liver disease is thought to be associated with obesity and oxidative stress; as a result, antioxidants seem to be one of the most promising pharmacologic treatment methods.[1] In a mouse model of non-alcoholic fatty liver disease and fibrosis, altered regulation of lipid metabolism, inflammation, fibrosis, and oxidation-associated expression, along with augmented lipoperoxidation have been observed.[2] Furthermore, administration of *Crepidiastrum denticulatum* extract and its active compound chicoric acid reduced oxidative stress by upregulating antioxidant enzymes and decreasing inflammation by inhibiting pro-inflammatory cytokines and nuclear factor kappa B (NF-κB) activation. Also, oxidative stress has been postulated to play a role in the development of bisphenol A-induced liver fibrosis.[3] It is clear that oxidative stress induces the activation of liver fibrogenic cells, thus promoting the expression of fibrosis-related genes, leading to hepatic fibrogenesis.[4] Therefore, the production of systemic oxidative stress markers may adversely affect liver tissue.

It was clearly observed that extracorporeal shock wave lithotripsy (SWL) used for the management of renal stones can cause systemic oxidative stress. An experimental SWL model on 69 rats clearly revealed a significant increase of malondialdehyde (MDA) levels and a decrease of superoxide dismutase activity (SOD) in blood as markers for oxidative stress.[5] In the previous report of this experimental SWL model, a significant increase in plasma oxidative stress index (OSI) was detected through measuring plasma total antioxidant status (TAS) and total oxidant status (TOS) in blood samples of rats.[6] Some histological alterations in renal tissues were reported. In this section of the study, the possible effects of systemic oxidative stress on liver tissues were investigated. Also, as a universal antioxidant, N-acetylcysteine (NAC) can prevent apoptosis and promote cell survival by activating the extracellular signal-regulated kinase pathway.[7] It is also proposed that NAC can modify DNA and may also have a preventive role for several processes, such as reducing endothelial dysfunction, inflammation, fibrosis, and invasion. The effects of NAC on liver tissue under systemic oxidative stress were also monitored in this experimental study.

The objectives of this study were to explore the effects of SWL associated systemic oxidative stress on liver tissues and to detect the possible protective role of NAC administration.

Material and methods

Study design and groups

A total of 18 female Wistar albino rats all aged 12 weeks with a range of weight of 175–250 g were used. Animals were housed in cages with a maximum of 9 rats. The animals were kept at a temperature range of 18–20°C and a 12 h/12 h light/dark cycle.

The groups were randomly constructed among animals used in the previous study.[6] Animals were arbitrarily assigned to 3 groups stratified by weight. Group III was further divided into 2 subgroups – short-term (group III-14 days) and long-term (group III-28 days). Group I (3 rats) constituted the control animals without any SWL and NAC. Group II (5 rats) underwent SWL and received intraperitoneal saline at a dose of 1 mL/kg/day until hepatectomy as the placebo treatment. Group III (10 rats) also underwent SWL, and these rats received intraperitoneal NAC at a dose of 300 mg/kg/day for 14 (short-term subgroup, 5 rats) or 28 days (long-term subgroup, 5 rats).

The control group had no intervention at all. Rats in groups II and III received a total of 2000 shock waves that were applied to the left kidney with an amplitude of 18 kV and a rate of 60 SW/min (Stonelith-V5 Lithotripter; PCK Medical Systems, Ankara, Turkey) under general anesthesia with ketamine HCl (1 mg/kg) and xylazine HCl (10 mg/kg).

All the animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985; National Institutes of Health, Bethesda, USA). The study was approved by the Local Animal Ethics Committee. According to the strict restrictions of the ethical committee, the animal number in each group was restricted to a minimum enabling statistical analysis.

Procedure

Blood samples and liver tissues were obtained. The blood samples were centrifuged at 12,000 rpm at 4°C for 10 min and then stored at 80°C for biochemical tests. The OSI was calculated by measuring plasma TAS and TOS using a novel, colorimetric and fully automated method for measuring total antioxidant response against potent free radical reactions.[8] The tests were performed using an auto-analyzer (Beckman Coulter AU480; Beckman Coulter, Brea, USA) by using appropriate kits (Rel Assay Diagnostics, Gaziantep, Turkey). The TAS results were expressed as mmol Trolox Eqiv./L, while TOS values were expressed as μmol H_2O_2 Eqiv./L. The OSI was calculated using the formula as OSI [AU] = TOS [μmol H_2O_2 Eqiv./L]/TAS [μmol Trolox Eqiv./L].

The final procedure was performed on the 14th day in group I, group II, group III-14 days. The remaining rats

(group-III-28 days) underwent surgery on the 28th day. A midline laparotomy was performed, and blood samples were collected from the vena cava using syringe for biochemical analyses. Hepatectomy was performed, and the specimens were fixed in formalin solution, embedded in paraffin and stained with hematoxylin and eosin (H&E) for microscopic examination to document histological changes. Myeloperoxidase expression and p53 were assessed with immunohistochemistry.

The histological investigation was evaluated by a single pathologist. Five histological alterations were graded to score any hepatic injury. These parameters were sinusoidal dilatation, periportal necrosis, steatosis, cellular necrosis, and inflammatory cell infiltration. Furthermore, p53 and myeloperoxidase staining were also classified. A scoring system was separately used for all these parameters as 0 (normal morphology or no staining), 1 (mild), 2 (moderate), and 3 (severe). The mean score was calculated for each group.

Statistical analysis

Non-parametric Kruskal–Wallis and Dunn's statistical tests were used for analysis. The value of $p < 0.05$ was considered statistically significant. The statistical calculations were performed using SPSS Statistics v. 18 (SPPS Inc., Chicago, USA).

Results

Oxidative stress

The comparison of the groups clearly demonstrated that the application of renal SWL developed a systemic oxidative response (Fig. 1). Shock wave lithotripsy caused a significant rise in median TOS values from 11.12 μmol H_2O_2Eqiv./L in the control group (group I) up to 15.38 μmol H_2O_2Eqiv./L in rats which received SWL (group II) ($p < 0.05$). N-acetylcysteine administration for 14 days (group III-14 days) provided a remarkable decrease in TOS (8.58 μmol H_2O_2Eqiv./L) ($p = 0.027$). This improvement in TOS was maintained (10.92 H_2O_2Eqiv./L) on the 28th day (group III-28 days) without further decrease. As a similar trend, antioxidant status (TAS) showed a significant alteration after SWL from 1.19 μmolTroloxEqiv./L (group I) down to 0.96 μmolTroloxEqiv./L (group II) ($p = 0.006$). N-acetylcysteine administration provided an increase in TAS values on the 28th day ($p < 0.05$). As a result of these measurements, OSI demonstrated a significant rise with SWL procedure ($p < 0.05$). N-acetylcysteine administration provided a significant decrease in OSI on the 14th day ($p = 0.013$), which continued on the 28th day.

Histological findings

Sinusoidal dilatation was found to be prominent in rats receiving SWL (Table 1). While the rats in the control group had no sinusoidal dilatation at all, SWL group had a mean score of 1.17 for this parameter (Fig. 2, $p < 0.05$). This histological alteration was similarly observed in the short-term NAC group. However, the mean score decreased to 0.33 in the 28 days in the NAC group ($p = 0.043$). Similarly, periportal necrosis was prominent in group II with SWL. It was not observed in the control group (Fig. 3). The mean score was 1 in the SWL group, and this value was decreased to 0.33 both in group III-14 days and group III-28 days ($p = 0.033$). The other histological markers were similar to the control group.

With regard to the immunohistochemical staining, p53 positivity was prominent in the SWL group (Table 2, Fig. 3). The mean score was increased from 0 to 1 in control and SWL groups, respectively ($p = 0.049$). This staining return control values with NAC administration.

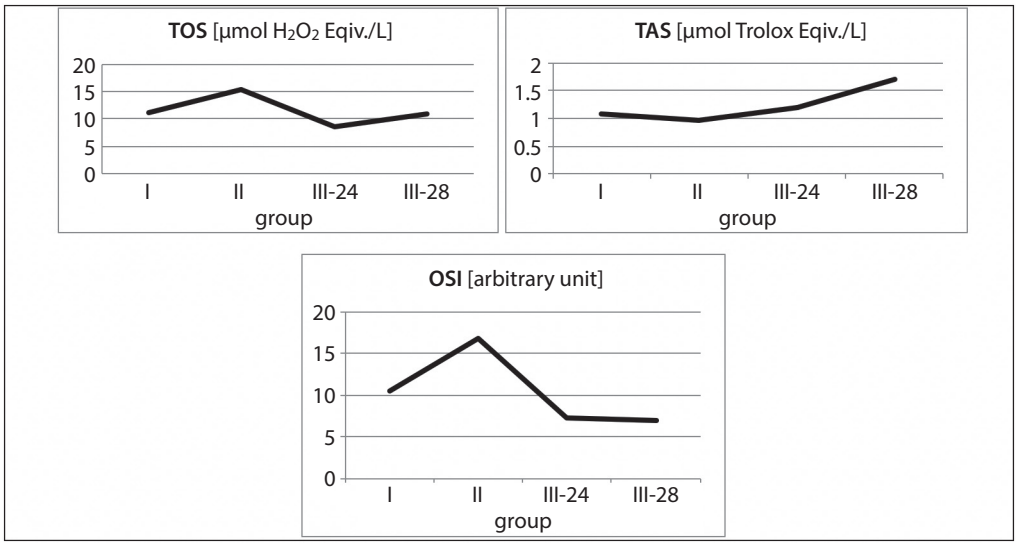

Fig. 1. The comparison of median systemic oxidative markers

group I – control group;
group II – SWL + saline;
group III – SWL + NAC 14-28 days;
TOS – total oxidant status;
TAS – total antioxidant status;
OSI – oxidative stress index;
SWL – extracorporeal shock wave lithotripsy;
NAC – N-acetylcysteine.

Table 1. Histological alterations in liver tissue

Variable	Group	n	Mean	Median	SD	Min	Max	p-value
Sinusoidal dilatation	I	3	0.00	0.00	0.37	0	1	0.043
	II	5	1.17	1.00	0.408	1	2	
	III-14 days	5	1.17	1.00	0.753	0	2	
	III-28 days	5	0.33	0.00	0.516	0	1	
Periportal necrosis	I	3	0.00	0.00	0.37	0	1	0.033
	II	5	1.00	1.00[a]	0.000	1	1	
	III-14 days	5	0.33	0.00[b]	0.516	0	1	
	III-28 days	5	0.33	0.00[b]	0.516	0	1	
Steatosis	I	3	0.00	0.00	0.000	0	0	1.000
	II	5	0.00	0.00	0.000	0	0	
	III-14 days	5	0.00	0.00	0.000	0	0	
	III-28 days	5	0.00	0.00	0.000	0	0	
Cellular necrosis	I	3	0.00	0.50	0.548	0	1	0.072
	II	5	0.50	0.50	0.548	0	1	
	III-14 days	5	1.17	1.00	0.408	1	2	
	III-28 days	5	0.50	0.50	0.548	0	1	
Inflammatory cell infiltration	I	3	0.00	0.00	0.00	0	00	0.084
	II	5	0.83	1.00	0.753	0	2	
	III-14 days	5	1.50	1.50	0.548	1	2	
	III-28 days	5	0.67	1.00	0.516	0	1	

group I – control group; group II – SWL + saline; group III – SWL + NAC; SD – standard deviation; SWL – extracorporeal shock wave lithotripsy; NAC – N-acetylcysteine; [a], [b], [ab] – different index letters indicate statistically significance for each column.

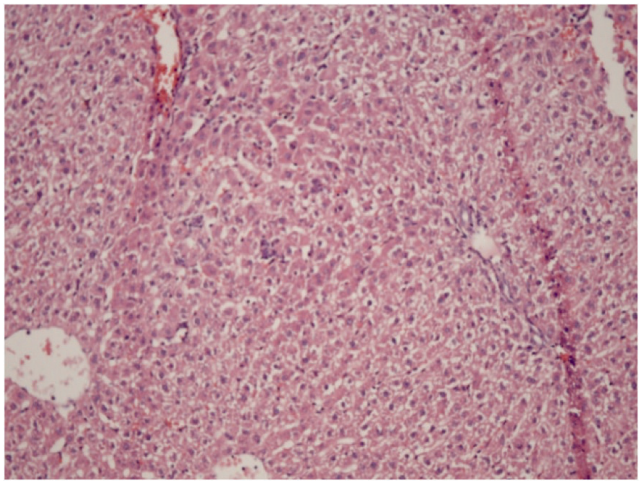

Fig. 2. Mild (grade 1) sinusoidal dilatation and lymphocyte infiltration (H&E staining, ×200 magnification)

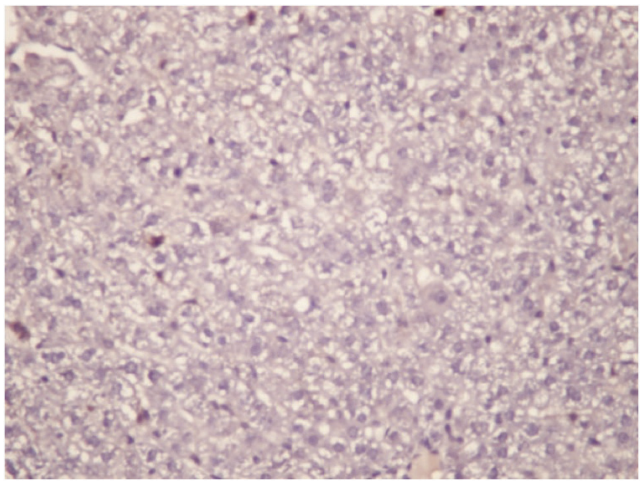

Fig. 3. Moderate (grade 2) periportal necrosis and nuclear p53 staining (H&E staining, ×400 magnification)

Although the expression of myeloperoxidase was higher in the SWL-treated rats (group II and III), the difference was not significant.

Discussion

The liver is first of all the principal detoxifying organ, acting in the clearance of waste products and toxic chemicals from the body. It also participates in almost all physiologic systems to maintain homeostasis.[9] Continuous exposure of the liver to adverse agents or conditions can cause hepatic injury. These events can then lead to inflammation and liver degeneration.

Shock wave lithotripsy remains a widely applied treatment modality for the majority of patients with renal stones.[10] However, several clinical and experimental models have clearly demonstrated that SWL is associated with increased systemic oxidative stress due to renal ischemia/reperfusion injury. A remarkable increase of MDA

Table 2. Results of immunostaining for p53 and myeloperoxidase in liver tissues

Variable	Group	n	Mean	Median	SD	Min	Max	p-value
p53	I	3	0.00	0.00[b]	0.00	0	1	0.049
	II	5	1.00	1.00[a]	0.632	0	2	
	III-14 days	5	0.33	0.00[b]	0.516	0	1	
	III-28 days	5	0.17	0.00[b]	0.408	0	1	
Myeloperoxidase	I	3	0.00	0.00[b]	0.00	0	1	0.533
	II	5	0.50	0.50	0.548	0	1	
	III-14 days	5	0.33	0.00	0.516	0	1	
	III-28 days	5	0.67	1.00	0.516	0	1	

group I – control group; group II – SWL + saline; group III – SWL + NAC; SD – standard deviation; SWL – extracorporeal shock wave lithotripsy; NAC – N-acetylcysteine; [a], [b], [ab] – different index letters indicate statistically significance for each column.

levels and a decrease of SOD in blood as markers for oxidative stress after SWL was documented in the rabbit SWL model.[5] Furthermore, it was postulated that astragalosides as a novel antioxidant agent can prevent shock wave-induced renal oxidative injury.[5] A well-designed clinical trial on 120 patients receiving SWL showed that oral antioxidant administration is associated with reduced mean serum concentration of MDA, higher levels of serum ascorbic acid and serum albumin, lower alpha-tocopherol/cholesterol ratio, and lower urinary albumin and β2 microglobulin levels.[11] The authors suggested that SWL generates free radicals through ischemia/reperfusion injury mechanism, and oral administration of antioxidants may have a protective role.[11] Similarly, SWL caused a significant rise in MDA, urine N-acetyl-beta-glucosaminidase (NAG) activity, and uric acid and white cell counts as systemic markers of oxidative stress.[12] A clinical observation also showed elevated plasma and urinary nitric oxide (NO) levels after SWL.[13] Plasma and urinary MDA levels were also remarkably elevated after SWL. Another clinical trial concluded that lipid peroxidation might be induced, and antioxidative defense mechanisms may be transiently impaired in erythrocytes after SWL, as a model of systemic consequences of SWL-induced oxidative stress.[14] All these studies indicate a systemic oxidative process after SWL. The initial results of this study also showed that SWL caused a decrease in TOS levels with subsequently higher TAS levels, indicating a remarkable increase in systemic oxidative stress index in blood samples.[6] This study also showed that NAC administration for 14 days provided a remarkable decrease in TOS, indicating a protective efficacy against oxidative stress.

An interesting finding in this trial is that systemic oxidative response after SWL is associated with some histological alterations in liver tissues. A major observation revealed that systemic oxidative stress caused a significant sinusoidal dilatation in rats receiving SWL. In the control group, no sinusoidal dilatation at all was seen. N-acetylcysteine administration has a protective role in terms of preventing sinusoidal dilatation in rats having NAC, particularly with longer administration. Alterations in hepatic sinusoids are regarded as a critical point in the development of certain liver diseases, including cirrhosis and portal hypertension.[15] It was shown that the hepatic sinusoids are the essential component of intrahepatic microcirculatory unit, where cells are intimately associated with one another and communicate through paracrine and autocrine effects.[16] Therefore, alterations in this microenvironment are related to the early steps of fibrogenesis and include sinusoidal remodeling, vasoconstriction, endothelial dysfunction, and angiogenesis.[17] Therefore, documentation of sinusoidal dilatation associated with increased oxidative status should be regarded as an important finding in this experimental study. Moreover, improvement in the sinusoidal dilatation with NAC administration indicates a possible relationship between oxidative stress and sinusoidal dilatation. It is postulated that oxidative stress plays an important role in the pathogenesis of non-alcoholic steatohepatitis and is likely involved in the progression of the disease from steatosis to non-alcoholic steatohepatitis and potentially cirrhosis.[17] In an animal model, the levels of total glutathione (GSH) and hepatic MDA were found to be increased significantly in rats with non-alcoholic steatohepatitis group.[18] It was reported that the administration of 20 mg/kg/day of oral NAC improved the level of GSH as attenuation of oxidative stress. Authors also observed a decrease in fat deposition and necroinflammation, indicating healing in liver histology caused by NAC. Similarly, periportal necrosis was also prominent in rats with increased oxidative stress after SWL. This was not observed in the control group. N-acetylcysteine also has a protective effect against developing periportal fibrosis. Periportal fibrosis is a recognized histological step in non-alcoholic steatohepatitis.[19,20]

With regard to the immunohistochemical staining, p53 positivity was prominent in the SWL group. This staining return control values with NAC administration. Silver nanoparticles administration caused a remarkable increase in the levels of MDA and total glutathione in adult

zebrafish.[21] Moreover, the mRNA levels of the oxyradical-scavenging enzymes catalase and glutathione peroxidase 1a were reduced. They also showed DNA damage by the expression of p53 protein in liver tissues. Similarly, lead significantly increased the levels of reactive oxygen species (ROS) and MDA in mice.[22] Also, severe DNA damage was obviously observed as an increased expression of p53. Therefore, all these studies including the current trial confirm that increased p53 staining may indicate oxidative stress related to ultrastructural changes in the liver. Although the expression of myeloperoxidase was higher in the SWL treated rats, the difference was not significant. The activity of this enzyme is also known to be a marker for oxidative stress.[23] The increased expression of this marker also suggests oxidative stress-related alterations in the liver.

A recent review clearly states that NAC, due to its antioxidant and anti-inflammatory roles, has important functions for liver diseases.[24]

It was shown that NAC can attenuate markers of inflammation and oxidative stress in hepatic damage. The results of both experimental and clinical trials show that supplementation of NAC in any form of administration and type of study is mostly satisfactory with promising endpoints. The current trial also confirms the protective role of NAC in liver tissue related to oxidative damage. However, clinical studies are urgently required to have a routine clinical utilization of NAC to prevent oxidative damage in liver tissue.

Conclusions

The current study showed that SWL causes systemic oxidative stress as expressed by a remarkably increased plasma oxidative stress index in blood samples of rats. The increased oxidative stress was shown to be associated with sinusoidal dilatation and periportal fibrosis in liver tissues. The expression of p53 and myeloperoxidase as immune stained markers of oxidative damage were also increased in the liver. Moreover, in this study, NAC was found to be effective in decreasing oxidative stress and in improving these histological and ultrastructural alterations. This experimental model provides important background for subsequent clinical trials on the protective role of NAC for liver diseases.

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Gender differences in variables associated with dipeptidyl peptidase 4 genetic polymorphisms in coronary artery disease

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Abstract

Background. In recent years, considerable effort has been devoted to identifying genes that contribute to the risk of coronary artery disease (CAD). Genetic factors can be used to identify individuals who have additional genetic risks. Genetic variations might contribute to cardiovascular disease differentially in men and women. Dipeptidyl peptidase-4 (*DPP-4*) may be involved in the development of atherosclerotic diseases.

Objectives. To examine the associations between genetic variations of *DPP-4* in men and women with CAD.

Material and methods. In this case-control study, blood samples of patients with angiographically documented CAD and of those without CAD were collected. We focused on the *DPP-4* gene (rs7608798 and rs3788979 polymorphisms) to assess the association of single nucleotide polymorphisms (SNPs) and the risk of CAD.

Results. We identified 1 SNP (rs3788979) that was significantly related to angiographic CAD in women (odds ratio (OR) = 2.437; p = 0.019). Moreover, the SNP (rs7608798) seemed to have a protective effect (OR = 0.291; p = 0.032). We did not find an association between CAD risk factors and *DPP-4* polymorphisms. Our study is the first to demonstrate that CAD pathogenesis is influenced by gender differences in polymorphisms in the *DPP-4* gene.

Conclusions. This study provides new information on the association of *DPP-4* polymorphisms with the risk of CAD in the Taiwanese population, especially in women. Further studies should be performed to verify this association.

Key words: women, coronary artery disease, *DPP-4* polymorphisms

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Introduction

The increasing prevalence of coronary artery disease (CAD) has become a worldwide health concern. It imposes a significant social and economic burden on society.[1,2]

Several risk factors may predispose an individual to CAD, such as smoking, high blood pressure, high cholesterol, obesity, and diabetes.[3] In recent years, considerable effort has been devoted to identifying genes that contribute to CAD risk.[4,5] Genetic factors can be used to identify people who have additional genetic risks. This raises the possibility of screening the population to detect important susceptibility loci once disease screening or an intervention becomes available. An obvious requirement for such screening would be validation through large-scale trials of the benefits of such early detection and treatment. A combination of conventional and genetic risk factors may be optimal for identifying populations at risk.

Dipeptidyl peptidase 4 (DPP-4) is an intrinsic membrane glycoprotein and a serine exopeptidase that cleaves X-proline dipeptides from the N-terminus of polypeptides. The activity of DPP-4 has received considerable attention as a therapeutic target, and DPP-4 inhibitors that extend the insulinotropic effect of glucagon-like peptide-1 are now widely used as antidiabetic drugs.[6]

The DPP-4 is expressed on the surface of several cell types[7] of a variety of tissues, including bone marrow, lung, spleen, pancreas, kidney, liver, intestinal, and epithelial cells.[7] Because of its many enzymatic activities, DPP-4 is not only involved in the pathogenesis of diabetes mellitus and glucose metabolism, but it also participates in modulating the cardiovascular system and it may be involved in the development of atherosclerotic diseases.[8–10] In vitro preclinical and clinical studies have shown that DPP-4 inhibitors may modulate atherosclerotic disease by reducing plasma lipids, suppressing inflammation and promoting vascular relaxation. However, the inhibition of DPP-4 may also exacerbate cardiovascular disease by enhancing sympathetic activation and angiogenesis.[8]

Three recent cardiovascular outcome trials of DPP-4 inhibitors showed that none of the DPP-4 inhibitors increased or decreased the predefined primary cardiovascular outcomes.[11–13] However, the main concern in the subsequent discussion has been about the higher incidence of hospitalization for heart failure seen with saxagliptin.[12] The question of why this ambiguous result in the cardiovascular outcome of DPP-4 inhibitors in human and animal studies may be explained by genetic polymorphism. Previous genetic studies revealed that polymorphisms in the defined DPP-4 loci are associated with serum lipid levels.[14] A polymorphism in the *DPP-4* gene in patients with known CAD may increase the risk of myocardial infarction (MI). It is associated with decreased plasma DPP-4 levels in patients with MI.[15] Due to the diverse results, more comprehensive study of the *DPP-4* gene polymorphism is needed. However, there is still limited data to confirm an association between *DPP-4* gene variants and CAD in Asian populations, especially in gender-specific gene variants. In this study, we hypothesized that the polymorphisms of the *DPP-4* gene might contribute differently in men and women. Therefore, we conducted a case-control study to examine the associations between single nucleotide polymorphisms (SNPs) in patients with or without CAD in a Taiwanese population.

