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Pursuant to the ordinance of the Rector of Wroclaw Medical University No. 12/XVI R/2023, from February 1, 2023, authors are required to pay a fee for each manuscript accepted for publication in the journal Advances in Clinical and Experimental Medicine. The fee amounts to 990 EUR for original papers and meta-analyses, 700 EUR for reviews, and 350 EUR for research-in-progress (RIP) papers and research letters.

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Contents

The impact of suction addition to simple water seal on the outcomes after pulmonary surgery: A meta-analysis

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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. Chest tube drainage during pulmonary surgery is fundamental to removing air and fluid, as well as for lung re-expansion. However, the advantages of adding external suction to the water seal are under debate.

Objectives. The aim of the study was to conduct a meta-analysis in order to assess the effects of adding suction to a simple water seal on the outcomes of lung surgery.

Materials and methods. A search of the literature up to November 2021 found 14 studies with 2449 lung surgery patients. Of these patients, 1092 received suction drainage and 1357 received a simple water-seal drainage. The studies reported the effects of adding suction to a simple water seal on postoperative outcomes after lung surgery. A random- or fixed-effect model determined the odds ratio (OR) or mean difference (MD) with 95% confidence intervals (95% CIs) to compare the outcomes.

Results. In patients undergoing lung surgery, suction resulted in a substantially longer chest tube duration (MD = 0.74, 95% CI: 0.90–1.40, p = 0.03, Z = 2.21) and a smaller postoperative pneumothorax (OR = 0.27, 95% CI: 0.13–0.59, p = 0.02, Z = 2.24) than a simple water seal. However, no differences existed in prolonged air leak (p = 0.91, Z = 0.12), air leak duration (p = 0.28, Z = 1.07) or length of hospital stay (p = 0.23, Z = 1.2) between the 2 approaches.

Conclusions. Suction led to considerably longer chest tube duration and lower postoperative pneumothorax, but no significant difference in sustained air leak, air leak duration or length of hospital stay was observed compared to a simple water seal in patients undergoing pulmonary surgery. Further research is needed to validate these findings and increase confidence, particularly regarding the postoperative pneumothorax results.

Key words: suction, pulmonary surgery, simple water seal, postoperative pneumothorax, prolonged air leak

Cite as
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Background

The fundamental principles of chest drainage systems are to allow air and fluid drainage, remove residual space in the pleura, allow lung re-inflation, and maintain negative pressure.[1] Currently, chest tube management applies 2 main techniques, non-suction-based drainage by a simple water seal or suction drainage via a water seal with external suction.[2] The applied technique depends on clinician preference,[3] and many believe that adding suction can minimize air leaks and, thus, expedite lung rehabilitation. In contrast, others believe that a simple water seal can reduce air leakage duration after lung surgery.[4–6] This difference in opinion arises from the question of whether the addition of external suction to the water seal is beneficial. The benefits of suction include fast removal of air, fluid leakage and lung expansion. However, the question of which technique is superior and leads to better patient outcomes remains debatable.[4]

Objectives

This meta-analysis aimed to investigate the impact of suction addition to a basic water seal on postoperative outcomes following lung surgery.

Materials and methods

The current investigation used an established protocol contingent on the statement of meta-analysis of studies in epidemiology.

Search strategy and selection of studies

The included studies reported the effects of suction addition to a water seal on lung surgical treatment and compared it with a simple water seal alone.

Only research conducted on humans was included, and there were no restrictions on language, size or study type. However, review articles, editorials and studies without a level of connection were all removed. The study protocol is depicted in Fig. 1. All articles included in the meta-analysis fulfilled the following criteria:

1. Designed as a prospective or retrospective randomized controlled trial (RCT);
2. The designated target population comprised patients undergoing lung surgery;
3. The intervention approach used suction and compared it to a simple water seal;
4. The investigation included comparisons of external suction with simple water seals.

The exclusion criteria were:

1. Studies with missing or incomplete data;

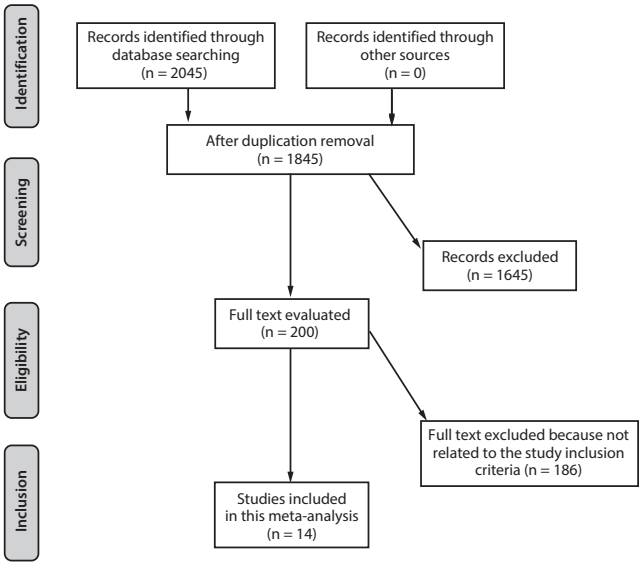

Fig. 1. Flowchart of the study

2. Studies designed with objectives other than an examination of the effects of suction compared to a simple water seal during pulmonary surgery;
3. Studies with methods other than suction and a simple water seal;
4. Studies without the investigation of the impact of comparative results.