Material and methods

This hospital-based case-control study recruited 607 patients who had undergone coronary angiography from the Chung Shan Medical University Hospital in Taichung, Taiwan. Two groups were identified for this study. For CAD group, a diagnosis of CAD was made for patients who had undergone coronary angiography and received percutaneous coronary angioplasty according to the recommendations for percutaneous coronary intervention. For the control (non-CAD) group, all participants were recruited from the same hospital; they had undergone coronary angiography, but the findings were negative for CAD, defined as coronary flow reserve is increased in blood flow in response to metabolic or pharmacological stimuli and a normal coronary angiogram without any suspected atherosclerosis. The patients' clinic characteristics were verified with a medical history review.

Diabetes mellitus was defined as having a fasting blood sugar level ≥126 mg/dL or taking any oral or injected antidiabetic drugs. Dyslipidemia was defined as having low-density-lipoprotein cholesterol (LDL-C) level >100 mg/dL or taking any anti-lipid lowering drugs. Hypertension was defined as having a systolic blood pressure (SBP) >130 mm Hg and a diastolic blood pressure (DBP) >80 mm Hg or taking any anti-hypertension drugs.

Whole-blood specimens collected from all patients were placed in tubes containing EDTA; they were immediately centrifuged and then stored at –80°C. The study protocols were approved by the institutional review of the Taichung Chung Shan Medical University Hospital. All methods were carried out in accordance with the approved guidelines and all participants provided written informed consent before participating in the study.

The allelic discrimination of the *DPP-4* rs7608798 and rs3788979 polymorphisms was assessed using an ABI StpOne Real-Time PCR System (Applied Biosystems, Foster City, USA), SDS v. 3.0 software (Applied Biosystems) and the TaqMan™ Genotyping assays.[17–19] The final volume for each reaction was 5 µL, containing 2.5 µL of TaqMan Genotyping Master Mix, 0.125 µL of TaqMan probe mix and 10 ng of genomic DNA. The reaction conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

We compared the differences in demographic characteristics between the CAD patients and the non-CAD patients

using the χ^2 test and Fisher's exact test. The odds ratios (ORs) with 95% confidence intervals (95% CIs) were estimated using logistic regression models between genotype frequencies and CAD risk, as well as clinical characteristics after controlling for other covariates. Values of $p < 0.05$ were considered statistically significant. The data was analyzed using SPSS statistical software v. 9.1, 2005 (SAS Institute Inc., Cary, USA).

Results

A total of 607 patients were recruited. Statistical analyses of the demographic characteristics of both the case group (391 patients with CAD) and the control group (216 patients without CAD) are shown in Table 1. Coronary artery disease was more common in men and in those with a history of hypertension and diabetes mellitus. Furthermore, gender, tobacco consumption and co-morbid conditions of hypertension or diabetes mellitus were significantly associated with CAD risk ($p < 0.05$ for each). To decrease the possible interference of several co-morbid factors, the OR was adjusted to control for risk related to gender, tobacco use and co-morbidities in each comparison using multiple logistic regression models.

The distribution frequency of *DPP-4* genotypes in both controls and CAD patients are presented in Table 2. The alleles with the highest distribution frequency were heterozygous A/G for rs7608798 and heterozygous G/A for rs3788979. There was no significant difference with respect to the rs7608798 and rs3788979 polymorphisms of *DPP-4* between the controls and the patients with CAD.

The gender distribution frequencies are presented in Tables 3 and 4. Heterozygous G/A rs3788979 in *DPP-4* was associated with CAD risk (OR = 2.437; p = 0.019) only in the female population. In this group, homozygous G/G rs7608798 seemed to have a protective effect (OR = 0.291; p = 0.032).

We further investigated the effects of the polymorphic genotypes of *DPP-4* on the clinical status of CAD (Table 5). No apparent significant association between *DPP-4* gene polymorphism and traditional CAD risk factors were found, except for elevated cardiac enzymes.

Discussion

Two SNPs in the *DPP-4* gene were found to have an association with CAD risk in our study: one was associated with a decreased risk in women, while the other was associated with an increased risk. The rs7608798, an intron variant in the *DPP-4* gene, was found to be associated with a decreased risk of CAD in the Chinese population for the first time. In contrast, rs3788979, also located in an intronic region of the *DPP-4* gene, increased the risk of CAD and showed a strong association signal for CAD in the *DPP-4* gene. This finding might seem to be partly consistent with previous observations of positive correlations between the rs3788979 SNP and the risk of MI in patients with atherosclerosis and of CAD in patients with diabetes.[15] Our study extends the findings to those

Table 1. Demographic characteristics and clinical parameters for controls (non-CAD) and patients with CAD

Parameter	Control group (n = 216) mean ±SD	CAD group (n = 391) mean ±SD	p-value
Age [years]	66.43 ±12.17	65.73 ±11.21	0.474
BMI [kg/m^2]	25.05 ±3.74	25.55 ±4.40	0.159
Parameter	**n (%)**	**n (%)**	**p-value**
Age >65 years	126 (58.3%)	210 (53.7%)	0.272
Male gender	130 (60.2%)	288 (73.7%)	0.001
Family history of CAD	49 (22.7%)	82 (21.0%)	0.623
Tobacco consumption	76 (35.2%)	171 (43.7%)	0.040
More than 3 risk factors of CAD	101 (46.8%)	231 (59.1%)	0.004
Atrial fibrillation	57 (26.4%)	39 (10.0%)	<0.001
Hypertension	134 (62.0%)	288 (73.7%)	0.003
Diabetes mellitus	73 (33.8%)	165 (42.2%)	0.042
Dyslipidemia	83 (39.0%)	158 (40.6%)	0.693
Recent (<24 h) severe angina	126 (58.3%)	285 (73.3%)	<0.001
Elevated cardiac markers	81 (37.5%)	222 (56.8%)	<0.001
Stroke	27 (13.4%)	42 (11.3%)	0.445
Congestive heart failure	73 (35.3%)	86 (22.8%)	0.001

The data is presented as numbers and percentages with χ^2 test/Fisher's exact test or means ± standard deviation (SD) with independent two-sample t-test. CAD – coronary artery disease; BMI – body mass index; $p < 0.05$ is statistically significant.

Table 2. Distribution frequency of *DPP-4* genotypes in 216 controls (non-CAD) and 391 patients with CAD

Genotype	Control group (n = 216)	CAD group (n = 391)	Adjusted OR (95% CI)	p-value
		DPP-4 rs7608798		
AA	85 (39.4%)	153 (39.1%)	1.00	–
AG	98 (45.4%)	195 (49.9%)	1.105 (0.772–1.584)	0.585
GG	33 (15.3%)	43 (11.0%)	0.724 (0.428–1.224)	0.228
AG+GG	131 (60.6%)	238 (60.9%)	1.009 (0.718–1.418)	0.957
		DPP-4 rs3788979		
GG	61 (28.2%)	91 (23.3%)	1.00	–
GA	106 (49.1%)	215 (55.0%)	1.360 (0.912–2.027)	0.131
AA	49 (22.7%)	85 (21.7%)	1.163 (0.721–1.876)	0.536
GA+AA	155 (71.8%)	300 (76.7%)	1.297 (0.889–1.893)	0.176

The odds ratios (OR) with their 95% confidence intervals (95% CI) were estimated with logistic regression; CAD – coronary artery disease; $p < 0.05$ is statistically significant.

Table 3. *DPP-4* genetic variation frequencies and CAD risk association among men

Genotype	Control group (non-CAD) (n = 130)	CAD group (n = 288)	OR (95% CI)	p-value
		DPP-4 rs7608798		
AA	54 (41.5%)	112 (38.9%)	1.00	–
AG	56 (43.1%)	138 (47.9%)	1.188 (0.758–1.862)	0.452
GG	20 (15.4%)	38 (13.2%)	0.916 (0.487–1.722)	0.786
AG+GG	76 (58.5%)	176 (61.1%)	1.117 (0.732–1.702)	0.608
		DPP-4 rs3788979		
GG	37 (28.5%)	75 (26.0%)	1.00	–
GA	66 (50.8%)	150 (52.1%)	1.121 (0.688–1.828)	0.646
AA	27 (20.7%)	63 (21.9%)	1.151 (0.633–2.094)	0.645
GA+AA	93 (71.5%)	213 (74.0%)	1.130 (0.711–1.795)	0.605

The odds ratios (OR) with their 95% confidence intervals (95% CI) were estimated with logistic regression; CAD – coronary artery disease; $p < 0.05$ is statistically significant.

Table 4. *DPP-4* genetic variation frequencies and CAD risk association among women

Genotype	Control group (n = 86)	CAD group (n = 103)	OR (95% CI)	p-value
		DPP-4 rs7608798		
AA	31 (36.0%)	41 (39.8%)	1.00	–
AG	42 (48.8%)	57 (55.3%)	1.026 (0.556–1.895)	0.934
GG	13 (15.2%)	5 (4.9%)	0.291 (0.094–0.902)	0.032*
AG+GG	55 (64.0%)	62 (60.2%)	0.852 (0.472–1.539)	0.596
		DPP-4 rs3788979		
GG	24 (27.9%)	16 (15.5%)	1.00	–
GA	40 (46.5%)	65 (63.1%)	2.437 (1.157–5.135)	0.019*
AA	22 (25.6%)	22 (21.4%)	1.500 (0.631–3.565)	0.359
GA+AA	62 (72.1%)	87 (84.5%)	2.205 (1.033–4.288)	0.038*

Odds ratios (OR) and 95% confidence intervals (95% CI) of female CAD patients associated with genotypic frequencies of *DPP-4*; CAD – coronary artery disease; * $p < 0.05$ is statistically significant.

Table 5. Comparison of demographics and co-morbidities between 2 genotypes of *DPP-4* rs7608798 in the group of CAD patients

Variable	DPP-4 rs7608798 AA (n = 153)	DPP-4 rs7608798 AG+GG (n = 238)	OR (95% CI)	p-value
Atrial fibrillation	15 (9.8%)	24 (10.1%)	1.032 (0.523–2.036)	0.928
More than 3 risk factors of CAD	90 (58.8%)	141 (59.2%)	1.018 (0.673–1.538)	0.934
Age >65 years	78 (51.0%)	132 (55.5%)	1.197 (0.797–1.799)	0.386
Family history of CAD	25 (16.3%)	57 (23.9%)	1.612 (0.957–2.717)	0.071
Hypertension	110 (71.9%)	178 (74.8%)	1.160 (0.733–1.834)	0.526
Diabetes mellitus	68 (44.4%)	97 (40.8%)	0.860 (0.570–1.296)	0.471
Tobacco consumption	65 (42.5%)	106 (44.5%)	1.087 (0.722–1.638)	0.689
Dyslipidemia	64 (42.4%)	94 (39.5%)	0.887 (0.586–1.343)	0.572
Recent (<24 h) severe angina	114 (75.0%)	171 (72.2%)	0.864 (0.543–1.374)	0.536
Elevated cardiac markers	97 (63.4%)	125 (52.5%)	0.639 (0.421–0.968)	0.034*
Stroke	17 (11.7%)	25 (11.0%)	0.927 (0.482–1.784)	0.821
Congestive heart failure	27 (18.5%)	59 (25.5%)	1.512 (0.906–2.522)	0.112

The odds ratios (OR) with their 95% confidence intervals (95% CI) were estimated with logistic regression; CAD – coronary artery disease; *p < 0.05 is statistically significant.

without diabetes, but we only found this positive correlation between the rs3788979 SNP and the risk of CAD in female patients.

In dominant inheritance mode, the carriers of genotype GA at the rs3788979 SNP had an increased risk of CAD in comparison with the carriers of AA after adjusting for other CAD risk factors.

Cardiovascular disease constitutes the leading cause of mortality, not only among men but also among women.[16] Gender differences in the incidence of CAD have attracted the interest of many clinical researchers. Some gender differences have been documented in regard to traditional cardiovascular risk factors. Age, hypertension, total cholesterol, and LDL-C have a great influence on men, while menopause, systolic arterial hypertension, smoking, diabetes, and triglyceride and high-density-lipoprotein cholesterol (HDL-C) levels are the main factors in women.[17] Genetic variations might not explain all of these disparities, but they could provide some further information on gender differences regarding the risk of cardiovascular disease.

Although the precise mechanisms that link DPP-4 and cardiovascular disease are not completely understood, various studies have suggested that DPP-4 inhibitors may also have cardiovascular protective effects.[18–20] On the other hand, some animal studies have shown that DPP-4 deficiency may cause dyslipidemia,[21] and another study revealed that elevated DPP-4 levels accelerate diet-related vascular aging and atherosclerosis.[22]

Moreover, a recent study reported that a genetic deficiency of DPP-4 improves cardiac function,[23] whereas inhibition of DPP-4 induced cardiac hypertrophy and impaired cardiac function and reduced monocyte migration to atherosclerotic plaque in response to tumor necrosis factor α (TNF-α) and soluble DPP-4[24]; another study reported that it also upregulates expression of adiponectin, which causes an anti-inflammatory effect,[25] indicating that DPP-4 may exert many functions, both positive and negative, directly and indirectly, on cardiovascular districts.

There were 2 animal studies which supported the role of DPP-4 in atherosclerosis among females. One showed that DPP-4 plays a role in the development of Western-diet-induced aortic stiffening, vascular oxidative stress, endothelial dysfunction, and vascular remodeling in female mice.[26] Another study, by inhibiting DPP-4, reduced Western-diet-induced, myocardial-cardiac-restricted overexpression of TRAF3-interacting protein 2 and further induced myocardial inflammation and fibrosis in female mice.[27]

We did not find an association of dyslipidemia or other traditional risk factors in the gene variants we studied. Further studies should be conducted in order to verify this association. However, the available literature verifies that the *DPP-4* gene is highly polymorphic among different populations and that some *DPP-4* loci are related to higher plasma lipid levels and cardiovascular risk.

Another study demonstrated that *DPP-4* variants in Chinese type 2 diabetes patients were associated with lipid levels. Patients with the rs7608798 SNP exhibited significantly lower serum TG levels in our study and, in the subgroup of women, a lower risk of CAD. For rs3788979, serum TG levels were significantly different among genotypes in our study, and the subgroup of women had a higher risk of CAD. Thus, some women with CAD may have higher lipid levels and cardiovascular risk due to their *DPP-4* polymorphisms. On the other hand, some women may have lower lipid levels and cardiovascular risk thanks to their *DPP-4* polymorphisms.

This study had several limitations. Firstly, we only investigated 2 genetic variants for each gene, so we cannot exclude the possibility that other polymorphisms are

associated with CAD risk. Another limitation of this study is the lack of a complete plasma lipid panel and allowing the use of statins. Furthermore, the control group consisted of patients with symptoms of CAD and negative coronarography findings, but microvessel disease cannot be excluded. It is unknown how the *DPP-4* genetic variants affect the function of DPP-4. The biological mechanisms on how DPP-4 influences CAD have not been thoroughly elucidated, although some mechanism has been hypothesized in several animal and human studies. Because of our relatively small sample size, multiple independent studies with larger sample populations are required to validate our findings.

Conclusions

Our study is the first to demonstrate that CAD pathogenesis is influenced by gender differences in polymorphisms of the *DPP-4* gene. This study provides new information on the association of DPP-4 polymorphisms with the risk of CAD in the Taiwanese population. Further studies should be performed to verify this association.

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Cerebral tissue oxygenation during cranial osteopathic CV4 procedure in newborns

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Abstract

Background. The cranial osteopathic manipulative medicine has been shown to alter regional cerebral tissue oxygenation ($cStO_2$) in adult patients; however, there are no reports regarding the neonatal population.

Objectives. To assess the influence of compression of the 4th ventricle (CV4) osteopathic procedure on $cStO_2$ in neonates.

Material and methods. Thirty-one patients born between 25 and 39 weeks of gestation were screened for inclusion in the neonatal unit. Twenty-two infants presenting with hyperstimulation of autonomous nervous system (ANS) according to the Neonatal Behavioral Assessment Scale were enrolled in the study. Near-infrared spectroscopy was used for continuous $cStO_2$ monitoring; pulse oximeter oxygen saturation (SpO_2) and heart rate (HR) measured with pulse oximetry were simultaneously monitored 10 min before CV4, during the therapy and 10 min after it was stopped.

Results. Patients' condition remained stable throughout the study. There were no significant differences in the mean $cStO_2$ values recorded before (69 ±8%), during (69 ±8%) and after CV4 (70 ±8%; $p > 0.05$). Mean SpO_2 was almost constant during the study (96 ±4% before, 95 ±3% during and 95 ±4% after the intervention). Heart rate was also stable pre-, during and post-therapy (153 ±21 min, 151 ±18 min and 151 ±20/min, respectively).

Conclusions. Compression of the 4th ventricle osteopathic procedure does not influence the $cStO_2$ in newborns. This method seems to be well-tolerated but its clinical efficacy needs to be further investigated in this group of patients.

Key words: neonates, cerebral oxygenation, osteopathic procedure

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Introduction

Immaturity of the nervous system is an important issue related to the pathophysiology and clinical care of preterm infants, as the autonomic nervous system (ANS) development is the most dynamic in the 3rd trimester of pregnancy.[1] Every year about 15 million infants are born preterm worldwide, including an estimated 25,000 neonates in Poland.[2,3] Preterm infants may require early developmental stimulation to alleviate the neurodevelopmental delay resulting from incomplete intrauterine development.[4] Methods such as Neurodevelopmental Treatment (NDT) Bobath therapy has become a standard of care, and it seems that the application of selected osteopathic procedures could also be considered.[4] Compression of the 4th ventricle (CV4) is an osteopathic technique used to influence the ANS.[5] An altered sympathetic–vagal balance reflecting the heart rate (HR) has been reported after application of this procedure.[6] In particular, increased parasympathetic and decreased sympathetic activity were observed.[7] Although CV4 has been practiced for over 80 years in adults (e.g., decreasing anxiety and relieving pain), it is still perceived as innovative in various aspects, and the knowledge regarding its effects in neonates is limited.[8,9] The procedure is easy to apply, short and seems well-tolerated by pediatric patients.[10] It is advised for the specialists who are aiming to apply the procedure to complete a five-year course rather than 3 weekend-long chiropractic training. In contrast to methods such as chiropractic techniques performed on the neck or spinal manipulative therapy, there were no safety concerns reported for osteopathic techniques.[11,12] According to previous observations, application of these methods may reduce the length of hospitalization of preterm newborns and lower the occurrence of gastrointestinal symptoms.[11,13–16] While osteopathic procedures will not replace established physiotherapeutic interventions in the newborn, they might be considered as supplementary or alternative methods. For example, CV4 has an advantage over NDT Bobath technique, because it requires less maneuvers and holding. Compression of the 4th ventricle procedure may be performed by experienced physical therapists and osteopaths according to the standards described in the literature and definitions established by the osteopathic medical profession of Educational Council on Osteopathic Principles (ECOP).[8,17–19] The therapist places one hand in the palm of the other, so that the thenar eminences are laid parallel, medial to the lateral angles of the occipital squama, next to occipitomastoid suture (Fig. 1). The weight of the patient's head rests on the thenar eminences of the therapist who applies gentle compression that leads to the approximation of lateral angles of occipital squama. The procedure is continued until a reduction in "cranial rhythm", defined as specific bone motion, is detected.[8,9,20] It is followed by the patients' cranial bone motion, which resembles the initial cycle.[8,9,20]

Compression of the 4th ventricle procedure was reported to reduce pain in infantile colic and otitis media.[20,21] It was also observed that CV4 decreases HR and systolic blood pressure (SBP) in patients with stage 1 hypertension, most likely due to altered sympathetic–vagal balance.[22] The technique was shown to alter regional cerebral tissue oxygen saturation ($cStO_2$) measured using near-infrared spectroscopy (NIRS) in the adult patients; however, there are no reports regarding the neonatal population.[23] The change in the $cStO_2$ is not always desirable, because it may be associated with reduced cerebral blood flow due to vasoconstriction, which was suggested in the previous studies.[23] This was proposed as a plausible explanation by Shi et al., assuming that the observed decrease in $cStO_2$ during CV4 technique in adults was most likely due to reduced oxygen delivery resulting from depression of cerebral blood flow.[23]

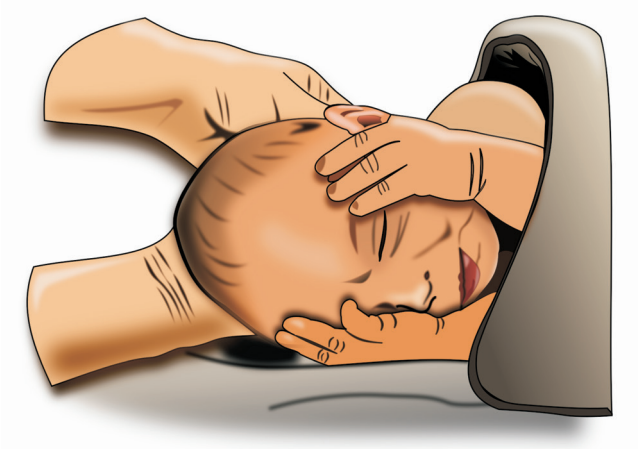

Fig. 1. Compression of the 4th ventricle procedure

The aim of this pilot study was to assess the influence of CV4 on $cStO_2$ in neonates undergoing the procedure. We focused on the following research questions:

1. Is $cStO_2$ altered by the CV4 procedure?
2. Are $cStO_2$ values within the reference range during and after CV4?
3. Are HR and SpO_2 changing during and after intervention?

Material and methods

During the study period, 41 infants were hospitalized in the neonatal unit. Thirty-one inborn infants (15 boys and 18 girls) born between 25 and 39 weeks of gestation (mean 31 ±4 weeks standard deviation (SD)) were screened for inclusion at 32–39 weeks postmenstrual age and 22 patients (8 boys and 14 girls) were enrolled. Mean birth weight of infants was 2300 ±920 g. Mean postconceptional age in the time of described intervention was 36 ±2 weeks. Patients' characteristics were summarized in Table 1. Patients' autonomic nervous system items were assessed using Neonatal Behavioral Assessment Scale (NBAS) on enrollment.[24,25] The NBAS was used for enrollment because it is a noninvasive, painless method, which enables

Table 1. Patients' characteristics

Parameter	Value
Week of gestation (mean; SD)	31; 4
Postmenstrual age at enrollment [weeks] (mean, SD)	36; 2
Birth weight [g] (median; IQR)	1529; 1140–2010
Body weight at enrollment [g] (median; IQR)	2190; 1860–2540
NBAS ANS (mean, SD)	4.4; 1.7

NBAS – Neonatal Behavioral Assessment Scale; ANS – autonomous nervous system; SD – standard deviation; IQR – interquartile range.

the assessment of ANS, habituation, reflexes, and even neurobehavior in infants until 8 weeks of life. We included stable infants with signs of hyperstimulation of ANS cluster in NBAS with more than 2 points in "tremulousness and startles" and score different (lower or higher) than 5 in "lability of skin color", which meant that they presented with tremors, startles and changes in skin color.[24–26] Infections, encephalopathy and congenital defects were exclusion criteria. Main causes for not including screened patients were unstable general condition (e.g., frequent desaturations) or insufficient NBAS scoring.

Written consent was obtained before the procedure from parents or legal guardians. Institutional Ethical Review Board at Poznan University of Medical Sciences (Poland) approved the study (decision No. 1009/18).

During the CV4 procedure, physiotherapist stood behind the infant, held the occipital bone and carefully approximated the lateral squama of the occipital bone towards the posterior occipital convexity and took the cranium into extension. The physiotherapist held this position for about 3–5 min until the cranial base was motionless and released cranium when its movement was noted again.[8,18,23] Compression of the 4th ventricle procedure was administrated only once by an experienced and certified physiotherapist according to established standards.[5] The neonatologist supervised each procedure, carried out monitoring and was observing for complications possible (e.g., apnea), although not expected based on previous reports.[13–15,27] All preterm infants were in stable condition throughout the study. No episodes of apnea, bradycardia or desaturation were observed while CV4 was performed.

Heart rate, peripheral oxygen saturation (SpO_2) and $cStO_2$ were continuously monitored 10 min before CV4, during the procedure and 10 min afterwards; this approach was similar to previous studies assessing short-term physiological effects of CV4.[8,9,23] The device used to measure both the HR and SpO_2 was Nellcor OxiMax N-600x (Coviden, Minneapolis, USA) placed on the neonate's foot. Cerebral tissue oxygenation was monitored with INVOS 5100C oximeter (Medtronic, Minneapolis, USA). The sensors were placed on the forehead.

Analyzed parameters were compared at the following time points: 1) 10 min, 5 min, 1 min, and 30 s intervals before CV4; 2) during the procedure; 3) 30 s, 1 min, 5 min, and 10 min.[8,18,23] Artifacts were removed manually and mean values of parameters at each time point were calculated based on 1 min of stable, continuous signal. Statistical analysis was performed using STATISTICA v. 13 software (StatSoft, Inc., Tulsa, USA). To describe experimental results, variables were represented as mean ± standard deviation (SD). Single-factor repeated-measures one-way analysis of variance (ANOVA; univariate tests results) for dependent variables was used, with Tukey's honestly significant difference (HSD) post hoc test.

Results

No significant differences were found between mean values and SD of $cStO_2$ before, during and after CV4 (Fig. 2). No $cStO_2$ desaturations below hypoxic threshold for Invos neonatal sensor (63%) were observed and mean values of $cStO_2$ were within a range considered as normal for the age group.[28,29] No statistically significant differences in mean SpO_2 value were observed before, during and after CV4 (Fig. 3). Mean HR values were also similar before, during and after CV4 (Fig. 4).

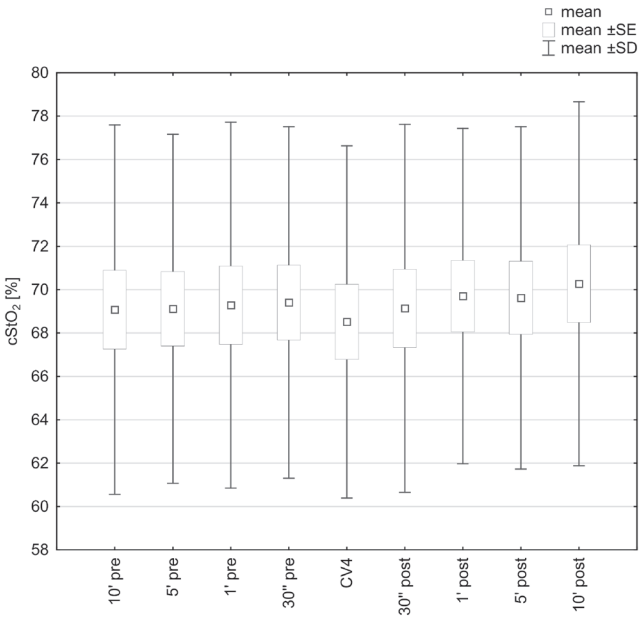

Fig. 2. Cerebral tissue oxygen saturation (%) before ("pre"), during and after ("post") CV4 procedure

Discussion

Patients in neonatal units are exposed to stressful diagnostic and therapeutic procedures, which may be of particular significance in preterm infants. Among efforts to alleviate these effects, CV4 may be considered to decrease stress and ANS hyperstimulation. In our study, NBAS ANS cluster assessment was performed at enrollment to investigate CV4 with a similar profile of hyperstimulation at baseline. The NBAS was firstly designed for full term;

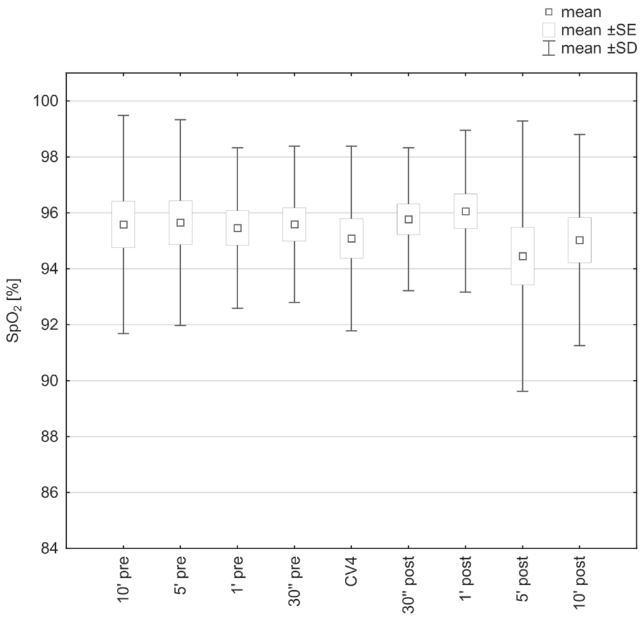

Fig. 3. Peripheral oxygen saturation (%) before ("pre"), during and after ("post") CV4 procedure

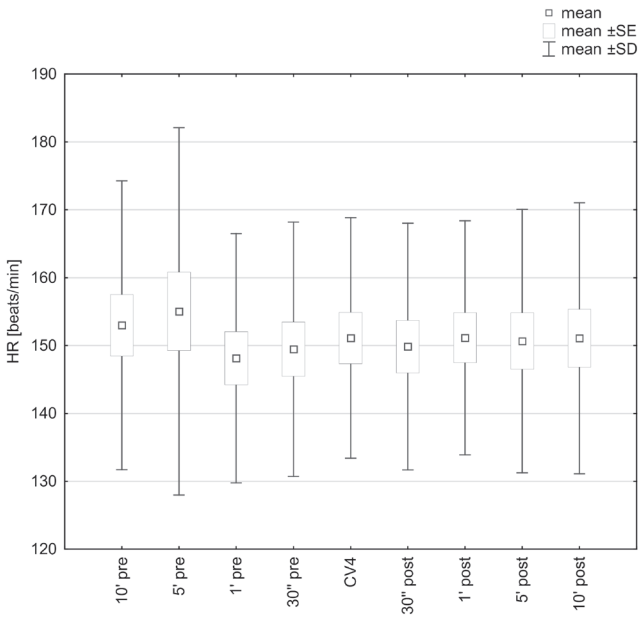

Fig. 4. Heart rate [beats/min] before ("pre"), during and after ("post") CV4 procedure

however, according to the available literature, this scale has also been used for very preterm infants.[26] In previous reports, CV4 was associated with reduced norepinephrine levels, improvement of sleep quality and latency, and pain relief in adult patients. Significant reduction in crying and sleep improvement has been shown in infants suffering from colic. Treated infants required less parental attention than the untreated group.[16,21]

An altered sympathetic–vagal balance, as seen in the HR, has been reported after use of the CV4 technique.[6] Increased parasympathetic and decreased sympathetic activity was observed.[22] The CV4 was found to decrease HR and decrease systolic blood pressure.[22] In the present study, we did not find any significant changes in HR. No significant effects on SpO_2 were observed either, which is similar to findings in adults reported by of Shi et al.[23]

According to the previous reports, CV4 may influence the nervous system. Application of this procedure was associated with the reduction of sleep latency or even altered electroencephalography tracing.[5] To our knowledge, this is the first study assessing the potential influence of CV4 on $cStO_2$ in the preterm infants. The procedure was shown to decrease $cStO_2$ in adults. It was suggested that these findings might have been caused by a reduction of the cerebral blood flow. According to a proposed theory, enhancing cranial extension and discouraging cranial flexion could create intracranial force to facilitate venous outflow from the dural sinuses into the internal jugular vein and to decrease internal carotid arterial blood flow.[23] However, in the presented study, no statistically significant differences in $cStO_2$ before, during and after single CV4 were found. Perhaps more pronounced effects of CV4 might be observed in neonates in worse clinical condition or with repeated procedures, as some authors suggest the impact to be greater in patients with excessive intracranial pressure or volume with repeated therapy bouts or multiple therapy sessions.[23] Recorded $cStO_2$ values were not only above the hypoxic threshold for the sensor, but they were also comparable throughout the study. No complications were observed during CV4, which is in accordance with previous studies regarding osteopathic treatment in infants.[13–16,21] Together with stable HR, SpO_2 and no apneic events, this seems to suggest that CV4 is a safe procedure in preterm infants.

Limitations of the study include a relatively small group of patients and a limited number of physiological variables studied. Among the strengths of this study, it should be highlighted that, in contrast to the previous studies, the patient's ANS status was assessed at baseline and only infants with clearly defined ANS hyperstimulation were enrolled.

Compression of the 4th ventricle seems to be a well-tolerated osteopathic procedure in neonates with no significant impact on $cStO_2$. Further investigations are needed to assess the potential clinical benefits of this procedure.

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Excess body fat increases the accumulation of advanced glycation end products in the skin of patients with type 1 diabetes

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Abstract

Background. The process of protein glycation described by Brownlee et al. is a crucial pathogenic mechanism in the development of chronic complications of diabetes.

Objectives. To assess advanced glycation end products (AGEs) in the skin of patients with type 1 diabetes (DM1) and excess body fat (EBF) accumulation.

Material and methods. The study group consisted of 227 DM1 patients (121 women and 106 men) whose mean age was 31 ±9.2 years; the mean duration of diabetes was 12 ±7.7 years; and the mean HbA1c was 8.9 ±1.8%. The inclusion criteria were as follows: age 18–65 years, DM1, and lack of acute inflammations and uncontrolled chronic diseases. The exclusion criteria were: anemia (hemoglobin (Hb) <11 g/dL), chronic kidney disease (CKD) (glomerular filtration rate (eGFR) <30 mL/min/1.73 m^2) and elevated aminotransferase levels (more than twice the upper normal limits). Total adipose tissue content was assessed using the electrical bioimpedance method, with the Tanita BC-418 MA analyzer (Tanita Corp., Tokyo, Japan). The Tanita ViScan AB 140 (Tanita Corp.) was used to evaluate visceral fat tissue (VTF). The content of glycation end products in the skin was assessed using a DiagnOptics AGE Reader device (type 214D00102; DiagnOptics, Groningen, the Netherlands).

Results. The group with normal body fat (NBF) consisted of 123 subjects, whereas 104 subjects had EBF. No significant statistical differences were found between the NBF and EBF groups with regard to age, duration of diabetes, current HbA1C value, and tobacco use. A significantly higher AGE score was observed in the EBF group.

Conclusions. Increased body fat affects the amount of AGE in the skin, which correlates with a higher risk of developing chronic diabetes complications.

Key words: advanced glycation end products, type 1 diabetes, excess body fat

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Introduction

The process of protein glycation, described by Brownlee et al. in the 1980s, is a crucial pathogenic mechanism in the development of chronic complications of diabetes.[1] Permanent, irreversible advanced glycation end products (AGEs) characterized by a brownish coloration and specific spectrophotometric properties (specific wavelength fluorescence) are created as a result of a non-enzymatic Millard reaction. They show substantial immunogenicity, as well as the ability to bind with certain types of cells through membrane receptors.[2] A correlation between protein glycation and micro- and macro-angiopathic diabetic complications has been observed.[3,4] Furthermore, AGEs and increased expression of their receptors for advanced glycation end products (RAGEs) are directly correlated with loss of vessel wall elasticity, crystalline aggregations in the eye, disintegration of endothelial cells, and increased thickness of epicardial fat tissue.[5,6]

The number of AGEs in an organism can be evaluated directly by assessing their concentration in blood, and indirectly using their spectrophotometric properties – skin autofluorescence (SAF) caused by the accumulation of protein glycation products. Evaluating the accumulation of AGEs in the skin based on SAF is a straightforward, non-invasive method that gives objective and reproducible results.[7] Elevated AGEs in the skin in patients with diabetes are related to an increased risk of cardiovascular complications and are a better death predictability factor than glycated hemoglobin (HbA1c) or lipid parameters.[8]

The use of intensive functional insulin therapy (IFI) in the treatment of type 1 diabetes (DM1) decreases the risk of micro- and macroangiopathy.[9] Sometimes the use of IFI is related to an increase in body mass and insulin resistance which, as a result, leads to the typical characteristics of metabolic syndrome.[10] Eighteen years after the Pittsburgh Epidemiology of Diabetes Complications Study (EDC), an increased prevalence of overweight and obesity was observed.[11] According to the World Health Organization (WHO) definition, metabolic syndrome was recognized by McGill et al. in 15% of DM1 patients; according to the International Diabetes Federation (IDF) definition, Uruska et al. found it in 20% of patients with DM1.[12,13] The highest frequency of metabolic syndrome (38% of women and 40% of men with DM1) was noted according to the National Cholesterol Education Program (NECP) criteria.[14] Observations have confirmed a relationship between metabolic syndrome and micro- and macroangiopathy in patients with DM1.[14]

The aim of the study was to assess AGEs in the skin of patients with DM1 and excess body fat (EBF) accumulation.

Patients and methods

The study group consisted of 227 DM1 patients (121 women and 106 men) treated in the Department of Internal Medicine and Diabetology at the Poznan University of Medical Sciences (Poland). Their mean age was 31 ±9.2 years; the mean duration of diabetes was 12 ±7.7 years. The study group presented poorly controlled diabetes; the mean HbA1c was 8.9 ±1.8%. The detailed characteristics of the study group are presented in Table 1. All the patients gave written informed consent to participate in the study, which was approved by the Bioethics Committee of the Poznan University of Medical Sciences (approval No. 1040/10).

Table 1. Characteristics of the study group. The results are presented as numbers and percentages as well as medians and interquartile ranges (IQR)

Characteristics of the study group	n = 227
Gender, F/M	121/106
Age [years]	29 (23; 36)
Duration of diabetes [years]	10 (6.0; 16)
HbA1c (%)	8.7 (7.7–9.6)
Creatinine [µmol/L]	74.3 (65.4; 85.7)
ALT [U/L]	17 (12; 25)
AST [U/L]	18 (15; 23)
TC [mmol/L]	5.1 (4.4; 6.0)
TG [mmol/L]	1.2 (0.9; 1.5)
HDL-C [mmol/L]	1.6 (1.3; 1.9)
LDL-C [mmol/L]	2.8 (2.4; 3.5)
Retinopathy, n (%)	74 (32.5)
Nephropathy, n (%)	24 (10.6)
Neuropathy, n (%)	39 (17.1)

ALT – alanine aminotransferase; AST – aspartate aminotransferase; HbA1c – glycated hemoglobin A1c; HDL-C – high-density-lipoprotein cholesterol; LDL-C – low-density-lipoprotein cholesterol; TG – triglycerides; TC – total cholesterol.

The inclusion criteria were as follows: age 18–65 years, DM1, and lack of acute inflammations and uncontrolled chronic diseases. The exclusion criteria were anemia (hemoglobin (Hb) <11 g/dL), chronic kidney disease (CKD) (glomerular filtration rate (eGFR) <30 mL/min/1.73 m^2) and elevated aminotransferase levels (more than twice the upper normal limits).

Anthropometric parameters such as height [m] and body mass [kg] were evaluated in all the subjects of the study. The body mass index (BMI) [kg/m^2], waist and hip circumference, and waist-to-hip ratio (WHR) were also determined. Systolic and diastolic blood pressure (SBP and DBP) were measured twice, sitting and after a five-minute rest. The measurement was performed with a sphygmometer using Korotkov's method [mm Hg]. The daily dosage of insulin was calculated the day before the study [number of units/kg/day].

A venous blood sample was drawn in order to note the following parameters: fasting glucose in the venous blood serum and glucose in the venous blood serum 2 h after breakfast using the standard method; HbA1c using high performance liquid chromatography (HPLC); lipid parameters (total cholesterol (THc) level, levels of high-density

lipoprotein (HDL) and low-density lipoprotein (LDL) fraction in the blood and triglyceride (TG) levels in the serum) using the enzymatic method; and aminotransferase activity (alanine aminotransferase – ALT, and aspartate aminotransferase – AST) in the serum using the standard method. The estimated glomerular filtration rate index (eGFR) was measured using the Modification of Diet in Renal Disease Study (MDRD) equation. All the laboratory tests were performed in the Raszeja Hospital Laboratory in Poznań.

The insulin resistance index – visceral adiposity index (VAI) and estimated glucose disposal rate (eGDR) – were calculated using the equations listed below:

– VAI in women: waist circumference/(36.58 + (1.89 × BMI)) × (TG/0.81) × (1.52/HDL);

– VAI in men: waist circumference/(39.68 + (1.88 × BMI)) × (TG/1.03) × (1.31/HDL);

– eGDR = 24.31–12.22 (WHR) – 3.29 (HA0/1) – 0.57 [mg/kg/min].[15]

An eGDR value below 7.5 mg/kg/min was considered an indicator of lowered tissue sensitivity to insulin action.[16]

Total adipose tissue content was assessed using the electrical bioimpedance method using a Tanita BC-418MA Body Composition Analyzer and a Tanita ViScan AB 140 (Tanita Corp., Tokyo, Japan). Total body fat (TBF) and visceral fat tissue (VFT) were assessed according to WHO age- and gender-adjusted criteria.[17]

The content of AGEs in the skin was evaluated using an AGE Reader Type 214D00102 (DiagnOptics, Groningen, the Netherlands). The device emits ultraviolet light at a wavelength of 300–420 nm, which illuminates 1 cm^2 of skin on the inside of the forearm, about 10 cm from the elbow); a built-in spectrometer registers light in the 300–600 nm range. The autofluorescence (AF) score is calculated automatically.

Statistical analysis

The statistical analysis of the results was performed using STATISTICA PL v. 13.3 software (StatSoft Polska sp. z o.o., Kraków, Poland). The normality of the distribution of the results was tested using the Kolmogorov–Smirnov test with the Lilliefors correction. The parameters analyzed did not have normal distributions; therefore, nonparametric tests were used for further analysis. The results were presented as numbers and percentages as well as medians and interquartile ranges (IQR). In the case of numerical variables, differences between subgroups were analyzed using the Mann–Whitney test. Differences in qualitative data were assessed with the χ^2 test. We used the multivariate regression method to analyze correlations between AGE level and selected parameters (such as sex, age, BMI, and the presence of chronic complications). In the correlation analysis, the Spearman correlation coefficients were used. A value of $p < 0.05$ was considered statistically significant.

Results

The normal body fat (NBF) group consisted of 123 subjects, whereas the EBF group included 104 subjects. Age and gender were taken into consideration in accordance with WHO norms. No significant statistical differences were found between the NBF and EBF groups with regard to age, duration of diabetes, current HbA1C value, daily dosage of insulin, and tobacco use.

The NBF and EBF groups were statistically different with regard to the following: anthropometric index (body mass, waist circumference, WHR), fasting blood glucose, blood glucose 2 h after eating, lipid parameters, and insulin resistance index (VAI, eGDR) (Table 2). A significantly higher AGE score was observed in the EBF group.

Statistically significant relationship was noted in the Spearmen correlation between lowered eGDR index (increased insulin resistance) and increased TBF, VBF and skin AGE (Table 3). The multifactor regression model showed the influence of VAI on the AGEs in the skin. It was gender-independent and disregarded chronic complications (Table 4).

Discussion

The Diabetes Control and Complications Trial (DCCT) published in 1993 revealed that intensive functional insulin therapy was the leading treatment to avoid microvascular complications (retinopathy and CKD) in patients with DM1.[18] Improved metabolic control during the first 2 years of DM1 treated with intensive functional insulin therapy modified β cell function and increased C-peptide, compared with conventional insulin treatment.[19] However, these intensively treated subjects become more vulnerable to severe hypoglycemia and weight gain, accompanied by efforts to lower blood glucose with multiple insulin injections. The adverse consequences of undesirable weight gain accelerate the development of chronic complications, and increase blood pressure and the parameters of the lipid profile. The greatest metabolic damage is caused by the accumulation of VFT, which directly affects endothelial function in patients with either DM1 or DM2. This accelerates the development of atherogenic dyslipidemia.[20,21] A study by den Engelsen et al. suggested that AGE accumulation in the skin can increase in populations with central obesity without diabetes.[22] In this study, AGE skin levels were measured in 816 non-diabetic obese patients and in 431 patients without central obesity; the mean AGE index increased with age and smoking, and was significantly higher in patients with central obesity.

Earlier studies highlighted the increased accumulation of AGE in the skin in patients with DM2. Samborski et al. also demonstrated increased AGE content in the skin in patients with DM1 in comparison to patients not suffering from diabetes.[23] However, a study by Dozio et al.

Table 2. Comparison of the group with excess body fat (EBF) to the group with normal body fat (NBF). The results are presented as numbers and percentages as well as medians and interquartile ranges (IQR)

Parameter	NBF (n = 123)	EBF (n = 104)	p-value
Gender, F/M	62/61	59/45	0.26
Age [years]	30.5 ±9.2	31.7 ±9.3	0.28
Duration of diabetes [years]	11.6 ±8.6	12.4 ±6.3	0.08
Retinopathy, n (%)	38 (16.7)	36 (15.8)	0.15
Nephropathy, n (%)	10 (4.4)	14 (6.2)	0.23
Neuropathy, n (%)	24 (10.6)	15 (6.6)	0.25
Smoking (%)	27.6	19.2	0.12
Daily insulin dosage [U/kg/day]	0.5 (0.4; 0.6)	0.5 ±0.2	0.71
FPG [mmol/L]	8.2 (5.4; 11.6)	9.2 (6.6; 12.1)	0.04
PPG [mmol/L]	9.5 (6.4; 12.5)	10.8 (8.3; 13.6)	<0.01
HbA1c (%)	8.7 (7.6; 9.8)	8.6 (7.7; 9.5)	0.93
HbA1c > 7%, n (%)	107 (87)	90 (86)	0.69
HbA1c ≤ 7%, n (%)	16 (13)	14 (14)	0.69
Creatinine [µmol/L]	70.7 (66.3; 85.7)	75.1 (65.4; 85.7)	0.8
eGFR [mL/min/m^2]	99.5 (88.4; 110.0)	97.7 (83.2; 105.6)	0.24
TCh [mmol/L]	5.2 (4.5; 6.0)	5.0 (4.4; 5.9)	0.6
TG [mmol/L]	1.0 (0.8; 1.4)	1.3 (1.0; 1.6)	<0.01
HDL-C [mmol/L]	1.7 (1.4; 2.1)	1.5 (1.2; 1.7)	<0.01
LDL-C [mmol/L]	2.8 (2.2; 3.3)	2.9 (2.5; 3.6)	0.03
BMI [kg/m^2]	23.0 (21.2; 25.0)	28.5 (27.2; 31.3)	<0.01
WHR	0.8 (0.8; 0.9)	0.9 (0.9; 1.0)	<0.01
TBF [kg]	13.5 (10.3; 16.8)	27.5 (22.3; 31.1)	<0.01
TBF (%)	19.6 (15.1; 27.0)	34.2 (25.5; 38.1)	<0.01
TRBF [kg]	7.1 (4.9; 9.0)	14.6 (12.6; 17.0)	<0.01
VBF, n	3.0 (1.0; 4.0)	7.0 (5.0; 9.0)	<0.01
VAI, n	2.1 (1.4; 3.0)	3.6 (2.3; 4.6)	<0.01
eGDR [mg/kg/min]	7.8 (6.0; 9.3)	7.0 (5.2; 8.3)	<0.01
AGEs, n	2.0 (1.7; 2.3)	2.1 (1.9; 2.4)	0.02

FPG – fasting plasma glucose; PPG – postprandial plasma glucose; HbA1c – glycated hemoglobin; BMI – body mass index; eGDR – estimated glucose disposal rate; eGFR – estimated glomerular filtration rate; VAI – visceral adiposity index; AGEs – accumulation of advanced glycation end products; TRBF – trunk body fat; VBF – visceral body fat; TBF – total body fat; WHR – waist-to-hip ratio; HDL – high-density-lipoprotein cholesterol; LDL – low-density-lipoprotein cholesterol; TCh – total cholesterol.

Table 3. Correlations between selected parameters and the content of glycation end products in the skin

Parameter	Spearman's correlation coefficient	p-value
TBF (%)	0.19	0.004
VBF (%)	0.25	0.001
eGDR [mg/kg/min]	–0.16	0.016

TBF – total body fat; VBF – visceral body fat; eGDR – estimated glucose disposal rate.

Table 4. Analysis of the associations between selected parameters and AGEs content (univariate regression analysis)

Parameter	β coefficient	p-value
Duration of diabetes [years]	0.18	0.042
VBF (%)	0.41	0.009
TBF (%)	0.11	0.001
eGDR [mg/kg/min]	–0.16	0.01

AGE – advanced glycation end-products; TBF – total body fat; VBF – visceral body fat; eGDR – estimated glucose disposal rate.

showed reduced content of soluble receptors for end products of protein glycation (sRAGEs) in obese women with waist circumferences >80 cm, higher fatty mass, epicardial adipose tissue, and VFT.[24] In our study group of patients with DM1, Spearman's correlation demonstrated that increased TBF correlates with increased accumulation of AGEs in the skin. A similar relationship was also found for VAI. The occurrence of obesity and excess weight in patients with DM1 is inextricably linked to insulin resistance. Increased insulin dose adjustment and increased numbers of mealtime insulin injections contribute to the development of this condition in patients treated with IFI. In a study

by Conway et al., the prevalence of obesity increased seven-fold with a nearly ten-fold increase in the frequency of intensive insulin therapy treatment.[11] Insulin resistance is mostly connected with hypoglycemia, poorer metabolic control and increased risk of chronic complications such as CKD, retinopathy and cardiovascular disease (CVD).[25] Our study also found a direct relationship between AGE content in the skin and the value of eGDR – the indirect insulin resistance index – in patients with DM1. Insulin, as a protein-structure hormone, is also subject to glycation processes. In a study by Jia et.al., it was shown that insulin glycation in the arginine position reduces the utilization of glucose by adipocytes and muscle cells.[26]

The AGEs of proteins are also produced in the bodies of healthy people. In patients with diabetes, they can be treated as a marker to assess the risk of complications. So far, the main marker used to assess metabolic control in diabetic patients is HbA1c. In a study by Monnier et al., it was shown that AGEs are more useful than HbA1c in the detection of late diabetic complications.[27] These results are similar to those in our study, in which the EBF group presented a statistically comparable value of HbA1c to the group with NBF. However, both groups were characterized by poor diabetes control. Patients from both groups required intensification of treatment through the use of insulin analogs and re-education regarding IFI.

According to the latest guidelines of the Polish Diabetes Association, it is also possible to use sodium-glucose co-transporter-2 (SGLT2) inhibitors to treat patients with inadequately controlled DM1 and DM2 (DEPICT1 and DEPICT2).[28] In clinical trials with SGLT2 inhibitors, statistically significant decreases in HbA1c, weight and total daily insulin dose were observed in both types of diabetes.[29,30] The difference in AGE content between these 2 groups may also indicate the higher usefulness and accuracy of SAF compared to HbA1c as a prognostic marker in patients with diabetes, especially DM1. Araszkiewicz et al. showed a correlation between AGEs in the skin and the mean value of HbA1c in patients with DM1.[4] As in our study, there was no correlation between one-time HbA1c and AGEs values. The relationship between earlier hyperglycemia and current skin AGE concentration may indicate the occurrence of metabolic memory in patients with DM1.

The AGEs are a stable parameter. A non-invasive assessment of AGEs in the skin may better reflect the toxic effects of hyperglycemia than the average glycemic values expressed as HbA1c. Proteins modified by AGEs may be more resistant to enzymatic degeneration, which promotes accumulation of AGEs in local tissue.[31] Moreover, AGEs have been localized in retinal blood vessels in DM2 patients and found to correlate with the degree of retinopathy.[32] Accumulation of these products in the body is also observed in patients with end-stage renal disease (ESRD).[33] A correlation between AGE concentration in tissues and the severity of atherosclerotic lesions has been demonstrated: AGEs bind with apolipoprotein B and induces LDL retention in the aortic wall.[34] A positive correlation has also been found between serum levels of AGEs and isovolumetric relaxation time measured during echocardiography in patients with DM1.[35] The AGEs also correlate with intima-media thickness (IMT) in DM1 patients, and increase with CVD, other autoimmune diseases and inflammatory processes.[36] Metabolic memory and overproduction of reactive oxygen species (ROS) play an important role AGE production, which may reflect metabolic control of diabetes over a longer period of time.[37,38] In DM1, AGEs are a reliable marker of past glycemic control and their accumulation is connected with diabetic microangiopathy.[39] In another study by Araszkiewicz et al., an association between SAF and long-term metabolic control and carotid IMT was revealed.[36] Although many studies have shown an association between AGEs and late diabetic complications, in our study, the EBF and NBF groups did not differ in diabetic complications, and there were no correlations between AGEs and diabetic complications.[7,40,41]

A correlation between AGE concentration in tissue, severity of atherosclerotic lesion and increased retention of AGE-LDL in the aortic wall has also been demonstrated.[42] The AGEs in DM2 are also inversely related to HDL anti-oxidative capacity.[34,43] Our study revealed differences in lipid profiles in the EBF and NBF groups, but there was no statistical correlation between lipid profile and AGE accumulation.

It has been shown that ageing correlates with high rates of AGE formation and accumulation.[44] Accumulation of AGEs is also a reliable biomarker of in vivo ageing. Accumulation of AGEs inside cells and tissues reflects a reaction between the intensity of inflammatory processes, modification of proteins like albumin or collagen, and modification of the proteasomal and lysosomal pathways.

In addition, high amounts of intracellular AGEs inhibit active immuno-proteasomes through the involvement of RAGEs and the Janus kinase 2/signal transducer and activator of transcription 1 (JAK2/STAT1) signaling pathway.[45] It is worth noting that the concentration of immunoproteasome is higher in aged cells.[46] In the aforementioned study by Araszkiewicz et al., a positive correlation was found between SAF and patient age.[31] In our study, this correlation was not confirmed.

A study by Schram et al. among patients with DM1 found no correlation between AGEs and mean arterial pressure.[47] Skin and blood AGEs were strongly and independently associated with pulse pressure. This is probably connected with arterial stiffness. In our study, we did not observe any differences in blood pressure in patients with or without EBF, nor between hypertension and AGEs.

Conclusions

Skin autofluorescence is simple to evaluate, and offers a valuable prognostic marker of the risk of developing chronic complications of diabetes. Increased body fat

content affects the amount of AGEs in the skin, which is associated with a higher risk of developing chronic diabetes complications. In patients with DM1, it is important to maintain proper body weight in order to avoid increasing the risk of chronic complications.

The study has some limitations. Firstly, no prospective observation was performed. Secondly, the results obtained are limited by the lack of a control group. However, AGEs are always higher in populations with diabetes, which is why we did not recruit a control group without diabetes. Moreover, an indirect method was used to assess insulin resistance (eGDR, not the gold standard glucose clamp technique); however, the 2 methods are comparable.

An advantage of the study is that it involved a homogenous group of patients with autoimmune disease treated with intensive functional insulin pen therapy.

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Face masks use during the COVID-19 pandemic: Differences in attitudes and practices between medical and non-medical students. A survey of 2256 students in Poland

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Abstract

Background. The use of face masks during the COVID-19 pandemic became ubiquitous. Such masks are believed to be effective in prevention of respiratory viral transmission.

Objectives. The study was undertaken to assess the use of face masks among students during the COVID-19 pandemic, with focus on similarities and differences between medical and non-medical students.

Material and methods. The study was based on the specially designed survey. The questionnaire, after consultation with 10 students, was created using Google Forms and posted on numerous Facebook groups for students in Poland. The recall period of the questionnaire was the previous 7 days. In 48 h, 2,315 answers were obtained. Fifty-nine questionnaires were excluded due to data incompleteness and failure to fulfil inclusion criteria. Therefore, 2,256 surveys (97.5%) were considered for final analysis.

Results. Medical students showed significantly different attitudes and practices concerning the use of face masks. Medical students used face masks more commonly and for longer periods of time. Moreover, they wore single-use masks more often and less frequently re-used them. Also, multiple use of single-use face masks and masks decontamination procedures were less common among medical students.

Conclusions. We suggest that medical students might be of help in educational campaigns for general public on proper use of face protection.

Key words: COVID-19, coronavirus, face masks, medical students

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Introduction

The COVID-19 pandemic resulted in increased use of face protection.[1–3] Face masks are believed to be effective in prevention of respiratory viral transmission.[2] This may be of importance for general health policy.

This study was undertaken to assess the use of face masks among students during the COVID-19 pandemic, with focus on similarities and differences between medical and non-medical students.

Material and methods

The study was based on the specially designed survey. The questionnaire was developed based on the interviews with 10 students. All the relevant issues raised during the interviews were put up together and considered for the questionnaire. Then, the proposed questionnaire was assessed by 2 independent experts in such studies, who gave their comments on the proper wording and understanding of each question. The recall period of the questionnaire was the previous 7 days. The final survey was created using Google Forms and posted on numerous Facebook groups for students in Poland. The data were collected during 48 h between April 12 (10:00 PM) and April 14, 2020 (10:00 PM). At that time, the use of face masks in Poland was not mandatory. Based on the decision of Polish government (from March 11, 2020) regular activities of schools and universities were suspended, and only tele-education was allowed and advised. A total of 2,315 answers were obtained. Due to the incompleteness of the data and failure to fulfil inclusion criteria (not completed by students), 59 questionnaires were excluded. Therefore, 2,256 surveys (97.5%) were considered for final analysis. The selected responses were subject to statistical analysis (STATISTICA v. 13; StatSoft Inc., Tulsa, USA). The χ^2 test was applied to determine statistical differences between the groups of medical and non-medical students. The resulting p-values were considered significant if $p < 0.05$.

Results

Among 2,256 respondent, there were 1,170 (51.9%) medical students and 1,086 (48.1%) students of other faculties, not related to health sciences (non-medical students). The mean age of the group was 20.9 ±1.6 years (age range: 19–27 years). A total of 1,366 students (60.5%) declared using face masks. Medical students wore face protection significantly more often ($p = 0.02$) than non-medical students (62.8% and 58.1%, respectively) (Table 1). More than $^1/_3$ of students (34.3%) used several types of face masks. Wearing various types of face protection was significantly more common ($p = 0.03$) in medical students (35.1%) than in students of other faculties (33.4%) (Table 1). Out of all types of face masks, cloth masks (46.2%) appeared to be worn most frequently, followed by surgical masks (39.3%) and respirators (N95 and FFPs (filtering face pieces); 13.3%), while other types were used only sporadically (Table 1). Medical students significantly more often ($p < 0.001$) used surgical masks. In contrast, non-medical students wore cloth masks more commonly ($p < 0.001$). There was no difference in the frequency of wearing other types of face masks between the 2 groups (Table 1). Single-use masks

Table 1. Face mask use among medical and non-medical students during the COVID-19 pandemic

Variable	Total	Medical students	Non-medical students	p-value*
Students using face masks				
yes	1366 (60.5%)	735 (62.8%)	631 (58.1%)	**0.02**
no	890 (39.5%)	435 (37.2%)	455 (41.9%)	
Types of face masks used				
surgical masks	744 (39.3%)	435 (42.4%)	309 (35.6%)	**<0.001**
cloth masks	874 (46.2%)	440 (42.9%)	434 (50.0%)	**<0.001**
respirators (N95 + FFP)	252 (13.3%)	141 (13.7%)	111 (12.8%)	0.45
half-face elastometric respirator	16 (0.8%)	7 (0.7%)	9 (1.0%)	0.42
full-face respirator	8 (0.4%)	3 (0.3%)	5 (0.6%)	0.35
Single-use face masks worn	996 (52.6%)	576 (56.1%)	420 (48.4%)	**<0.001**
Several types of face masks used	469 (34.3%)	258 (35.1%)	211 (33.4%)	**0.03**
Duration of face masks used per day				
up to 1 h	689 (50.4%)	354 (48.2%)	355 (56.3%)	0.06
up to 2 h	1145 (83.8%)	600 (81.6%)	545 (86.4%)	**0.009**
up to 3 h	1259 (92.2%)	665 (90.5%)	594 (94.1%)	**0.004**
more than 5 h	56 (4.1%)	37 (5.0%)	19 (3.0%)	0.06
Multiple use of single-use face masks	332 (24.3%)	172 (23.4%)	160 (25.5%)	**0.02**
Decontamination of face masks (all types)	1004 (73.5%)	506 (60.8%)	498 (78.9%)	**<0.0001**
Decontamination of multi-use face masks	1267 (92.8%)	678 (92.2%)	589 (93.3%)	0.43

p-values in bold are statistically significant.

were used significantly more often ($p < 0.001$) by medical students than non-medical ones (56.1% and 48.4%, respectively). Consequently, decontamination of face masks was markedly a more common procedure ($p < 0.0001$) among non-medical students; however, no differences were noticed in terms of multi-use masks (Table 1). As for the duration of the daily face mask wear, non-medical students used them more commonly for shorter periods during the day (less than 1 h). By contrast, medical students significantly more often used face protection for longer periods of time ($p = 0.009$ for cut-off point of 2 h per day and $p = 0.004$ for cut-off point of 3 h per day) (Table 1). Some students (24.3%) used single-use masks several times. The multiple use of disposable respiratory protective devices was significantly less frequent ($p = 0.02$) in medical students' group (Table 1).

Discussion

Since the outbreak of COVID-19, in Asian countries, especially in China, the use of facial masks has become ubiquitous.[4,5] People at low risk of infection were instructed to use disposable masks for medical usage and even people at very low risk were recommended to use non-medical masks, such as cloth masks.[1,5] With the spread of infection, as a protective action, on March 11, 2020, the Polish government decided to suspend regular activities in all schools, including universities. Centers for Disease Control (CDC; Atlanta, USA) advised to screen mouth and nose with a cloth face cover when around others during the COVID-19 pandemic.[6] In our country, more and more people decided to wear face protection when they were outdoors. On the April 9, 2020, the Polish Ministry of Health announced that starting from the April 16, 2020 covering the nose and mouth when being in public space was obligatory. It is worth to note that our study was performed when wearing the face masks by general population was not obligatory.

Differences in the health-related attitudes, behaviors and practices between medical and non-medical students have been previously documented.[7–9] To the best of our knowledge, this is a first report on students' attitudes concerning the use of face masks during viral pandemic. It is worth mentioning that the conditions for both groups were more or less similar (all universities were closed; medical students did not attend clinical classes). Our results clearly showed that medical students used face masks more commonly and for longer periods of time. Moreover, they wore single-use masks more often and less frequently reused them. It is not surprising that the decontamination procedures of the masks were significantly less common among medical students, since they used more disposable masks. Based on our results, we suggest that medical students, most probably due to their awareness, knowledge and personal interest, might be of help in educational campaigns for general public on proper use of face protection.

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Clinical value of soluble ST2 in cardiology

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Abstract

We are constantly looking for new parameters and markers that can help in the assessment of patients with various diseases, including cardiac disorders; this can translate into better care and improved prognosis. Suppression of tumorigenicity 2 (ST2) has recently gained interest as a potential biomarker in many fields: it is involved in many inflammatory diseases and allergies, including asthma, rheumatoid arthritis and inflammatory bowel disease, and it participates in cardiovascular pathophysiology. Suppression of tumorigenicity 2 is being investigated as a promising biomarker in heart diseases. The interaction of interleukin 33 (IL-33) and ST2L is part of a cardioprotective pathway that prevents fibrosis and inhibits inflammatory response, hypertrophy and apoptosis of cardiomyocytes. In this review, we try to summarize the current knowledge about the usefulness of soluble ST2 (sST2) in cardiology. Clinical data show promising results for the possibility of using sST2 in various diseases, such as arrhythmia, hypertension, myocarditis, acute aortic syndrome, and coronary artery disease (CAD). This novel biomarker may also play a role in heart transplantation and perioperative care.

Key words: heart, biomarker, ST2, cardiac biomarkers, sST2

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Introduction: sST2 as a new biomarker

In 2001, the Biomarkers Definitions Working Group, a part of the National Institutes of Health (NIH, Bethesda, USA), has updated the definition of a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention".[1] New parameters and markers that can help improve the assessment of patients with various diseases, including cardiac disorders, are constantly being sought; they can translate into better care and improved prognosis. Suppression of tumorigenicity 2 (ST2) has recently gained interest as a potential biomarker in many fields. A member of the interleukin 1 (IL-1) receptor/Toll-like superfamily, ST2 is known as interleukin 1 receptor-like 1 (IL1RL-1) and when it was originally described in 1989, it was considered an "orphan" receptor.[2] Interleukin 33 (IL-33), a member of the IL-1 family of cytokines, was described as a ligand for the suppression of tumorigenicity 2 in 2005.[3] The discovery of IL-33 helped to understand the signaling axis of IL-33 and ST2. The ST2L, after IL-33 binding, has an inhibitory effect on the inflammatory response related to Th2 (Fig. 1).

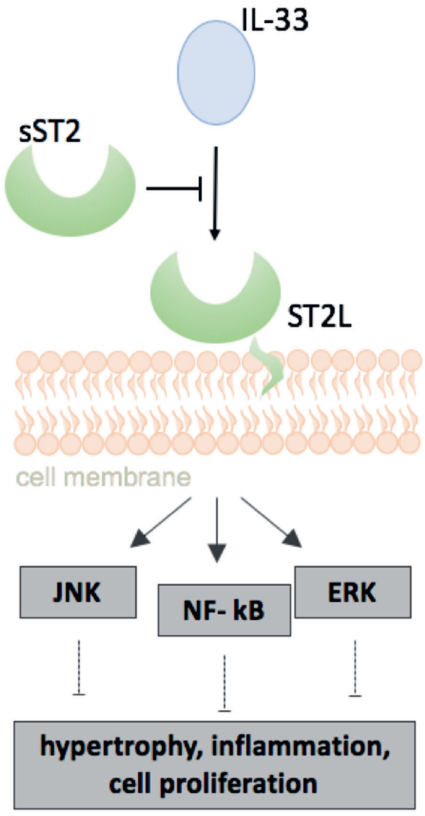

Fig. 1. Signaling axis of IL-33 and ST2

IL-33 – interleukin 33; sST2 – soluble suppression of tumorigenesis 2; ST2L – membrane-bound receptor; JNK – c-Jun N-terminal kinases; NF-kB – nuclear factor kappa-light-chain-enhancer of activated B cells; ERK – extracellular signal-regulated kinases.

Suppression of tumorigenicity 2 participates in inflammatory processes and functions in relation to immune diseases. To the best of our current knowledge, ST2 has 4 isoforms: the 2 main ones are a membrane-bound receptor form (ST2L) and a soluble form (sST2), while the other 2 are ST2V[4] and ST2LV.[5] Differences in their structure and quantity result from a dual promoter system to drive differential mRNA expression.[6] The ST2L membrane protein consists of 3 extracellular domains, a single transmembrane domain and an intracellular domain.[6] Because sST2 lacks the transmembrane and intracellular domains, it circulates freely in the blood. Interleukin 33, due to tissue damage or necrosis, is released into the extracellular space, where it binds to the ST2 receptor and then recruits the IL-1 receptor accessory protein (IL-1RAcP), which leads to the activation of the NK-kB signaling pathway.[7] The soluble form of ST2, after binding to IL33, inhibits the inflammatory response associated with Th2 lymphocytes, thereby blocking the protective effect of IL-33 (Fig. 1).

Interleukin 33 is biologically active and may be released by living and necrotic cells, the latter as a result of tissue damage playing the role of an endogenous danger signal.[8] Multiple organs and cell types in humans express IL-33.[3] Together with ST2, IL-33 is involved in many inflammatory and allergic diseases, including asthma,[9] rheumatoid arthritis[10] and inflammatory bowel disease,[11] and it participates in cardiovascular pathophysiology. Weinberg et al. described the expression of ST2 in cardiac cells as a "response" to myocardial stress and biomechanical overload.[12] This discovery focused researchers' attention on the role of ST2 in the cardiovascular system. Further studies revealed that in cardiac diseases, the main source of sST2 may be vascular endothelial cells rather than myocardium.[13] The interaction of IL-33 and ST2L is part of the cardioprotective pathway that prevents fibrosis and inhibits the inflammatory response, hypertrophy, and apoptosis of cardiomyocytes.

Soluble ST2 and coronary artery disease

Circulating ST2 is a promising biomarker being investigated in ischemic heart disease. Several studies have investigated the association of sST2 serum levels with the prognosis of coronary artery disease (CAD). Myocardial infarction (MI) with ST-segment elevation (STEMI) is associated with increased myocardial expression of sST2 and elevated levels of serum sST2 due to transmural myocardial injury and stretching of the left ventricle.[14] During this process, cardiac fibroblasts and cardiomyocytes release IL-33 and sST2.[14] In 2002, Weinberg et al. showed that sST2 was elevated after experimental MI in mice. A similar situation was observed in vivo, in patients 1 day after MI.[12] Several studies have reported that an increased sST2 level in the initial phase of STEMI is a significant

prognostic biomarker for predicting mortality and heart failure (HF).[15,16] Using this assay can help identify patients with a higher risk of developing adverse cardiac events while in intensive care due to MI.[16] O'Donoghue et al. showed that sST2 level in patients hospitalized with acute MI is a significant predictor of short-term cardiovascular death or the development of HF.[17] A high concentration of sST2 is an important predictor of major adverse cardiovascular events (MACE), including cardiovascular mortality and HF in a one-year follow-up period in MI patients.[18] Moreover, sST2 may be used as a predictor of late ventricular remodeling after MI.[19] Although baseline sST2 level may be valuable in predicting MACEs in STEMI patients receiving primary PCI,[20] there are no guidelines that recommend sST2 testing as a predictor biomarker in STEMI patients.

The concentration of sST2 may be used to predict cardiovascular risk. An increased level of this biomarker was an independent predictor of long-term all-cause mortality for stable CAD.[21] Zhang et al., in a recently published study, confirmed that sST2 concentration was not related to the severity of coronary artery atherosclerosis detected with angiography.[22] However, they found an association between complex coronary lesion morphology and sST2 level, especially in a group of patients with unstable CAD. The authors suggested that sST2 concentration may be useful as a marker in the detection of unstable and complex atherosclerotic plaques.[22]

Soluble ST2 and acute aortic syndrome

Another group of diseases in which the sST2 biomarker can be used is acute aortic syndromes (AAS). Wang et al. showed that AAS might result in the release and elevation of serum sST2 concentration.[23] Moreover, they indicated the potential superiority of sST2 over D-dimer in AAS. Morello et al. confirmed higher levels of serum sST2 in patients with AAS, but, unlike previous studies, showed poor diagnostic utility.[24] Further studies are needed in this field to confirm the usefulness sST2 as a biomarker in aortic diseases.

Soluble ST2 and myocarditis

Soluble ST2 may also be used as a biomarker in patients with myocarditis for predicting the development of HF. In a recent study, Coronado et al. showed higher sST2 concentrations in both men and women with myocarditis,[25] but sST2 level only correlated with worse HF symptoms based on New York Heart Association Functional Classification (NYHA) class in men ≤50 years old. This was the first work to show the potential usefulness of sST2 in myocarditis; more studies in this field are required.

Soluble ST2 and arterial hypertension

Soluble ST2 may be useful as a diagnostic biomarker for cardiac remodeling in patients with arterial hypertension (AH). It has been shown that plasma concentration of sST2 in the general population is associated with systolic blood pressure (SBP).[26] Hypertensive patients with HF and left ventricular hypertrophy (LVH) had higher plasma sST2 concentrations than those without LVH. This biomarker has the potential to distinguish AH patients with or without LVH.[27] Similar findings were reported in a study by Ojji et al., where sST2 may help differentiate patients with LVH in the HA population.[27] Farcas et al. confirmed that in hypertensive patients' serum, sST2 levels increase along with LV mass and the severity of myocardial dysfunction.[28] Moreover, sST2 level can help in predicting LV diastolic dysfunction in hypertensive patients, an indicator which displayed a significant positive correlation with impaired relaxation parameters (transmitral E/A ratio) measured echoardiographically.[28]

Soluble ST2 and atrial fibrillation

In the most common clinically relevant arrhythmia – atrial fibrillation (AF) – sST2 is still under investigation. Chen et al. showed that sST2 levels were higher in patients with AF, both persistent and paroxysmal, than in patients with sinus rhythm.[29] In patients with CAD, serum levels of sST2 predict the risk of new-onset AF.[30] Okar et al. demonstrated the possible utility of sST2 as a new biomarker to predict the reoccurrence of paroxysmal AF in patients after cryoballoon catheter ablation.[31] Soluble ST2 may be applied as an independent biomarker for predicting HF in patients with AF.[29] The CASABLANCA study showed that sST2 adds an independent prognostic value in the population at a high risk of HF for predicting progression to the development of symptomatic HF.[32] The role of soluble ST2 in AF still has to be precisely explored; additional studies should be conducted to validate these findings.

Soluble ST2 and heart failure

Soluble ST2 is a biomarker for the prognosis of acute and chronic HF, and may be used for predicting all-cause and cardiac mortality among HF patients, and even in the general population. Elevated concentrations of sST2 are associated with poorer LVF and higher NYHA functional class. According to some studies, age, sex, body mass index (BMI), etiology of HF, and comorbidities such as renal dysfunction has less of an influence on sST2 levels than on natriuretic peptides.[33] Soluble ST2 is considered a valuable biomarker used in the prediction and monitoring of HF and

was included in the 2017 American College of Cardiology/American Heart Association update of HF guidelines.[34] The first study of sST2 measurement in patients with suspected or proven HF was the PRIDE (Pro-BNP Investigation of Dyspnea in the Emergency Department) substudy.[35] Soluble ST2 was measured in almost 600 patients with dyspnea with and without HF; the concentration of sST2 was significantly higher in the group of patients with HF-related dyspnea.[35] Despite higher levels of sST2 in patients with acute decompensated HF, there was no statistically significant value in the diagnosis of HF,[35] which was confirmed in further studies. The sST2 levels in the PRIDE study were significantly higher in patients who had died within the one-year follow-up period than in survivors. Moreover, there was an association between sST2 concentration and mortality rates: higher levels predicted a higher risk. Mueller et al. reported similar results that higher sST2 concentration at initial presentation in patients with acute heart failure (AHF) indicated an increased risk of mortality in the future.[36]

After proving that the baseline sST2 values at admission predict outcomes, serial measurements were investigated for its significance. Boisot et al. were the first to note changes in sST2 concentration in hospitalized patients with AHF.[37] Serial measurements of sST2 correlated with other biomarkers, according to the RELAX-AHF trial, and are useful for prognosis in AHF.[38] The TRIUMPH study showed that baseline levels and repeated sST2 measurements, performed in patients with AHF, are strong and independent factors of adverse outcome in this group.[39]

Soluble ST2 is gaining attention as a potential tool in the management of chronic heart failure (CHF) and as a prognostic marker in such patients. Concentrations of sST2 in a group of patients with CHF were generally higher than in a healthy population.[35] Soluble ST2 is an indicator of prognosis in CHF. Patients with higher concentrations of sST2 correlated with worse functional status and more advanced HF. Higher baseline levels of sST2 were associated with worse prognosis and an increased risk of sudden cardiac death in patients with CHF.[40,41] Soluble ST2 concentration carries information in addition to levels of N-terminal pro-B-type natriuretic peptide (NT-proBNP). Lupón et al. investigated different biomarker combinations to determine their role in CHF. They developed a calculator that takes into account the concentration of sST2 together with NT-pro-BNP and high sensitive troponin T (hs-TnT) to estimate the risk of death and/or HF hospitalization within 5 years.[42] The Barcelona Bio-HF Risk Calculator takes into account biomarkers in combination with well-established risk factors such as gender, age, etiology of HF, LVEF, NYHA functional class, estimated glomerular filtration rate (eGFR), diabetes mellitus (DM), sodium and hemoglobin levels, and the treatment administered – βa-blocker or angiotensin converting enzyme inhibitor/angiotensin receptor blocker (Table 1). According to meta-analysis of Emdin et al., concentration of sST2 is an independent prognostic value for all-cause mortality and hospitalization in patients with CHF.[43] Moreover, in a group of patients with dilated cardiomyopathy, sST2 correlated with all-cause mortality and combined outcome of death, left ventricle assist device (LVAD) implantation and cardiac transplantation.[44]

Table 1. Variables used in the Barcelona Bio-Heart Failure Risk Calculator (BCN Bio-HF Calculator)

Clinical variable	Treatment	Biomarkers
Age	loop diuretics	hs-cTnT
Sex	ACE-I or ARB	ST2
NYHA functional class	β-blocker	NT-proBNP
LVEF	statin	
Na		
Hemoglobin		
eGFR		

NYHA – New York Heart Association Functional Classification; LVEF – left ventricular ejection fraction; Na – sodium; eGFR – estimated glomerular filtration rate; ACE-I – angiotensin-converting enzyme inhibitors; hs-cTnT – high-sensitive cardiac troponin T; ST2 – suppression of tumorigenicity 2; NT-proBNP – N-terminal prohormone of brain natriuretic peptide.

Moreover, significant interactions between sST2 level and HF treatment were suggested. In CHF patients treated with higher doses of β-blockers (>50 mg of metoprolol succinate extended-release equivalent), sST2 concentrations dropped.[45] Also, the higher the dose of β-blockers, the greater reduction in risk was found.[45] Similar results were reported in the Valsartan HF Trial (Val-HeFT): a group treated with valsartan and a β-blocker had lower levels of sST2.[46] Higher sST2 concentrations may indicate the increased risk of sudden cardiac death in patients with HF and reduced ejection fraction,[47] which may help to identify the individuals who can benefit from an implantable cardioverter-defibrillator (ICD).

Soluble ST2 and cardiosurgery

Suppression of tumorigenicity 2 has the potential to be used in cardiac surgery. Patel et al. found that postoperative elevated plasma sST2 levels were associated with an increased incidence of cardiovascular event or mortality in adult patients who had undergone cardiac surgery.[48] These findings were independent of pre-existing conditions such as CHF or acute kidney injury (AKI). Moreover, promising publications on the usefulness of sST2 in heart transplantation are appearing. Increased sST2 levels may be an indicator of acute allograft rejection in heart transplant recipients.[49] It is suggested that the rejected graft is a source of serum sST2. Concentrations of sST2 are associated with an increased risk of antibody-mediated rejection after heart transplantation.[50] This discovery opens the possibility for biopsy-free monitoring of anti-rejection therapy in heart transplant recipients.

Discussion

Cardiac fibroblasts and cardiomyocytes produce circulating ST2 in response to stress and overload.[12] The IL-33/ST2L axis has a cardioprotective effect and may, in the future, help create targeted therapy.[35] Soluble ST2 is a biomarker which has the potential to be used for diagnosis and treatment monitoring in patients with various cardiovascular diseases. It is proving to be an especially important biomarker for both acute and chronic HF.[35,36,40] Clinical data show promising results for the possible use of sST2 in various diseases, such as arrhythmia, hypertension, myocarditis, acute aortic syndrome, and CAD.[30,28,22] This novel biomarker may play a role in heart transplantation and perioperative care of patients after cardiac surgery. This paper reviewed the literature on the possible applications of measuring sST2 in cardiology, where CAD and atherosclerosis are just one area of interest to researchers. In our opinion, the greatest interest in new biomarkers is currently focused on the area of HF. Heart failure is a significant and growing medical problem. The search for new biomarkers in this area is the opportunity to construct new hypotheses for HF pathogenesis and the opportunity to develop new therapies. The largest subsection is devoted to this problem. The ACC/AHA guidelines demonstrate the importance of this biomarker designation.[34]

Conclusions

Soluble ST2 is a promising biomarker in cardiology, especially in HF. However, further studies of the usefulness of sST2 in particular cardiac diseases are needed.

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The protective effect of hypothermia on postoperative cognitive deficit may be attenuated by prolonged coronary artery bypass time: Meta-analysis and meta-regression

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Abstract

There is controversy about whether hypothermia during coronary artery bypass grafting (CABG) surgery is effective in reducing postoperative cognitive deficit (POCD). The objective of this study was to determine the effect of hypothermia on POCD and to undertake a meta-regression to determine whether moderator variables mediate the relationship between hypothermia and POCD. We searched the Web of Science, PubMed database, Scopus, and the Cochrane Library database (up to June 2017), and systematically reviewed a list of retrieved articles. Our final review includes only randomized controlled trials (RCTs) that compared administration of hypothermia (34°C). Statistical analysis of the risk ratio (RR) and corresponding 95% confidence interval (95% CI) was used to report the overall effect. Mantel–Haenszel risk ratio (MH RR) and corresponding 95% CI was used to report the overall effect and meta-regression analysis. Eight RCTs were included in this study, with a total of 1,474 patients. The POCD occurred in 36.06% of all cases. A wide range of hypothermia (28–34°C) did not reduce the occurrence of POCD (RR = 0.983 (95% CI = 0.881–1.143); Z = –0.304; P = 0.761; I^2 = 38%). Shorter CPB time reduced the occurrence of POCD (MH log risk ratio = –0.011 (95% CI = –0.021––0.0008); Z = –2.123; P = 0.033). Postoperative cognitive deficit is a common event among CABG patients. Contrary to deep hypothermia, mild hypothermia was significantly effective in reducing the risk of POCD. The neuroprotective effect of hypothermia on POCD may be attenuated by prolonged cardiopulmonary bypass (CPB) time.

Key words: coronary artery bypass grafting, meta-analysis, hypothermia, postoperative cognitive deficit

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Introduction

Postoperative cognitive deficit (POCD) has long been understood as a major complication after cardiac surgery.[1,2] Despite the fact that reported occurrence of POCD varies widely across studies, coronary artery bypass grafting (CABG) with the use of cardiopulmonary bypass (CPB) is associated with an increased risk of morbidity and mortality.[3,4] The underlying cause of POCD has not yet been properly identified.[5] It is probable that microemboli are responsible for brain injury after CPB surgery.[6]

Hypothermia is a widely used protocol to reduce cognitive deficit after CPB surgery.[7] It reduces cerebral metabolic rate as well as the inflammatory response and apoptosis.[8] Several studies investigated the effect of hypothermia on cognitive deficit among patients with CABG and they did not find any advantage of hypothermia over normothermia for improving cognitive deficit,[4,9–14] resulting in ambiguity in terms of the role of temperature management during CPB surgery. Therefore, clinical practice guideline of temperature management advised that arterial outlet blood temperature should be limited to below 37°C to avoid cerebral hyperthermia. Although the avoidance of hyperthermia was emphasized, no threshold temperature during hypothermia is recommended due to the insufficient published evidence.[15] Therefore, a hypothesis may be advanced that there may be other variables that need to be explored.

Due to the effect of hypothermia on neuroprotection, the widespread use of this technique and inconclusive findings from randomized controlled trials (RCTs), our aim was to systematically review and summarize evidence relevant to the effect of hypothermia on the occurrence of POCD and perform a meta-regression to discover whether moderator variables mediate the relationship between hypothermia and POCD.

Methods

Data sources

The PubMed, the Web of Science, Scopus, and the Cochrane Library databases were searched for clinical trials up to June 2017, using the search terms: ("coronary artery bypass surgery" OR "cardiopulmonary bypass surgery" OR "thoracic surgery" OR "cardiovascular surgery" OR "cardiac surgery" OR "CABG") AND ("cognitive dysfunction" OR "cognitive function" OR "cognitive decline" OR "neurocognitive") AND ("temperature" OR "*thermia" OR "*thermic" OR "body temperature" OR "perfusion temperature" OR "hypothermic cardiopulmonary bypass surgery" OR "rewarming") AND ("randomized controlled trial" OR "controlled clinical trial" OR "randomized" OR "randomly" OR "trial"). The reference lists of the retrieved studies including systematic reviews and meta-analyses were also checked.

Study selection

Only RCTs were incorporated into this meta-analysis in which patients undergoing CPB surgery received hypothermic (≤34°C) and normothermic (>34°C) CPB surgery. We excluded non-English-language studies as well as studies that did not administer the neuropsychological test battery and studies that did not measure cognitive deficit after CPB surgery.

Data extraction and clinical endpoint

The occurrence of any cognitive deficit following CPB surgery (a decline >1 standard deviation (SD) in the postoperative score in comparison to the preoperative score) was defined as primary outcome.[16] The decline must be verified using the 1 or more neuropsychological tests. The occurrence of POCD in time periods within the first 30, 30–90 and 90–360 days were defined as the very early, early and late occurrence of cognitive deficit, respectively.[1]

Studies that met the inclusion criteria were assessed by one reviewer and another reviewer verified the studies. The POCD in intervention and control groups was the main outcome. The potential moderator variables including male gender [%], age [years], temperature thresholds [°C], mean duration of CPB [min], hypertension (HTN) [%], diabetes mellitus (DM) [%], reduced left ventricle ejection fraction (rLVEF) [%], follow-up period [days], and loss to follow-up [%] were collected. Hypothermia can be categorized as mild and deep hypothermia. A 3°C difference between normothermic and hypothermic group is considered as mild hypothermic protocol and more than 3°C difference is defined as deep hypothermic protocol. Disagreements between authors were resolved through discussion. The Jadad five-point scale was used to assess the quality of RCT; this scale scores a maximum of 2 points for blinding, 2 points for randomization and 1 point for the description of withdrawals and drop-outs (that is, patients lost to follow-up).[16] The Q and I^2 statistics were used to assess the statistical heterogeneity among the included RCTs. Heterogeneity was considered for variables with Q statistics ($p < 0.05$). It means that the amount of total variance is more than we would expect based on within-study error, so random effect model was presumed. A random-effect model was used in meta-regression because there was significant between-study variation.[17] The degree of heterogeneity was determined using I^2 statistics and it also represents the proportion of variation in treatment which is independent from sampling error. The I^2 statistics result between 30% and 60% is moderately heterogeneous.[18] The relationship between 1 or more moderators and a dependent variable was assessed using meta-regression.[19] Effect size as dependent variable and moderator variable as independent variable were analyzed to identify potential predictors of effect size. Q-model statistics with $p < 0.05$ showed that the relationship between

moderator variable and treatment effect is stronger than we would expected by chance. Variables with Z statistics ($p < 0.05$) were interpreted as their slope is probably not 0, and the treatment effect is more effective when moderator variable changes.[17] Mantel–Haenszel (MH) log risk ratio was regressed on the following variables: between-group proportion difference of male gender [%], between-group mean difference of age [years], CPB time [min], and temperature threshold [°C]. Hypertension [%], DM [%] and rLVEF [%] were not reported in all trials. Difference was defined as the difference between normothermia and hypothermia groups (normothermia – hypothermia). Negative value of MH log risk ratio is in favor of the neuroprotective effect of hypothermia. Data are presented as a mean (SD) for continuous variables and as proportions (%) for categorical variables. Statistical analyses were conducted using the Comprehensive Meta-Analysis v. 2 statistical software package (Biostat Inc, Englewood, USA). Dichotomous results were analyzed using the Mantel–Haenszel method. The risk ratio (RR) and its 95% confidence interval (95% CI) were calculated.

Results

Search results

From a total of 715 citations identified, 29 articles were fully evaluated. A total of 1,747 patients in 8 RCTs met the inclusion criteria and all of them were included in the final analyses.[20–27] Steps of the search strategy are presented in Fig. 1.

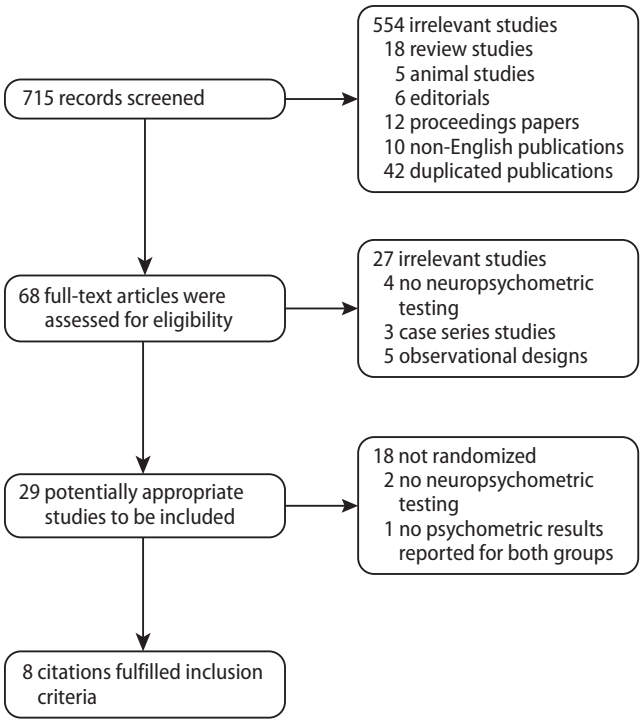


Fig. 1. Flow diagram of the selection process of eligible articles

Study characteristics

All RCTs scored above moderate and 2 studies scored high on the Jadad scale (Table 1). The Wechsler Adult Intelligence Scale was the most frequently used scale to assess the severity of POCD. The overall occurrence of POCD was 36.06%. The occurrence of POCD was 48.23%, 15.21% and 48.49% for very early, early and late periods, respectively. Male patients had a higher proportion than female patients in all RCTs. Mean surgery time (CPB) ranged from 71 min to 127.5 min and surgery time difference between hypothermic and normothermic groups was up to 27.3 min. No significant between-group difference was reported in the baseline characteristics of patients (except for CPB time in the study by Plourde et al.). The key characteristics of the included studies are presented in Table 1. Data and conclusions are summarized in Table 2.

Overall effect of hypothermia

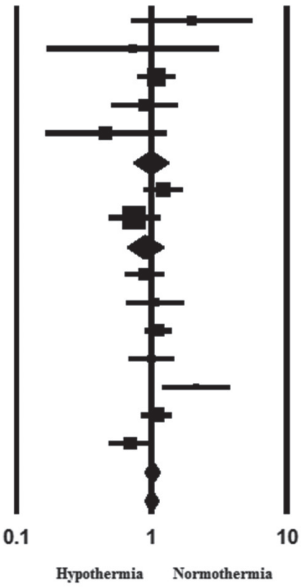
The overall effect of hypothermia was estimated through a meta-analysis. The effect of hypothermia on POCD was calculated through MH RR as 0.983 (95% CI = 0.881–1.098). The MH RRs for very early, early and late cognitive deficit were 1.004 (95% CI = 0.883–1.143), 0.968 (95% CI = 0.722–1.299) and 0.887 (95% CI = 0.656–1.198) (Fig. 2). The findings of the pooled analyses are summarized in Fig. 2. The overall heterogeneity of the RCTs was estimated as 38% (Q-model = 20.912; p = 0.075). A funnel plot for publication bias analysis is not presented due to the fact that it is not recommended with analysis of fewer than 10 studies.[19]

Meta-regression

The overall effect of moderator variables was estimated through meta-regression analyses. The model was significant for the association of CPB time difference as well as temperature difference with cognitive deficit. Between-group temperature difference was calculated through MH log RR as 0.053 (95% CI = 0.007–0.098); Z = 2.302, p = 0.021, Q-model = 5.303, degree of freedom (df) = 1, p = 0.021 (Fig. 3A). The mild temperature difference (about 3°C) is significantly associated with a lower rate of POCD in comparison with deep hypothermia (>3°C) (Fig. 3A). Figure 3B shows that the MH log RR for CPB time difference between groups was −0.011 (95% CI = −0.021–−0.0008); Z = −2.123, p = 0.033, Q-model = 4.509, df = 1, p = 0.033 (Fig. 3B) and it shows that shorter CPB time is significantly associated with a lower rate of POCD in either group.

Stepwise regression analyses were performed to examine the association between MH log risk ratio and potential predictors including temperature and CPB time difference. The R^2 was 43.8%. Regression model was significant, R = 0.693, R^2 = 0.431, p = 0.006. The CPB time was

Study name	Subgroup within study	MH risk ratio	lower limit	upper limit	Z-value	p-value
McLean et al. 1994	early	1.974	0.708	5.505	1.300	0.194
Mora et al. 1996	early	0.729	0.169	3.135	−0.425	0.671
Regragui et al. 1996	early	1.076	0.781	1.482	0.447	0.655
Heyer et al. 1997	early	0.889	0.507	1.560	−0.411	0.681
Boodhwani et al. 2007	early	0.458	0.164	1.282	−1.487	0.137
	early	0.968	0.722	1.299	−0.215	0.830
Kaukinen et al. 2000	late	1.222	0.874	1.708	1.172	0.241
Nathan et al. 2007	late	0.745	0.484	1.145	−1.342	0.179
	late	0.887	0.656	1.198	−0.783	0.433
McLean et al. 1994	very early	0.886	0.638	1.230	−0.724	0.469
Mora et al. 1996	very early	1.064	0.653	1.734	0.249	0.804
Heyer et al. 1997	very early	1.122	0.899	1.400	1.019	0.308
Plourde et al. 1997	very early	1.000	0.684	1.462	0.000	1.000
Kaukinen et al. 2000	very early	2.143	1.212	3.788	2.621	0.009
Boodhwani et al. 2007	very early	1.091	0.845	1.410	0.670	0.503
Nathan et al. 2007	very early	0.694	0.482	0.998	−1.970	0.049
	very early	1.004	0.883	1.143	0.067	0.946
	overall	0.983	0.881	1.098	−0.304	0.761



	Z-value	P-value	Q-value	df(Q)	P-value	I^2
Very early	0.067	0.946	12.749	6	0.047	52.938
Early	−0.215	0.830	4.534	4	0.339	11.772
Late	−0.783	0.433	4.148	1	0.042	75.89
Overall	−0.304	0.761	20.912	13	0.075	37.835

Fig. 2. Comparison of patients on hypothermia and normothermia

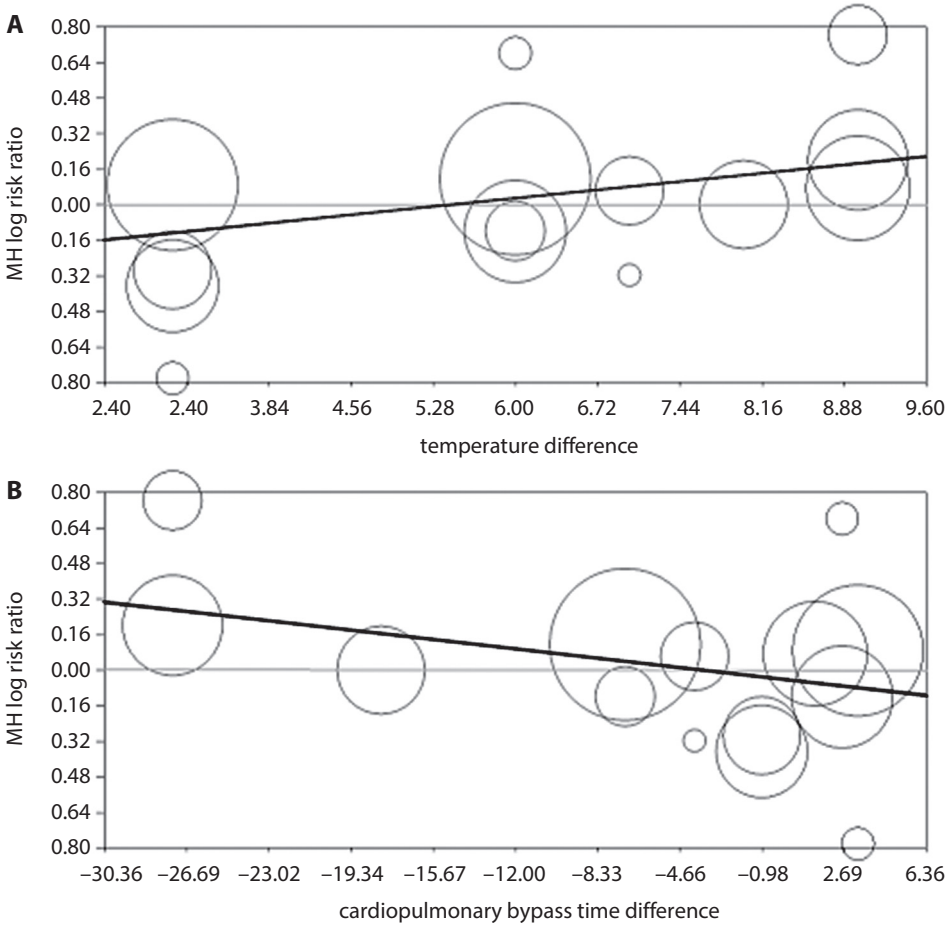


Fig. 3. A. Plot of MH log risk ratio against temperature difference between groups. Positive values in the horizontal axis indicate a greater mean or proportion for the normothermic group (difference = normothermia – hypothermia). Negative values in the vertical axis favor the neuroprotective effect of hypothermia (below the horizontal grey line). B. Plot of MH log risk ratio against cardiopulmonary bypass time difference between groups. Negative values in the horizontal axis indicate a greater mean or proportion for the hypothermic group (difference = normothermia – hypothermia). Negative values in the vertical axis favor the neuroprotective effect of hypothermia (below the horizontal grey line)

Table 1. Baseline characteristics of patients in eligible studies. Intervention – hypothermia; control – hyperthermia

Parameter	McLean et al.[20]		Mora et al.[21]		Regragui et al.[22]		Heyer et al.[23]		Plourde et al.[24]		Kaukinen et al.[25]		Boodhwani et al.[26]		Nathan et al.[27]	
	intervention	control	intervention	control	intervention	control	intervention	control	intervention	control	intervention	control	intervention	control	intervention	control
Temperature [°C]	28	>34	28	≥34	28	37	28	34	28	36	28	≥36	34	37	34	37
Number of points	77	78	70	68	31	29	46	53	24	30	18	18	133	134	65	66
Age, mean (SD)	59.3 (9.5)	58.1 (9.5)	61 (11)	65 (10)	57.3 (1.6)	58.7 (1.5)	64.4 (11.9)	63.5 (10.4)	55.9 (9.2)	58.3 (8.7)	57.7 (7.4)	58.5 (7.5)	68.2 (6)	69.3 (6)	68 (5)	67 (5)
Males (%)	–	–	57 (81)	51 (75)	27 (96.4)	27 (93.1)	35 (76.08)	46 (86.79)	23 (95.83)	27 (90)	17 (94.44)	14 (77)	119 (85)	116 (85)	54 (83)	59 (89)
Diabetes	–	–	19 (27)	16 (24)	1 (3.5)	1 (3.44)	–	–	–	–	3 (16.66)	0 (0)	46 (34.5)	47 (35)	16 (24)	18 (26)
rLVEF (%)	22 (28.5)	32 (41)	–	–	–	–	–	–	–	–	–	–	10 (7)	16 (12)	12 (18)	10 (15)
CPB duration [min], mean (SD)	100.1 (26.8)	97.5 (26.8)	86 (26)	82 (23)	80.4 (3.6)	85.8 (4.4)	112.8 (29.5)	105.7 (24.1)	89 (24)	71 (21)	127.5 (36.5)	103.2 (20.2)	77.2 (22.5)	80.5 (23.4)	86 (26)	85 (27)
Procedure	CABG with CPB		CABG with CPB		CABG with CPB		CABG with CPB		CABG with CPB		CABG with CPB		CABG with CPB		CABG with CPB	
Temperature control	nasopharyngeal		bladder		nasopharyngeal		nasopharyngeal		nasopharyngeal		nasopharyngeal		nasopharyngeal		nasopharyngeal	
Follow-up [days]	before operation, 4–5 and 90 days postoperatively		on the day before surgery and 7–10 and 28–42 days after surgery		the day before and 42 days after operation		on the day before surgery and 6 and 42–63 days after surgery		before surgery and 6–7 days after surgery		before surgery and 5 and 330–690 days postoperatively		before surgery and 7 and 90 days postoperatively		before surgery and 7 and 1825 days (5 years) postoperatively	
Drop-out (%)	n = 4 (1.3%) – 2/154 (1.3%) in very early assessment; – 2/154 (1.3%) in early assessment; – 2/78 (1.3%) dropouts in normothermic group: 1 death, 1 agitated.		n = 106/276 (38.4%) – 52/138 (37.68%) in very early assessment; – 54/138 (39.13%) in early assessment; – not reported for each group.		n = 1 (1.66%) – 1/32 (3.22%) dropouts in hypothermic group: 1 death.		n = 84/198 (73.73%) – 13/99 (13.13%) in very early assessment; – 73/99 (73.73%) in early assessment; – long distance from home to the site of care; – not reported for each group.		n = 8 (14.81%) – 5/24 (20.83%) dropouts in hypothermic group: 2 bleeding, 1 refused, 1 not cooled, 1 acute abdomen; – 3/30 (10%) dropouts in normothermic group: 1 stroke, 2 bleeding.		n = 5/72 (6.94%) – 4/36 (11.11%) in very early assessment; – 1/36 (2.77%) in late assessment dropouts in hypothermic group: 1 stroke, 1 nausea, 1 severe mental depression; – 2/18 (11.11%) dropouts in normothermic group: 1 stroke, 1 confused.		n = 31 (12%) – not reported for each group.		n = 63 (27.13%) – 29/223 (13%) in very early assessment; – 92/223 (41.25%) in late assessment; – 29/65 (44.61%) dropouts in hypothermic group: 13 death, 2 too ill to participate, 7 refused, 7 unable to contact; – 34/66 (51.51%) dropouts in normothermic group: 8 death, 6 too ill to participate, 10 refused, 10 unable to contact.	
Jadad score	3		4		3		3		5		3		3		5	

rLVEF – reduced left ventricular ejection fraction; CABG – coronary artery bypass surgery; CPB – cardiopulmonary bypass

Table 2. Summary of data and conclusions from eligible studies

Studies	Design	Trial procedure	NP Test	Endpoint	Conclusion
McLean et al.[20]	parallel groups, randomized, prospective, double-blind trial	– hypothermic (28°C) vs normothermic (>34°C); – blood cardioplegia at 37°C in a continuous manner at 50–150 mL/min for the normothermic patients and 5–8°C in an intermittent fashion with reinfusions >400 mL for the hypothermic patients.	– Buschke Selective Reminding Procedure; – Wechsler Memory Scale (WMS); – trail-making test; – Wechsler Intelligence Scale (WAIS-RDS); – Grooved Pegboard Test (Pegs).	– a decline ≥1 SD in the postoperative score in comparison to the preoperative score; – POCD if occurred in 1 or more tests.	Unable to demonstrate any neuroprotective effect from moderate hypothermia.
Mora et al.[21] 1996	parallel groups, prospective, randomized, and double-blind	– hypothermic (≤28°C) vs normothermic (≥37°C); – the flow rate was adjusted based on nasopharyngeal temperature.	– Wechsler Memory Scale (WMS); – Wechsler Adult Intelligence Scale, Revised (WAIS-R); – Grooved Pegboard Test (Pegs).	– a decline ≥1 preoperative group SD for that test.	Hypothermia decreases the risk of perioperative cognitive deficit.
Regragui et al.[22]	parallel groups, prospective, randomized, and double-blind	– hypothermic (28°C) vs normothermic (37°C); – the flow rate was adjusted based on nasopharyngeal temperature; 2.4 $Lm^{-2}min^{-1}$ for the normothermic group and 1.8 $Lm^{-2}min^{-1}$ for hypothermic group.	– Wechsler Intelligence Scale (WAIS); – Wechsler Memory Scale (WMS); – control tests: General Health Questionnaire (GHQ-30) and Hospital Anxiety and Depression Scale (HAD).	– regression coefficients for the 2 temperature (37°C vs 28 °C) dummy variables were examined using multivariate testing; – POCD if occurred in 1 or more tests.	Hypothermia (28°C) conferred no additional benefit in terms of cognitive function.
Heyer et al.[23]	parallel groups, a randomized, double-blind	– hypothermic (28°C) vs normothermic (34°C); – the coronary arteries were perfused with cold blood cardioplegic solution.	– Mini-Mental State Examination; – Halstead–Reitan Trails Test; – right and left index finger tapping test; – Grooved Pegboard Test; – Buschke Selective Reminding Test.	– at least 25% decrease in postoperative performance compared with preoperative results; – POCD if occurred in 1 or more tests.	No detectable difference in postoperative cerebral dysfunction.
Plourde et al.[24]	parallel groups, a randomized, double-blind trial	– hypothermic (28°C) vs normothermic (36°C); – the flow rate was adjusted based on nasopharyngeal temperature; 2.4 $Lm^{-2}min^{-1}$ for the normothermic group and 2 $Lm^{-2}min^{-1}$ for hypothermic group.	– Wechsler Adult Intelligence Scale-Revised (WAIS-R); – associate learning task (paired words); – Wechsler Memory Scale (WMS); – Rey–Osterrieth Complex Figure (DLRY); – Rey–Osterrieth Complex Figure Test (ROCF); – Controlled Oral Word Association Test (COWA).	– a decline ≥1 SD in the postoperative score in comparison to the preoperative score; – POCD if occurred in 1 or more tests.	Temperature during CPB for coronary operations does not influence postoperative cognitive function.
Kaukinen et al.[25]	parallel groups, a randomized, double-blind trial	– hypothermic (28°C) vs normothermic (36°C); – cardioplegia was delivered, either as continuous warm blood cardioplegia in the normothermia group or intermittent cold (5–7°C) blood cardioplegia in the hypothermia group.	– Wechsler Adult Intelligence Scale (WAIS); – memory tests modified to Finnish practice; – control tests: Zung Self-Rating Anxiety Scale and Zung Self-Rating Depression Scale.	– a 20% or more decrease from the preoperative test result; – POCD if occurred in 1 or more tests.	The cognitive decline following CABG operation performed with hypothermic or normothermic CPB was similar.
Boodhwani et al.[26]	parallel groups, a randomized, double-blind trial	– hypothermic (34°C) vs normothermic (37°C); – high-efficiency thermal pad were applied. The patients either cooled to 34°C or warmed to 37°C.	– Rey Auditory Verbal Learning Test; – trails A and B; – Grooved Pegboard Test; – Symbol Digit Modalities Test (SDMT); – Wechsler Adult Intelligence Scale-Revised (WAIS-R); – Wechsler Memory Scale (WMS); – letter and category fluency.	– a decline ≥1 preoperative group SD for that test; – POCD if occurred in 1 or more tests.	Mild hypothermia does not decrease the incidence of neurocognitive deficits.
Nathan et al.[27]	parallel groups, a randomized, double-blind trial	– hypothermic (34°C) vs normothermic (37°C); – all were cooled to 32°C during application of the aortic cross-clamp. Patients were rewarmed to a temperature of either 34°C or 37°C.	– Buschke Selective Reminding Test; – Wechsler Adult Intelligence Scale (WAIS); – Grooved Pegboard Test; – Symbol Digit Modalities Test (SDMT).	– a decline ≥1 preoperative group SD for that test; – POCD if occurred in 1 or more tests.	Cognitive decline was not different between temperature groups.

POCD – postoperative cognitive deficit.

significantly associated with MH log risk ratio – unstandardized B CPB time was −0.62 (0.018; p = 0.006) and unstandardized B constant was −0.157.

Sensitivity analysis

Only Plourde et al. reported a significant difference of CPB time between groups (CPB time of hypothermic group was longer than that of normothermic group).[24] Slope was significant after exclusion of the study by Plourde et al. and it resulted in a reduction from −0.011 to −0.045, meaning that the association between CPB time and POCD was not influenced by the results of the study conducted by Plourde et al[24]. There are no influential studies in any of the analyses estimated from "Remove-One" sensitivity analysis.

Discussion

To date, this is the first meta-regression to evaluate the association between the neuroprotective effect of hypothermia and moderator variables. We demonstrated that shorter CPB time had a favorable influence on POCD. This finding may have critical implications for the therapeutic management of patients undergoing CABG surgery.[1]

It is well-recognized that hypothermia is neuroprotective.[28] Hypothermia provides cerebral protection against ischemia, reduces brain metabolism under hemodynamic instability, and inhibits free radical and oxidative enzymatic activities and attenuation of the excitatory and inhibitory neurotransmitters, resulting in a protective balance against brain damage.[13] In detail, extracellular amino acids play a pivotal role in neural damage.[28,29] However, glutamate, aspartate, gamma-aminobutyric acid, taurine, glycine, and alanine showed a significant increase in response to the ischemia in normothermia. Therefore, inhibition of excessive effluxes of both excitatory and inhibitory amino acids contributes to the neuroprotective effect of hypothermia against ischemia.

In harmony with other reviews on the association of hypothermia and POCD,[4,9,12–14] our meta-analysis showed that a wide range of hypothermia (28–34°C) does not reduce POCD after CABG surgery. However, there is no statistically significant difference between the 2 groups, risk ratio for very early (1.004), early (0.968) and late (0.887), showing that the trend is compatible with that reported by Brown et al.[6] They demonstrated that there is a rapid decline in the embolic load as time from surgery increases (particularly in the first few days after surgery). In our study, there is a trend toward a lower occurrence of cognitive deficit among patients after CPB surgery in hypothermic group (35.45% compared to 36.66%), suggesting that a moderator variable may interfere with a significant decrease in the POCD in hypothermic group.

Meta-regression demonstrated shorter CPB time is associated with a lower occurrence of POCD in either group, meaning that CPB surgery time is an independent predictor of POCD. In the regression model, patients with longer surgery time had a significantly increased risk for the occurrence of POCD. Bearing in mind that only 2 (25%) studies had a shorter surgery time for the hypothermic group, it may explain why the findings of these studies have been inconclusive across all previous reviews. Figure 3B shows that the neuroprotective effect of hypothermia can only resist up to 4 min longer mean duration of normothermic CPB. This finding is supported by other studies, indicating that an increase in CPB surgery time is associated with an embolic load increase.[6,30,31] The key role of CPB surgery time in association with the risk of microemboli development and POCD has been well-discussed in the cardiac surgery studies.[30–32] Brown et al. reported that a 30.5% increase in the embolic load is contributed by each 60-minute increase in CPB surgery time.[6] Patients with valve plasty surgery experience even more severe embolic load (145.3%/h). In this line, Salis et al. showed that a 30-minute increment in CPB surgery time is a significant independent risk factor for postoperative death (OR = 1.57).[33] In addition, Bucerius et al. reported that CPB surgery time longer than 120 min is an independent risk factor for stroke (OR = 1.42).[34] Therefore, it has to be said that postoperative outcome of patients with CABG surgery is considerably associated with CPB surgery time.

In this review, absolute mean CPB surgery time difference between normothermic and hypothermic groups was up to 27.3 min and mean CPB surgery time ranged from 71 min to 127.5 min. However, all RCTs (except that of Plourde et al.[24]) reported that the average of CPB surgery time does not differ significantly between groups. Hypothermic groups experienced greater mean CPB surgery time in 6 studies.[20–25,27] Therefore, the effect of hypothermia on POCD may be confounded by the great impact of prolonged CPB time on emboli formation. Also, mean statistics can be highly affected by the extreme values and it is not an appropriate statistic for highly skewed distributions. An analysis of 5,000 patients undergoing CABG surgery showed that the distribution of CPB duration is positively skewed (0.18) and data is dispersed (coefficient of variation (CV) = 40%), showing that 25% of surgeries lasted longer than 135 min (up to 643 min).[33] In this review, each 30-minute increment in CPB surgery time increases the risk of death 1.53 times, which shows that CPB surgery time is closely related to the final outcomes such as death, stroke and POCD. In the included studies, there is a high dispersion in data due to the fact that CV of CPB surgery time ranged from 20% to 30%. It is known that it is not appropriate to use mean statistics in skewed data, particularly when there is a strong association between dispersed cases and final outcomes. Therefore, dispersity may be the reason why the neuroprotective effect of hypothermia is not significant in the meta-analysis. It is concluded that insignificant difference between CPB time is not sufficient to compare occurrence of POCD

between groups, which is why studies need to be stratified for the CPB time as well.

Meta-regression showed that mild temperature difference (–3°C) is related with a lower POCD in hypothermic group, meaning that mild hypothermic protocol (34°C) showed a lower POCD in comparison with deep hypothermic protocol (>34°C). In fact, other hypothermic protocol did not offer any advantage over normothermic group. Figure 3A shows that the mild temperature difference (about 3°C) is significantly associated with a lower rate of POCD, meaning that the neuroprotective effect of hypothermia decreases as temperature difference between normothermic and hypothermic group rises. This is in line with other studies showing that modest reductions in temperature have been shown to protect brain neurons and decrease lactate production.[35] Ooboshi et al. indicated that mild brain hypothermia (3°C reduction) may be superior to attenuate effluxes of excitatory amino acids and protect of hippocampal neurons.[28] Besides, a meta-analysis of the relationships between hypothermia and the neuroprotection effect after cardiac arrest showed that mild hypothermia (32–34°C) improves neurological outcomes in resuscitated patients from cardiac arrest.[29]

Different time periods are used to examine cognitive status among RCTs (7 days, 4–6 weeks, 3 months, or longer). The follow-up period after CPB surgery ranged from 4 days to 5 years in the current studies. In the first week, the occurrence of POCD is at the highest rate, followed by a significant reduction over the first 6 months.[3,6,12,36] In this line, Cormack et al. indicated that a trend developed towards a decrease in the occurrence of POCD across all 4 neuropsychological tests in the 1st year following CPB surgery.[1] They also mentioned there is a decline in half of the neuropsychological tests in the 1st week after CPB surgery, specifically in those tests that assess psychomotor function.[1] Apart from that, the practice effect may occur during the neuropsychological tests, so the effect size may be confounded by the patients' scores which showed practice effects over multiple testing sessions.[2] However, the practice effect may improve scores towards a decline in the occurrence of POCD. Brown et al. also reported that there is a consistent reduction in the microemboli load as time passes after CPB surgery,[6] implying that there may be multiple factors associated with trend towards decline in POCD.

The variability in the definition of POCD may contribute to the heterogeneity among RCTs.[37] A 1 SD decline in the postoperative score in comparison to the preoperative score is the most commonly used measure to define POCD in the literature. It uses a fixed amount of decline for each patient and is specific to each study. It also estimates between the higher (20% drop) and lower (IRs) estimates of cognitive decline. Therefore, a 1 SD decline criterion may miss up to 1/3 of the occurrences of POCD.[37] Finally, this error may be ignored because both hypothermic and normothermic groups have been classified with same methodology in each individual study, so it may have no significant impact on the effect size. Neuropsychological testing is still considered as the main method for the measurement of POCD. Therefore, the result must be interpreted cautiously because the neurocognitive assessment is accompanied by inherent variations in measuring POCD.

Patients lost to follow-up ranged from 1.3% to 73.73% in the current review. Loss to follow-up was not associated with the occurrence of POCD through meta-regression; in Heyer et al., it was 73.73% in early assessment.[23] The validity of studies is questionable if more than 20% of patients are lost to follow-up.[38] After exclusion of the study by Heyer et al., there was no significant association between hypothermia and POCD and the association between CPB time and occurrence of POCD also remained significant.[23] These authors reported that loss to follow-up rate does not significantly differ between groups in order to lessen the bias caused by a high drop-out rate.

Further investigation of the effect of hypothermia on POCD with a focus on important moderators is necessary to provide a better understanding of the profile risks. We could not analyze the association between the patient's profile risk factors such as age, gender, DM, HTN and rLVEF, and POCD, since the studies did not report the patient's profile risk factors completely.

Several factors may be associated with cognitive deficit, but very few have been proven to have the significant effect on cognitive deficit as much as CPB surgery time. A higher occurrence of POCD is reported among patients undergoing CABG surgery with valve plasty.[6] All included RCTs did not recruit patients with valve plasty surgery, which is why it is not considered as a source of bias in our meta-analysis. All included studies used on-pump CABG with conventional extracorporeal bypass, so the results may not be confounded by surgery technique. Generally, on- and off-pump CABG groups do not differ in terms of the occurrence of cognitive deficit. A meta-analysis reported comparing off-pump compared to on-pump CABG does not show any significant effect on POCD.[14,39]

There are several limitations of this meta-analysis. Studies have been conducted from 1994 to 2007; therefore, it was impossible to contact all the authors for additional data. The skewness of CPB time could be calculated if we had access to raw data and median CPB time. Further investigation of the effect of hypothermia on POCD with focus on CPB time is necessary. Stepwise regression model showed that CPB time and temperature contribute to 43.8% of the overall results, so patient risk profiles may be involved in the neuroprotective effect of hypothermia on POCD.[5,12]

Conclusions

Meta-analysis of the available evidence showed that POCD is a common event among CABG patients. However, hypothermia was not significantly associated with a lower

occurrence of POCD; mild hypothermia may be more effective than deep hypothermia in decreasing the risk of POCD. Shorter CPB time was associated with a lower occurrence of POCD, so the neuroprotective effect of hypothermia on POCD may be attenuated by prolonged CPB time.

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Secondary skin neoplasms in patients after autologous and allogeneic hematopoietic stem cell transplantation procedures

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Abstract

The increasing number of hematopoietic stem cell transplantation (HSCT) procedures and lower transplant-related mortality has led to a growing population of survivors facing long-term increased risk of secondary malignancy, including cutaneous neoplasms. In this review, we aim to discuss the incidence, risk factors and preventive strategies for secondary skin neoplasms after autologous and allogeneic HSCT. Cutaneous neoplasms, such as basal cell carcinoma, squamous cell carcinoma and melanoma, are among the most common solid cancers arising in patients after HSCT. Besides risk factors established in the general population, primary disease, chronic graft-versus-host disease (CGvHD), prolonged immunosuppression, especially with the use of cyclosporine and azathioprine, radiation exposure, light skin color, male sex, and young age at transplantation play a role in the development of cutaneous neoplasms in HSCT recipients. Skin cancer development after HSCT may be explained by cumulative effects of chemotherapy and radiotherapy-induced DNA damage, prolonged immunosuppressive conditions and chronic mucosal inflammation, particularly after allogeneic HSCT. Delayed immune recovery and persistent immunodeficiency in patients with graft-versus-host disease (GvHD) may also contribute to carcinogenesis. Regular dermatological surveillance and prompt recognition of precancerous and cancerous lesions is crucial for patient's prognosis and management.

Key words: hematopoietic stem cell transplantation, skin neoplasms, graft-versus-host disease, basal cell carcinoma, squamous cell carcinoma

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Introduction

Hematopoietic stem cell transplantation (HSCT) is potentially curative for malignant hematological neoplasms and other non-malignant conditions. The introduction of new protocols has led to the improvement of survival,[1] but challenges connected with long-term health problems of patients have emerged.[2] Secondary neoplasms are divided into 3 types: post-transplant lymphoproliferative disease (PTLD), hematologic malignancies and solid cancers.[3] Post-transplant lymphoproliferative and hematologic diseases occur earlier after a transplant and their incidence stabilizes after 10 years, whereas solid malignancies are characterized by long latency period and no plateau even after 15 years.[4–6]

In this review, we aim to discuss the incidence, risk factors and preventive strategies for secondary skin neoplasms after autologous (autoHSCT) and allogeneic hematopoietic stem cell transplantation (alloHSCT). Additionally, we present clinical and dermoscopic aspects of secondary skin malignancy in patients hospitalized in our Bone Marrow Transplantation Department (Fig. 1–5).

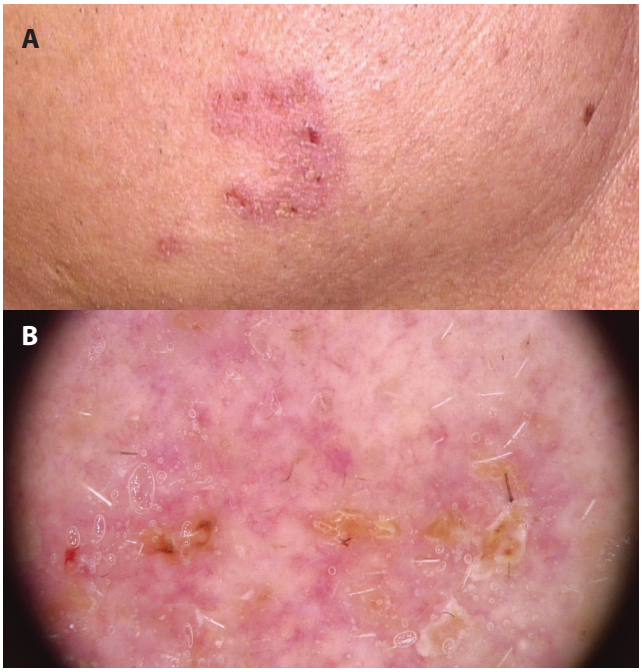

Fig. 1. Superficial variant of basal cell carcinoma (BCC) in a 52-year old male patient in +122 day after allogeneic hematopoietic stem cell transplantation (alloHSCT) for myelofibrosis. A. Clinical image of BCC presenting as a large, erythematous plaque with superficial scaling and multiple small erosions covered with crusts. B. Videodermoscopy in non-polarized light with 50-fold magnification showed the presence of multiple thin, short teleangiectasias with numerous small erosions covered with yellowish crusts

Incidence of secondary skin neoplasms

Studies usually reported incidence rate and a standardized incidence ratio (SIR) (observed cancer cases in a HSCT cohort to expected cancer cases in the general population of similar age and gender). Table 1 shows data on secondary solid neoplasms including skin neoplasms reported in analyzed studies.[4,5,7–31] Secondary solid tumors have been reported to appear at twice the rate expected in the general population,[5,19] although some reports cited over 11-fold heightened risk.[7] Cumulative incidence of secondary solid cancers varies from 1% to 15.9% at 10 years,[22,25] reaching 17.6% at 30 years.[29]

Cutaneous malignant neoplasms are among the most common neoplasms, accounting for 0–58.5% of secondary neoplasms.[32] Twenty-year cumulative incidence is 6.5% for basal cell carcinoma (BCC) and 3.4% for cutaneous squamous cell carcinoma (SCC).[15] The median time from HSCT to diagnosis is 7.3–9.4 years for BCC[13,15,17,23] and 2.1–7.0 years for SCC.[11,13,15,17,19,23] Half of the reported melanomas occurred after 1–4 years[19] and SIRs of melanoma are between 3.5–8.3.[4,14,19]

Risk factors

The pathogenesis of secondary neoplasms after HSCT is multifactorial. Numerous risk factors have been proposed and distinct pathways may be involved in the pathogenesis of different solid tumors.[33]

Patient-related factors

There are conflicting results on the impact of age at transplantation. Younger patients were reported to be at risk,[5] especially when irradiation-based conditioning was used.[6,15,18,19] Old age entailed higher risk, especially in the setting of autoHSCT.[4,13,25,26]

Notable difference in skin cancer incidence was noted in the Asian population; some of the studies did not report on skin cancer after auto- or alloHSCT,[12] despite high incidence of other solid cancers, including SCC in oral cavity.[12] However, in other studies SIRs for skin cancers were reported as high as 7.2–40.23.[23,27] Those discrepancies are consistent with low background incidence of skin malignancies in local cancer epidemiology and may be partly explained by gene–environment interaction.[12]

Genetically determined skin pigmentation plays an important role in BCC susceptibility and light complexion was reported as a risk factor in some of the analyzed studies.[18,25,30] Male sex was also shown to be a risk factor in some studies.[5,19,26]

Primary disease-related factors

Diagnosis of Fanconi anemia, dyskeratosis congenita or Li-Fraumeni syndrome confers an increased risk of secondary cancers, also in the setting of HSCT.[34] In malignant hematologic neoplasms patients, acute myeloid leukemia/myelodysplastic syndrome patients were found to have a tendency towards the development of secondary

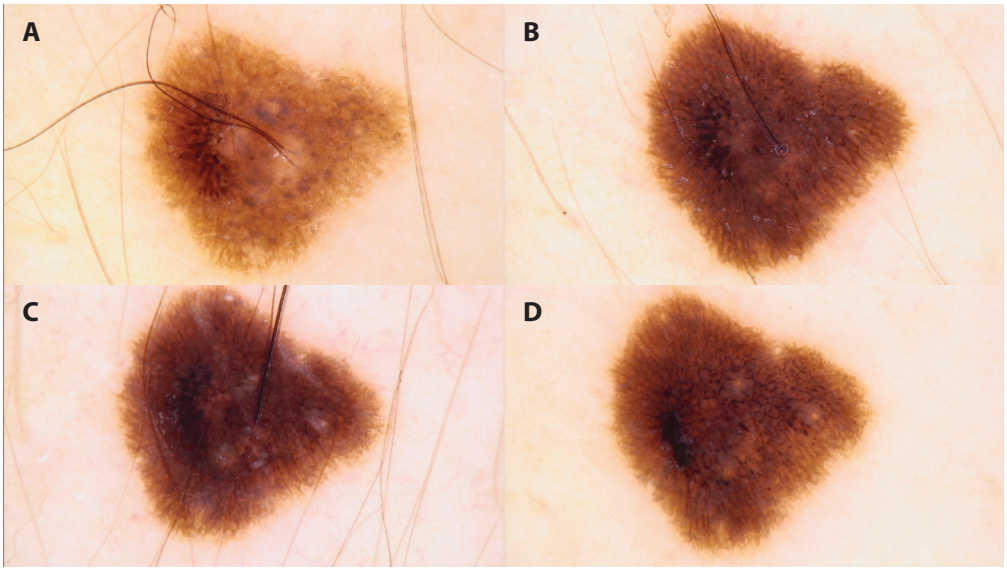

Fig. 2. Dermoscopy in non-polarized light of the monitored melanocytic nevus located on the abdominal skin in a 20-year-old female patient (III phototype of the skin according to the Fitzpatricks scale), treated with alloHSCT procedure for myelodysplastic syndrome (videodermoscopy, non-polarized light, ×50 magnification).
A. Just before alloHSCT: during skin examination the melanocytic nevus 7 mm in diameter was found on the abdominal region. The nevus was included in the close short-time follow-up because of the presence of atypical network: sharply cut-off, with thickening of the pigmented network and presence of irregularly distributed grayish globules.
B–D. Dynamic changes observed after the alloHSCT procedure (days +50, +72, +95): pigmented network has become darker, irregularly thickened, forming peripheral short streaks and structureless irregularly distributed areas. In adhesive tape test, the black lamella was not torn off. The complete excision was postponed due to immunosuppression, agranulocytosis and thrombocytopenia. Finally, histopathologic examination revealed the diagnosis of compound nevus

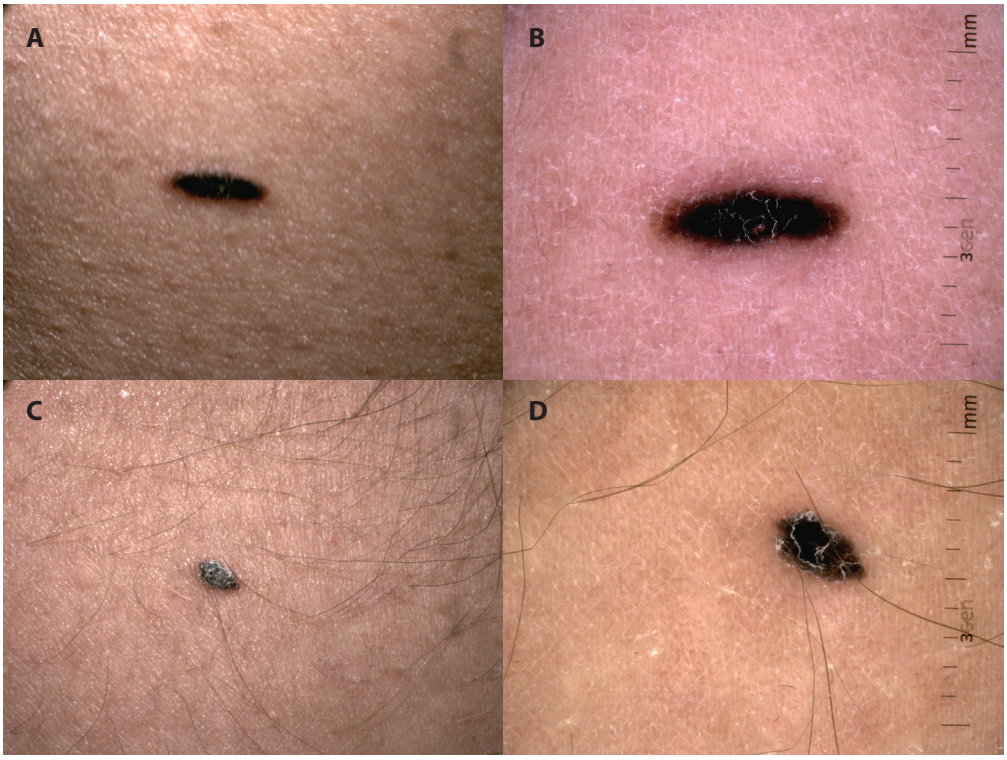

Fig. 3. Macroscopic (A, C) and dermoscopic (B, D) images of melanoma simulators- synchronously appearing multiple, small dark nevi in male 21-year-old patient with the IV phototype according to Fitzpatrick as a symptom of the aggravated nevogenesis after alloHSCT (day +184) for ALL. Dermoscopy in non-polarized light revealed multiple, small dark nevi with structureless dermoscopic pattern

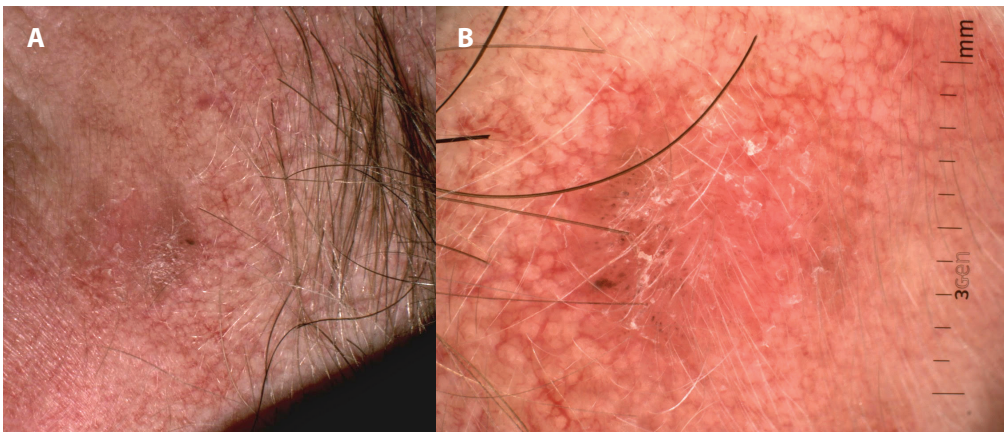

Fig. 4. Clinical and dermoscopic aspect of histopathologically confirmed squamous cell carcinoma in situ in a 54-year-old patient with cutaneous chronic GvHD after alloHSCT for myelofibrosis (day +750).
A. Suspicious erythematous, velvety solitary skin lesion.
B. Dermoscopy in polarized light showed the presence of central scaling with prominent brownish dots distributed linearly at the periphery of the lesion. Background consisted of multiple linear curved vessels forming a pseudonetwork, suggesting the diagnosis of actinic keratosis

Table 1. Outline of the studies analyzed

Study (first author, date)	Years	Type of HSCT	Population and country	Number of points	Number of secondary neoplasms (solid and skin cancer given if reported)	Increased risk compared with general population	Cumulative incidence	Risk factors	Time from transplantation-diagnosis; median (range)
Curtis 1997[5]	1964–1992	allo	IBMTR registry	19229	80 solid, 71 NMSC (not included in analysis), 8 invasive SCC, 9 melanomas	SIR 2.7 (all), 5 (melanoma), 8.3 for those who survived >10 years after HSCT	2.2% at 10 years, 6.7% at 15 years	younger age (<10 years RR 36.6, 10–29 years RR 4.6 compared with >30 years), cGvHD (invasive SCC RR 22.6), male sex (all invasive skin SCC occurred in men), higher doses of TBI (≥10 Gy single-dose, ≥13 fractionated; melanoma RR 8.2), T-cell depletion (melanoma RR 4.5)	not reported
Kolb 1999[8]	before 1986–1996	auto and allo	EBMT registry	1036	53 solid, 14 skin (8 BCC, 4 SCC, 2 melanoma)	SIR 3.8	3.5% at 10 years, 11.5% at 15 years	older age, treatment of GvHD with CsA (RR 2.5), thalidomide (RR 3.4)	not reported
Kulkarni 2000[9]	1973–1997	allo	UK	725	12 solid, 2 BCC	SIR 10.0 (all), skin 13.0 (skin)	0.4% at 2 years, 1.7% at 5 years, 6.4% at 10 years and 6.6% at 15 years	use of additional cranial or cranio-spinal irradiation, ALL, cGvHD (all patients with SCC)	7 years (2–17 years)
Bhatia 2001[10]	1976–1998	auto and allo	USA	2129	29 solid, 9 NMSC (3 SCC and 6 BCC)	SIR 5.3 (excluding BCC and SCC)	1.6% at 5 years, 6.1% at 10 years (all)	TBI, cGvHD (all SCC patients had GvHD), age <34 years	not reported
Baker 2003[4]	1974–2001	auto and allo	USA	3372	147 all, 62 solid, 19 BCC and SCC, 8 melanoma	SIR 8.1 (all), 2.8 (solid tumors), 8.3 (melanoma)	6.9% (all), 3.8% (solid) at 20 years	older age ≥20 years (solid, RR 2.0)	not reported
Curtis 2005[11]	1964–1996	allo	CIBMTR registry	24011	58 SCC (19 skin SCC), 22 melanoma, 103 BCC (excluded form analysis)		1.1% at 20 years	long duration of cGVHD therapy, azathioprine, particularly when combined with cyclosporine and steroids, severe GvHD	not reported
Shimada 2005[12]	1981–2000	auto and allo	Japan	809	19 solid, 0 skin	SIR 2.8	1.9% at 5 years, 4.2% at 10 years	extensive cGvhD, older age	range: 12–139 months
Hasegawa 2005[13]	1970–1993	allo	Canada	557	additional 31 (all), 4 SCC, 5 BCC	SIR 5.13 (all)	4.2% at 10 years, 6.17% at 15 years (all)	older age	6.8 years (0.5–21.9 years)
Brown 2005[14]	1982–1997	auto	USA	605	42 solid, 39 NMSC (26 BCC, 13 SCC), 5 melanoma	SIR 5.9 melanoma (calculated)	21% at 10 years (all), 10% at 10 years (excluding MDS/AML)	older age	not reported
Leisenring 2006[15]	1969–2003	allo	USA	4810	237 with at least 1 NMSC (158 BCC, 95 SCC)	not reported	6.5% (BCC), 3.4% (SCC) at 20 years	TBI (most strongly if <18 years), light skin color (BCC), aGvHD (SCC), cGvHD (both BCC and SCC)	BCC 7.9 years (0.5–32.0 years), SCC 6.3 years (0.3–24.8 years)
Cavalier 2006[16]	not reported	allo	USA	49	6 patients (14 SCC, 2 BCC, 2 melanoma); 1 patient had 10 recurrent SCCs	not reported	not reported	not reported	2–26 months
Gallagher 2007[17]	1985–2003	allo	Canada	926	30 solid, 4 SCC, 8 BCC	SIR 1.85	3.1% at 10 years	older age at HSCT, female donor	6.8 years (BCC 7.6 years, SCC 2.1 years)
Schwartz 2009[18]	1969–2006	auto and allo	USA	6306	282 BCC	not reported	not reported	TBI, younger age at HSCT, whites, cGvHD (non-irradiated patients)	not reported

Table 1. Outline of the studies analyzed – cont

Study (first author, date)	Years	Type of HSCT	Population and country	Number of points	Number of secondary neoplasms (solid and skin cancer given if reported)	Increased risk compared with general population	Cumulative incidence	Risk factors	Time from transplantation-diagnosis; median (range)
Rizzo 2009[19]	1964–1996	allo	CIBMTR registry	28874	189 solid, 18 melanoma, 19 invasive skin SCC (BCC excluded from analysis)	SIR 2.09 (all), 3.5 (melanoma), 4.2 (skin)	2.5% at 10 years, 5.8% at 15 years, 8.8% at 20 years (all neoplasms); 1% at 10 years, 2.2% at 15 years, and 3.3% at 20 years (solid cancers)	cGvHD (skin RR 11.0), male sex (skin RR 11.9), irradiation – TBI (non-SCC tumors RR 2.3), T-cell depletion	not reported
Abou-Mourad 2010[20]	1981–2002	allo	Canada	429	20 solid, 11 skin	not reported	not reported	not reported	6.3 years (0.12–17.3 years)
Chen 2011[21]	1984–2004	allo	Taiwan	170	8 solid, 0 skin	not reported	2.89% at 10 years, 3.82% at 15 years	cGvHD, age >40 years	10 years (5.2–20.8 years)
Majhail 2011[22]	1986–2005	allo	CIBMTR registry	4318	66 solid, 4 melanoma	SIR 1.4 (all), 1.38 (melanoma)	1.2% at 10 years (AML) 2.4% at 10 years (CML)	cGvHD	6 years
Yokota 2012[23]	1984–2005	allo	Japan	2062	30 solid, 6 skin (1 SCC, 3 BCC, 1 melanoma, 1 myxofibrosarcoma)	SIR 2.16 (all) 40.23 (skin)	0.9% at 5 years, 2.4% at 10 years, 6.7% at 15 years	cGvHD, lymphoma	5.6 years
Shimoni 2013[24]	1999–2012	allo	Israel	931	27 patients, 8 skin (7 SCC, 1 melanoma)	SIR (excluding skin SCC) 2.0 (all)	5.6% at 10 years (all), 1.5% at 10 years (skin)	fludarabine-based conditioning, moderate and severe GvHD, diagnosis of chronic mieloproliferative or non-malignant disease	43 months (7 months–11.5 years)
Krishnan 2013[25]	1989–2009	auto	USA	841	53 solid, 13 BCC, 14 SCC, 4 melanoma	not reported	7.4% at 5 years, 15.9% at 10 years (all), 1.9% at 5 years, 4.7% at 10 years (only NMSC, calculated)	age >55 years, non-Hispanic white race	not reported
Bilmon 2014[26]	1992–2007	auto	Australia	7765	298 all, 56 melanoma (BCC and SCC excluded)	SIR 1.4 (all), 2.6 (melanoma)	5.28% at 10 years (all), 4.22% at 10 years (solid)	age ≥45 years, male sex	3.0 years (0.1–14.6 years)
Atsuta 2014[27]	1990–2007	allo	Japan Transplant Registry	17545	269 solid, 13 skin	SIR 1.8 (solid), 7.2 (skin)	0.7% at 5 years, 1.7% at 10 years, 2.9% at 15 years	cGvHD, older age >30 years	not reported
Omland 2016[28]	1999–2014	auto and allo*	Denmark	3302	11 melanoma, 8 SCC, 53 BCC	allo: SIR 3.1 (BCC), 18.3 (SCC), 5.5 (MM), auto: SIR 1.4 (BCC)	allo: 5.3% at 10 years, auto: 4.7% at 10 years (BCC)	TBI (BCC RR 3.9)	not reported
Michelis 2017[29]	1970–2015	allo	Canada	2415	209 all, 32 BCC, 26 non-metastatic SCC, 5 SCC with meta, 10 melanoma	SIR 2.07 (excluding non-metastatic NMSC)	6.3% at 10 years, 13.5% at 20 years, 17.6% at 30 years	older age >55 years compared to ≤40 years, RIC	not reported
Song 2017[30]	1994–2013	allo	USA	85	1 BCC, 4 SCC	not reported	not reported	not reported	not reported
Inamoto 2018[31]	1990–2013	auto and allo	Japan	31867	713 all, 28 skin	not reported	not reported	not reported	5.9 years (1.0–25.0 years)

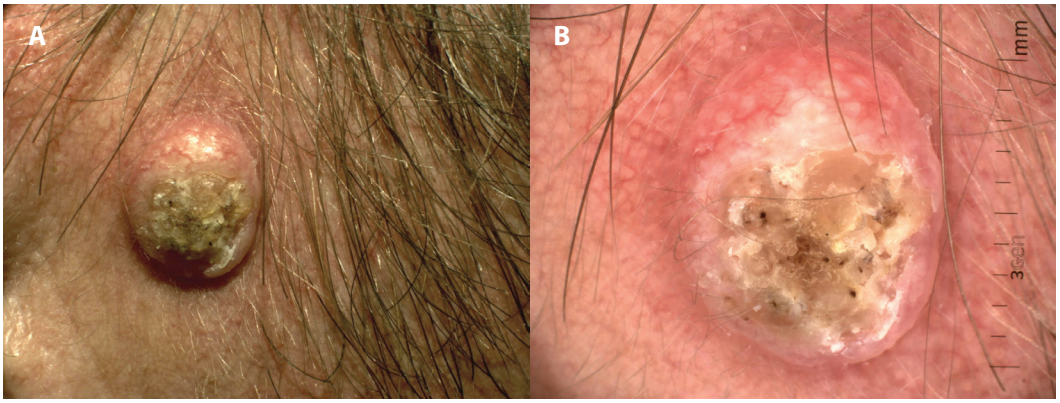

Fig. 5. Raised skin lesion in a 54-year-old male patient suffering from cutaneous chronic GvHD after alloHSCT for myelofibrosis (day +750). Histopathologic examination after total surgical excision of the presented lesion confirmed the diagnosis of keratoacanthoma. A. A rapidly growing skin lesion appeared within a one-month period as a dome-shaped 1 cm nodule with the central keratin masses. B. Dermoscopy in polarized light revealed the presence of central keratin masses with several blood spots and several branched serpentine vessels located at the periphery

neoplasms.[16] Multiple myeloma was also linked with secondary neoplasms.[25,26] Rizzo et al. reported lower risk for patient with chronic myeloid leukemia compared to acute leukemia,[19] but this claim was not confirmed in other studies.[20]

Transplantation-related factors

Initially, in the HSCT procedure myeloablative conditioning using total body irradiation (TBI) or high-dose alkylating agents was performed. Presently, non-myeloablative conditioning that utilizes transient intensive immunosuppression enabling engraftment of allogeneic material without myeloablation is frequently used. Reduced intensity and toxicity conditioning (RIC/RTC) were also developed. The incidence of secondary neoplasms is not lower in the setting of non-myeloablative or RIC[16,24]; in some reports, the incidence was even higher.[29] Presently, the widespread use of haploidentical transplantation with post-transplant cyclophosphamide is noted, but there is scarce data on secondary neoplasms in this population.

All alkylating agents are considered carcinogenic, and the effect varies among the drugs in this group. There is concern regarding the high neogenic potential of melphalan; however, its use in conditioning was not associated with a higher risk of second malignancies than cyclophosphamide and TBI.[9] Also, neither cyclophosphamide, busulfan conditioning, cyclophosphamide-based mobilizing therapy, nor epipodophyllotoxins were found to increase the risk of secondary neoplasms in autoHSCT recipients treated for multiple myeloma.[25] Fludarabine is associated with the risk of secondary malignancies,[24] as its active anti-metabolite incorporates into DNA, resulting in the inactivation and inhibition of DNA repair. It is speculated that the combination of fludarabine with alkylating agents as given in RIC/RTC may have a synergistic carcinogenic effect.[24]

Radiation

The impact of radiation seems to depend on the age of exposure; younger people were reported to have almost a ten-fold higher risk of secondary neoplasms, while for older patients it was only slightly elevated.[19] In a study focusing on BCC, TBI was reported as a risk factor, especially in patients <18 years, with relative risk exceeding 20 for those <10 years and with light complexion; however, the impact declined with age and there was no increased BCC risk after TBI conditioning in patients over the age of 40.[18]

The risk of radiation-related malignancies and other long-term adverse effects, particularly in children, has led from fractionation and reduction of doses to the development of non-radiation-based regimens. A study involving a population of patients without TBI exposition, utilizing busulfan/cyclophosphamide, reported lower rates of secondary cancers, although still 1.4-fold higher than general population.[22] However, the role of TBI remains unresolved, with some publications providing strong evidence of increased risk from TBI exposure,[15] and others failing to identify this association.[9,20,29]

Immunosuppression and graft-versus-host disease

After auto- and alloHSCT, a period of lymphopenia and cell-mediated immune deficiency occurs and can persist for months. Adequate numbers of CD4+ T-lymphocytes have been postulated to inhibit malignant transformation of precursor skin lesions, and their lack is reflected in the most common cancer related with AIDS – Kaposi sarcoma.[35] There are also reports of telomere shortening after alloHSCT, which can cause significant genomic instability, leading to malignant transformation.[36] What is more, low-grade inflammation may not be clinically perceived; in a study by Vasallo et al., "normal-looking"

skin in 76% of patients around day 100 after HSCT showed inflammatory abnormalities in histopathological assessment.[37] In the case of graft-versus-host disease (GvHD) with persistent inflammation, ulceration and scarring may itself be a risk factor for carcinogenesis. Repeated cell division correlates with chromosomal abnormalities in keratinocytes, including aneuploidy and haploinsufficiency for p53 due to loss of chromosome 17.[36]

Immunosuppressed patients are at risk of developing cutaneous neoplasm[35] and immunosuppression is a mainstay of GvHD treatment. Recent meta-analysis encompassing 50951 HSCT recipients showed that chronic GvHD (cGvHD) was associated with increased incidence of BCC (RR 1.95) and SCC (RR 5.31) but had no effect on melanoma and acute GvHD (aGvHD) alone was not associated with increased risk of skin cancer.[38] A large case-control study of 24011 HSCT recipients[11] reported that the risk of SCC was almost three-fold higher in those with cGvHD and even higher with previous aGvHD. Therapy of GvHD with azathioprine, cyclosporine, steroids, psoralen, ultraviolet A (PUVA), and others increased the risk for SCC 18 to 50-fold. Several other studies consistently reported GvHD as a risk factor.[16,18,27]

Azathioprine in GvHD treatment was identified as the major risk factor for the development of secondary cancers after alloHSCT.[11] Possible mechanisms include incorporation of activated azathioprine forms into DNA, making it susceptible to mutagenic oxidation, especially after radiation therapy.[39] Cyclosporine, a calcineurin inhibitor, commonly used in immunosuppression after transplant, is a carcinogen causing induction of phenotypic changes and enhancement of invasiveness of cells through transforming growth factor ß (TGF-β) mechanisms.[40] Topical pimecrolimus use was questioned due to its similarity to oral calcineurin inhibitors. In the pediatric population, it was linked to the development of precancerous and cancerous lesions.[30]

Voriconazole, a recommended antifungal agent both in prophylaxis and treatment, was found to increase the risk of skin cancer and precancerous lesions in HSCT recipients.[30,41] A multistep mechanism of carcinogenesis involving acute phototoxicity, then actinic keratosis followed by SCC was suggested.[42]

Oncogenic viruses

Oncogenic viruses in the context of prolonged immunosuppression also participate in pathogenesis of tumors after HSCT. Human papillomavirus (HPV) infection was suggested to be involved in the development of non-melanoma skin cancer.[35] Cancers associated with oncogenic viruses express oncoproteins inactivation p53, which is associated with the development of BCC and SCC. HPV DNA, especially types 5 and 8, was detected more frequently in SCC of transplant recipients than in non-immunosuppressed patients.[43] However, in 1 study, none of the oral SCCs showed evidence of HPV infection.[21]

Hematopoietic stem cell transplantation and nevi

In a recent study comprising pediatric patients,[30] the patients after HSCT had significantly more nevi and 16.5% of HSCT recipients developed cancerous or precancerous lesions.[30] The majority of nevogenesis occurs in childhood; in a study of adult and pediatric HSCT recipients, an increase in the nevus count was found only among those aged <20 years at HSCT.[44] In addition, children may be at a higher risk of thymic dysfunction after HSCT, with impaired immunosurveillance, possibly contributing to the development of secondary malignancies and nevogenesis.[30] In another study, the number of nevi was not significantly increased after HSCT,[44] although a group of patients who were conditioned with a combination of 2 alkylating drugs at high doses and younger patients tended to have a higher count of nevi. Conversely, there was a trend in favor of a lower count of nevi in patients presenting with cutaneous cGvHD. Alloimmunity, chronic skin inflammation with overproduction of pro-inflammatory cytokines or pigmentation, areas of depigmentation, leukoderma, and fibrosis in cGvHD may be responsible for the perceivable decreased number of nevi, although this observation requires further investigation.[44,45]

Differences between primary and secondary skin cancers

Risk factors for the development of skin cancer in the general population, including fair skin type, advanced age, exposure to UV radiation, and genetic predisposition, seem to play a role in the initiation and progression of carcinogenesis in skin of HSCT recipients.[15,18,25,30] However, SCC after transplantation may not share all conventional risk factors; in an evaluation of SCC of the buccal cavity, no excess risk was linked to alcohol or tobacco use.[11]

Secondary malignancies after alloHSCT tend to behave more aggressively in these patients than primary ones in immunocompetent individuals, and they have a higher risk of metastasis[46] and are often multiple.[47,48] Adjusted overall survival probabilities were lower in patients with subsequent cancer compared with those with primary cancer in the general population for colon, central nervous system and bone/soft tissue cancers after allogeneic HSCT.[31] Michelis et al.[29] found that 40 of 209 patients (19%) with secondary malignancy developed another one, including 13 patients with local skin cancers recurrences and 12 patients presenting with SCC or BCC before other solid malignancy. In the study, 22% of long-term survivors' deaths were attributable to secondary neoplasms. Of note, 4 out of 5 metastatic cutaneous SCC carcinomas in this study were reported to be a cause of death.[29]

In a study by Inamoto et al.,[31] secondary cancers occurred in alloHSCT recipients at a younger age than primary cancers in the general population (median 55 compared to 67 years).

Differences between solid organ transplantation and HSCT

The risk of secondary skin malignancies is high in solid-organ transplant recipients and has been extensively studied.[49] The incidence of cutaneous SCC in solid-organ transplant recipients is 65- to 250-fold greater than in the general population, and this cancer has greater morbidity and mortality in solid-organ recipients than in the general population.[50] The risk factors include cumulative ultraviolet radiation exposure, long-term use of immunosuppressive agents and infections by human papillomaviruses. Several guidelines and risk prediction tools have been established for this population of patients.[49] Recent research observed a reduction in cumulative incidence of secondary cancers when sirolimus was used instead of cyclosporine.[51] Emerging possible chemoprophylaxis include retinoids, antioxidants, difluormethylornithine, and cyclooxygenase-2 (COX-2) inhibitors.[49,51]

For solid-organ transplant recipients, the duration of immunosuppressive therapy is usually lifelong, whereas in HSCT recipients, it may be discontinued after transplantation if they do not develop GvHD. Thus, prolonged immunosuppression and GvHD are usually linked. On the other hand, solid organ recipients rarely develop GvHD, which itself causes processes of tissue destruction and possible tumor development. Omland et al. compared HSCT recipients with renal transplant patients; alloHSCT recipients had a three-fold higher risk of melanoma, similar risk of BCC and lower risk of SCC.[28]

Limitations of the studies

There are several limitations in the published studies. Reports with long follow-up reflect the then used transplantation strategies, which have since greatly changed. In older reports, bone marrow as a source of stem cells and HLA identical matched sibling donor with myeloablative conditioning were predominantly used. Presently, peripheral blood cells are dominant as a graft source, and alternative conditioning regimens – RIC and haploidentical transplantations – are commonly utilized.[52] Furthermore, immunosuppression strategies and treatment of GvHD have changed. Some studies reported combined results of allo- and autoHSCT, and others included a considerable number of pediatric patients among the adults, resulting in heterogeneity of population. Most established diagnoses of second neoplasms from hospital records or from patient's self-reports potentially underestimate risk, particularly in patients without other post-transplant complications. What is more, there is a lack of information on cancer stage at diagnosis, localization of lesions and treatment details.

National cancer registries rarely include information on non-melanoma skin cancer. Only some of the studies included an assessment of the risk in comparison to the general population. The majority of studies did not include non-melanoma skin cancers in the analysis, citing low mortality and unknown incidence of BCC and SCC, so it was not possible to assess excess risk, SIR or specific factors for development of secondary skin cancers. Retrospective studies lack assessment of Fitzpatrick's skin phototype and detailed patient history. No prospective study with pre-transplant skin assessment focusing on risk of skin neoplasms have been published. Other issues are long latency period necessary for the occurrence of these complications and a relatively low numbers of events. Follow-up of some of the studies may be too short to predict the actual incidence of skin cancer.

Additional studies with systematic data collection and comprehensive reporting with extended follow-up are needed to characterize the incidence and actual risk for developing skin cancer.

Screening and preventive measure recommendations

Patients after HSCT should follow the general population recommendations: avoidance of carcinogenic agents such as tobacco and alcohol, and use of sun-protection measures. Specific guidelines for prophylaxis are consensus-based and include whole skin and mucous membranes assessment by dermatologist every 12 months.[3,53,54] In patients with a history of cutaneous malignancies or GvHD, screening interval should be shortened to at least 6 months.[55] Patients should be educated about prevention and recognition of skin cancers.[55] The role of the dermatologist in the care of HSCT recipients is important and includes also diagnosis and treatment of cutaneous GvHD; thus, the development of dedicated dermatology service for allogeneic HSCT was proposed.[56] Efforts to prevent GvHD and to improve immune reconstitution after transplantation may be an effective strategy of preventing secondary tumors. During the assessment of skin lesions in HSCT recipients, it is important to consider a possible differential diagnosis that includes a plethora of GvHD manifestations, cutaneous manifestation of primary neoplasms, infectious lesions, and others.

Heightened awareness and more vigilant skin surveillance are warranted for patients with GvHD who received TBI-based conditioning, and those with hereditary disorders associated with cancer risk, such as Fanconi anemia. Discontinuation of voriconazole may be considered in patients experiencing chronic phototoxicity. Suspicious lesions should be addressed promptly, with management complying with standard practice, but the treatment plans should include previous history.[57] There are no studies on specific skin cancer preventative measure in the population of patients after HSCT. It would be valuable to find whether preference of mTOR inhibitors, such

as sirolimus, is protective, as was shown in solid-organ transplant recipients.[51]

Studies on HSCT recipients have reported generally high adherence rate to cancer screening; however, it was reported that autoHSCT survivors were less likely than alloHSCT to have a skin examination in the previous year.[58] Physicians should have lower thresholds to investigate new concerning signs or symptoms of malignancy in patients after HSCT than for the general population. Many transplantation centers expect to receive notification if their survivors develop second cancers.[57] Regular dermatological surveillance and prompt recognition of precancerous and cancerous lesions is crucial for a patient's prognosis and management.

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Adalimumab in the treatment of non-infectious uveitis

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Abstract

Non-infectious uveitis (NIU) is a serious sight-threatening condition whose pathogenesis is often autoimmune in nature. It may manifest in any age group, though adults aged 20–50 are the group most often affected. It causes 5–10% of visual impairment worldwide. The epidemiology of some specific uveitis diseases varies worldwide, because they are influenced by genetic, environmental and socioeconomic factors. It can occur only in the eye or as a symptom of a systemic condition. The most common cause of NIU is HLA-B-27-associated anterior uveitis (4–32%). The standard treatment for NIU is a local, topical and systemic steroid therapy in combination with immunomodulatory therapy. However, recently, a new drug – adalimumab, which is a tumor necrosis factor α (TNF-α) inhibitor – was approved by FDA in the treatment of NIU and is increasingly used to treat various conditions. Adalimumab has been proven in many studies to be safe and effective in the treatment of NIU associated with diverse systemic diseases.

Key words: non-infectious uveitis, adalimumab, biologic therapy, TNF-α inhibitors

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Introduction

Uveitis is a common cause of blindness worldwide,[1] which in turn may be caused by infectious or non-infectious factors.[2] Non-infectious uveitis (NIU) is often associated with an underlying systemic, vision-threatening disease. It is characterized by inflammation in the uveal tract and generally occurs more often in the developed world. The most common causes of NIU are HLA-B27-associated anterior uveitis, sarcoidosis, Vogt–Koyanagi–Harada syndrome, sympathetic ophthalmia, birdshot chorioretinopathy (BSCR), multifocal choroiditis, serpiginous choroiditis, and Behçet's disease (BD).[2]

Currently, the mainstay in the treatment of uveitis is steroid therapy, focused on suppressing the severity of inflammation.[1,3] Although corticosteroids are often effective, ocular and/or systemic adverse effects limit their long-term use.[4,5] Other therapeutic options for primary or secondary NIU include traditional immunosuppressants, such as cyclosporine (CsA), methotrexate (MTX), azathioprine (AZA), sulfasalazine (SSZ), and mycophenolate mofetil (MMF). However, a significant proportion of uveitis cases cannot be controlled only using corticosteroids and immunosuppressants.[6] A new promising drug approved for the treatment of NIU in adults is adalimumab.[5,7] Adalimumab is a recombinant human immunoglobulin monoclonal antibody that specifically binds to tumor necrosis factor α (TNF-α).[1,6,8] Although there are other TNF-α inhibitors, such as etanercept and infliximab, adalimumab has been proven to have an advantage in therapeutic treatment, probably because of its higher affinity to bind with TNF-α than etanercept or infliximab.[9]

Tumor necrosis factors are a group of cytokines that are produced by activated macrophages, CD4+lymphocytes and natural killer cells. These cytokines are responsible for inducing inflammation and apoptosis and for inhibiting viral replication.[10,11] Moreover, TNF-α is found to upregulate vascular endothelial growth factor (VEGF) production in choroidal endothelial cells, and VEGF is responsible for macular edema in uveitic patients. This explains the successful outcome of adalimumab treatment, reducing VEGF-α levels in the plasma by inhibiting TNF-α production in the treatment of uveitic macular edema.[11,12] The pro-inflammatory cytokine TNF-α is thought to play a key role in uveitis inflammation, and aqueous humor and serum levels of TNF-α are upregulated in uveitis patients.[6,13] Tumor necrosis factor α binds to specific membrane receptors (TNF-α receptors I and II). In the eye, TNF-α receptors I and II are located at the surface of the pigment epithelium cells of the iris, ciliary body and retina.[11]

Adalimumab binds to TNF-α, inhibiting its pro-inflammatory and apoptotic action

Apart from its beneficial effect, adalimumab may lead to severe side effects, the most frequent of which are infections. In a long-term analysis of 23,458 patients, there were reported cases of cellulitis, pneumonia, appendicitis, herpes zoster, urinary tract infection, gastrointestinal tract abscess, and gastroenteritis. Other severe side effects, such as tuberculosis, opportunistic infections, demyelinating disorders, lupus-like syndrome, and congestive heart failure, were rare. The incidence of malignancies among patients treated with adalimumab was similar to that of malignancies in a reference population. The overall rates of lymphoma were greater in a rheumatoid arthritis (RA) group treated with adalimumab,[14] but RA increases the risk of lymphoma itself[15] and the data showed that the incidence of lymphoma among RA patients treated with adalimumab was the same as among the RA patients who had never been treated with anti-TNF-α.[16]

Clinical studies of adalimumab in non-infectious uveitis

Ming et al. analyzed 3 randomized clinical trials and 20 non-randomized clinical trials in order to summarize the current evidence of the efficacy and safety of adalimumab in treating patients with NIU.[3] The primary outcome of the analysis was a decrease in inflammatory activity. The inflammatory activity of uveitis was found to have decreased by 74% and 79% in their combined analysis of short and relatively long-term follow-up periods, respectively. In addition to this effectiveness, the meta-analysis revealed other promising results, demonstrating that adalimumab is a reasonable choice in the treatment of uveitis. It controlled the worsening of visual acuity (88.8% of involved eyes), reduced the use of corticosteroids, significantly so in ¾ of the patients, and was generally well-tolerated, thus proving to be safe. Patients with juvenile idiopathic arthritis, BD and HLA-B-27-associated uveitis were studied.

A multicenter retrospective study was conducted by Bitossi et al. to assess the long-term ocular control of adalimumab in a population with non-infectious primary or secondary uveitis.[6] The main goal of the study was to evaluate its steroid-sparing effect and co-treatment with disease-modifying anti-rheumatism drugs (DMARDs). A total of 106 eyes were included. The effective ocular control was 83.7% and 83.3%, after 6 and 12 months, respectively. Patients with poor baseline visual acuity remained stable or improved, while those with good vision acuity worsened. Other findings of the study were that the steroid dosages were reduced and that ocular control and the steroid-sparing effect were not influenced by the concomitant use

of DMARDs. Mild or moderate side effects were reported in 6.6% of the patients.

The most significant studies on adalimumab in NIU are the VISUAL studies – multicenter randomized, controlled trials.[4,17] The VISUAL I, VISUAL II and VISUAL III studies involved 217, 229 and 371 patients, respectively. In the first 2 rounds of the study, participants (aged ≥18 years) with active, non-infectious intermediate or posterior uveitis or panuveitis were enrolled. The risk of treatment failure for participants in the adalimumab group was lower than that of the participants in the placebo group, by 44% in VISUAL I and by 48% in VISUAL II. The incidence of adverse effects in both studies was comparable between the placebo and adalimumab groups. The most common adverse effect was injection site reaction. There were also 2 cases of malignancies each in VISUAL I and VISUAL II; 2 participants had tuberculosis in VISUAL I and 3 in VISUAL II. One case of demyelinating disorder, lupus and lupus-like reaction was reported. However, the most relevant conclusion of these studies is that adalimumab lowered the risk of uveitic flare or visual acuity loss in the participants with active and controlled uveitis who were at risk of the long-term side effects of corticosteroids (CS).

The VISUAL III study lasted 78 weeks. As before, the main goal of the study was to assess the efficacy, safety and steroid- and immunomodulator-sparing effect of adalimumab. To determine its efficacy, anterior chamber activity, vitreous activity and mean best-corrected visual acuity (BCVA) were analyzed at 0, 12 and 78 weeks. The results revealed an improvement in ocular control inflammation and mean BCVA, but only in patients who had active uveitis initially. The BCVA remained stable over time in patients with inactive uveitis. For patients with active uveitis at study entry, the observed mean daily corticosteroid dose had decreased by 75% at week 78 and the mean dose of immunomodulators had decreased by 26% at week 78 in comparison with week 8.

Adalimumab in the treatment of selected disorders

HLA-B-27-related uveitis

Most patients with HLA-B27-associated uveitis have an associated spondyloarthropathy (AS); the archetypal disease is ankylosing spondylitis. Other forms of spondyloarthropathy include reactive arthritis (Reiter's syndrome), arthritis associated with inflammatory bowel disease, psoriatic arthritis, undifferentiated spondyloarthropathy, and juvenile forms of these diseases. The most typical HLA-B27-related uveitis is a sudden onset, unilateral, recurrent, anterior uveitis.[18] The pathogenic mechanism underlying HLA-B27 uveitis is strongly associated with pro-inflammatory cytokines, among which TNF-α plays a key role. Pérez-Guijo et al. proved that by analyzing samples of the aqueous humor concentration of TNF-α among HLA-B27-positive patients and B27-negative patients. The concentration of TNF-α in the aqueous humor of HLA-B27-positive patients was significantly higher than in those who were HLA-B27-negative.[19]

The most relevant study on anterior uveitis in active AS was an open-label, uncontrolled study on 1,250 patients.[20] It was conducted to assess the effect of adalimumab on the frequency of anterior uveitis in active spondylitis. Adalimumab was administered every week for 12 weeks. Adalimumab effectiveness was assessed by measuring the rate of anterior uveitis flare. The effect was at least a 50% reduction in the anterior uveitis flare rate during adalimumab treatment in patients with active AS.

Kim et al. analyzed the medical records of 143 patients diagnosed with HLA-B27-positive AS who were taking an anti-TNF-α agent for at least 1 year.[21] Among all the patients enrolled in this study, 45 were treated with adalimumab, while the others were administered infliximab and etanercept. Almost ¾ of the patients receiving adalimumab had a history of uveitis. The examination was done at baseline and after 1, 2 and 12 months of treatment. During the follow-up, uveitis inflammation decreased after 17.9 ±6 days among all of the patients, as assessed according to the Standardization of Uveitis Nomenclature (SUN) Working Group. Almost 80% of the patients had no recurrence of uveitis during anti-TNF-α treatment. A decrease was observed in the number of systemic medications in 93.3% of the patients. Minor side effects were noted in 7 patients, while 1 case of tuberculosis was diagnosed.[16]

Sarcoidosis

Sarcoidosis is a multisystemic, idiopathic disorder that is characterized by the formation of immune granulomas in the involved organs.[22] The eye is often the first organ affected by the disease, which can lead to severe visual impairment. Any part of the uvea can be involved in the pathogenic process, as well as other parts of the eye and its adnexa. Most sarcoid uveitis is bilateral and chronic.[23]

Erckens et al. enrolled 26 patients with sarcoidosis who were being treated with adalimumab due to refractory posterior uveitis.[24] Before adalimumab was administered, all of the patients were treated with oral corticosteroids, and MTX as the second-line agent if there was no response to corticosteroids. The patients were examined at baseline and after 6 and 12 months of treatment. At baseline, they manifested various intraocular inflammatory signs, such as papillitis, macular edema, choroidal involvement, vasculitis, and vitritis. After 6 months, vitreous involvement and vasculitis resolved in all 26 patients; papillitis was resolved in 7 patients (of the 8 in whom it was present at baseline), while 1 had a partial response; macular edema resolved in 5 of 8 patients, while the remaining 3 had a partial response; choroidal involvement was initially present in 15 patients and resolved in 10 of them,

while the remainder had a partial response. The final result was improvement in 85% of patients and stabilization in 15%; there was no difference in the results after 6 or 12 months. Only 1 patient had a serious side effect at the injection site, which caused a subcutaneous mass to form.

In another open-label, multicenter study, 17 patients were enrolled to assess anti-TNF-α therapy among patients with uveitis related to sarcoidosis and refractory to standard treatment. The outcomes assessed after 2 years were visual acuity, macular thickness, intraocular inflammation, and a corticosteroid- and immunosuppressive-sparing effect. All of them show improvement 2 years after the onset of adalimumab therapy.[25]

Behçet's disease

Behçet's disease is an idiopathic, chronic, multisystem inflammatory vasculitis characterized mainly by recurrent oral aphthous ulcers, genital ulcers and severe intraocular inflammation.[26] Any part of the uvea can be involved in the pathogenic process, and the frequency of ocular involvement is in the range of 50–70%. Clinical manifestations of acute inflammation are typically self-limiting over time with relapsing episodes of varying intensity. The frequency and duration of relapses are unpredictable and follow no discernible pattern of onset. Occasionally, the cardiovascular system, the central nervous system and the gastrointestinal tract can be involved in the pathogenic process.[26] The strongest genetic factor related to BD is HLA-B-51 antigen. The presence of HLA-B-51 may lead to BD in both the adaptive and innate immune responses. An increased expression of pro-inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α, among patients with BD has been reported, which proves the role of an innate immune response in the pathogenesis of BD.[27]

In a multicenter retrospective, observational study, the medical histories of 44 patients (66 eyes) with BD-related uveitis were analyzed.[28] The medical data of this study was analyzed at baseline, after 3 months of treatment and after 12 months. Almost all of the patients (97.5%) had previously been treated with steroids; some of them (32.5%) were treated with other biological agents, and some of them had taken DMARDs (77.5%). The steroid treatment was continued with adalimumab in 92.5% of patients and with DMARDs in 42.5% of patients. Significant improvement was demonstrated in all of the factors under study, the number of flares, BCVA and optical coherence tomography (OCT) findings regarding central macular thickness and vasculitis, so visual, clinical, functional, and morphological improvement was observed. The other relevant finding of this study was that a combination of adalimumab and other immunosuppressive agents had no superior benefit over monotherapy. Patients co-administered with DMARDs had more flares, but this could also be due to a higher activity of the disease at baseline, making it necessary to administer the combination therapy early on.

Birdshot chorioretinopathy

Birdshot chorioretinopathy is a non-infectious, bilateral panuveitis. The pathogenic mechanism of BSCR is not well-understood; several pathways have been proposed, but there is definitely a strong association between the presence of the HLA-A29 molecule and BSCR.[29] Almost all patients are Caucasian adults, and women are affected more often than men.[30] The characteristic features of BSCR are yellow-white choroiditis spots. Most of the patients also have cystoid macular edema, caused by diffuse retinal vasculitis.[30]

Huis Het Veld et al. analyzed the medical records of 19 patients (38 eyes) with HLA-A29- positive BSCR who received adalimumab treatment.[31] None of the patients enrolled in this study had responded to previous standard systemic immunomodulatory therapy. The analyzed records were from 1 year before adalimumab treatment, at baseline and after 1 year of treatment. The most relevant observation was an improvement in visual function after 1 year of adalimumab therapy. As in the previously mentioned studies, adalimumab was generally well-tolerated and allowed the dosages of co-administered immunosuppressants to be lowered. However, the ocular inflammation control was poorer than that of the other previously mentioned diseases. Only 2 of 9 patients experienced a complete remission of inflammation (as measured using OCT and fluorescein angiography).

In another retrospective case series study, the medical records of 3 patients were analyzed.[32] All of the patients were HLA-A29-positive. Before adalimumab was introduced, all patients were treated with oral prednisolone (>10 mg) and 2 second-line immunosuppressive agents. The most relevant outcome was a significant decrease in central macular thickness after 6 and 12 months of adalimumab treatment. The BCVA improved in 4 eyes after the introduction of adalimumab, and improvement was noted in at least 1 electrodiagnostic parameter. The reduction in central macular thickness allowed the dosages of systemic immunosuppressive drugs to be lowered.

Sympathetic ophthalmia

Sympathetic ophthalmia is a rare, bilateral, non-necrotizing granulomatous uveitis. The incidence ranges from 0.2% to 0.5% following injury and 0.01% following intraocular surgery.[33] The eye which has undergone trauma or surgery is the exciting eye, and the other eye is the sympathizing eye. The clinical manifestations may be anterior, cells and flare in the anterior chamber or mutton fat keratic precipitates, or posterior, vitritis with yellowish-white subretinal lesion, papillitis, choroiditis, exudative retinal detachment, or choroidal granulomas. A diffuse, granulomatous inflammatory response of the uveal tract is caused mainly by infiltration of the t-lymphocytes.[34] So far, steroidotherapy alone or combined with other immunosuppressive agents,

such as CsA or AZA, has been the mainstay of sympathetic ophthalmia treatment.[33] However, Kim et al. and Hiyama et al. published some case reports proving that adalimumab can lead to successful outcomes in the treatment of sympathetic ophthalmia.[35,36] So far, these are the only 2 papers concerning treatment with adalimumab in sympathetic ophthalmia to be published. Kim et al. published the case report of a young girl who developed sympathetic ophthalmia after an accidental injury to one eye. After initial treatment by local and systemic steroidotherapy and a minor decrease in inflammation activity, the girl developed weight gain and cushingoid habitus. The steroid treatment was tapered off and methotrexate was initiated. The patient continued to have inflammation, so adalimumab was administered. Within 3 months, the inflammation had completely resolved, and the steroids were discontinued. After 6 months of stability on adalimumab, methotrexate was tapered off and discontinued over the course of 6 months. After 18 months of adalimumab therapy, no evidence of recurrent inflammation was observed.[35] Hiyama et al. reported 2 cases of sympathetic ophthalmia following trabeculectomy.[36] In both cases, the initial treatment was steroids, but in the 1st case, although the serous retinal detachment resolved, the steroids had to be discontinued because of diabetes mellitus. Methotrexate, the second-line treatment, was not tolerated, and while CsA was introduced, the serous retinal detachment relapsed. Lastly, adalimumab was administered, which led to a remission of serous retinal detachment and control of inflammation for 7 months. In the 2nd case, serous retinal detachment did not resolve completely during steroid therapy, and steroids had to be discontinued because of the primary open-angle glaucoma. The second-line treatment, CsA was not tolerated, so adalimumab was introduced, leading to a remission of the serous retinal detachment and control of the ocular inflammation for 7 months.[36]

Conclusions

Based on the results of several clinical studies, adalimumab is a safe and promising therapy in NIU that preserves vision and controls ocular inflammation in the majority of associated systemic diseases. However, there are still some types of refractory uveitis, like BSCR, where complete remission of the inflammation is rarely achieved.

Moreover, adalimumab is also a reasonable choice as a steroid-sparing agent, limiting the ocular and systemic side effects that are associated with chronic corticosteroid use, as well as an immunosuppressant-sparing agent. In the majority of patients, it allows the disease to be controlled with minimal corticosteroid use. Even the side effects of adalimumab are rare and mild to moderate, though there is still a risk of severe side effects, such as infections and demyelinating disorders, which require cautious qualification for the treatment.

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