Identification

A search protocol was devised based on the PICOS criteria, which were identified as follows: P (population): patients undergoing pulmonary surgical procedures; I (interventional/experimental): comparison of suction with a simple water seal; C (comparison): suction compared to a simple water seal; O (outcome): chest tube length, postoperative pneumothorax, prolonged air leakage, duration of air leak, and hospital stay duration; and S (study protocol/design): no limitations.[7] During a systematic and comprehensive search of electronic engines, including Embase, Google Scholar, Cochrane Library, PubMed, and OVID, articles published up to November 2021 were retrieved. The search used a combination of selected keywords and words related to suction and pulmonary surgery, water seal, chest tube use and its duration, postoperative pneumothorax, prolonged air leak, air leak duration, and hospital admission duration, as summarized in Table 1. All selected research publications were collated in a single EndNote file (Clarivate, London, UK), with duplicates omitted. Examination of the titles and abstracts excluded publications that did not report an association between the effect of adding suction and a water seal and the consequences after pulmonary surgery. The retrieved studies were then investigated for relevant data.

Table 1. Search strategy for each database

Database	Search strategy
Pubmed	#1 "suction" [MeSH terms] OR "pulmonary surgery" [all fields] OR "simple water-seal" [all fields] #2 "chest tube duration" [MeSH terms] OR "postoperative pneumothorax" [all fields] OR "prolonged air leak" [all fields] OR "air leak duration" [all fields] OR "length of hospital stay" [all fields] #3 #1 AND #2
Embase	#1 "suction"/exp OR "pulmonary surgery"/exp OR "simple water-seal"/exp #2 "chest tube duration"/exp OR "postoperative pneumothorax"/exp OR "prolonged air leak"/exp OR "air leak duration"/exp OR "length of hospital stay"/exp #3 #1 AND #2
Cochrane Library	#1 "suction": ti,ab,kw OR "pulmonary surgery": ti,ab,kw OR "simple water-seal": ti,ab,kw (word variations have been searched) #2 "chest tube duration": ti,ab,kw OR "postoperative pneumothorax": ti,ab,kw OR "prolonged air leak": ti,ab,kw or "air leak duration": ti,ab,kw or "length of hospital stay": ti,ab,kw (word variations have been searched) #3 #1 AND #2
OVID	#1 "suction" OR "pulmonary surgery" OR (simple water seal or lung adj2 (disturb* or dysfunction*)): tw,kw,kf #2 "chest tube duration" OR "length of hospital stay" (acute adj2 (postoperative pneumothorax or pneumothorax rate)): tw,kw,kf #3 (air leak duration* or prolonged air leak): tw,kw,kf
Web of Science	#1 ("suction" OR "simple water seal") AND "pulmonary surgery" (word variations have been searched) #2 "chest tube duration" AND "postoperative pneumonthorax" (word variations have been searched) #3 "length of hospital stay" OR "prolonged air leak" OR "air leak duration") (word variations have been searched)

MeSH – medical subject headings; ti,ab,kw – terms in either title or abstract or keyword fields; exp – exploded indexing term; tw – title or abstract; kw – author-provided keyword exact; kf – word in a provided keyword; TS – topic search.

Screening

A pre-designed form summarized the study- and participant-related properties, including the last name of the first author, study time frame, region, year of publication, target population, study protocol, subject numbers, demographic data, and properties of clinical treatments applied. Additionally, the form included the assessment period, quantitative and qualitative techniques of evaluation, information resources, and outcome assessment, as well as whether statistical analysis used mean difference (MD) or odds ratio (OR) with 95% confidence intervals (95% CIs).[7] If a study met the inclusion criteria and conformed to relevant guidelines, 2 authors independently retrieved the information. In case of disagreement, the corresponding author made the final decision. When there was variability in the data retrieved from one of the trials, based on the examination of the association between suction and water seal impacts on the postoperative outcomes of patients undergoing lung surgical procedures, data were extracted separately. For the assessment of bias, 2 authors independently evaluated the procedural quality of the selected trials.

Risk of bias

The Cochrane risk-of-bias tool for randomized trials v. 2 (RoB 2; Cochrane, London, UK) evaluated bias risk and procedural quality. Risk of bias evaluation criteria categorized a study as a low risk of bias if it fully met quality standards. If the quality requirements (one or more) were only partially met or were unclear, the publication was assigned to the moderate risk of bias category. High-risk publications did not fulfill the quality standards. Any inconsistencies were resolved by re-investigating the original article.

Eligibility

The main finding focused on the impact of suction addition to a simple water seal on lung surgery outcomes. A summary was created based on the evaluation of the suction addition effect and simple water seal approach on chest tube duration, postoperative pneumothorax, prolonged air leak, air leakage duration, and hospital stay duration.

Inclusion

Sensitivity analyses were only applied to studies that demonstrated an effect of suction addition to a simple water seal on postoperative outcomes of lung surgery. Suction and a simple water seal were compared in terms of subgroup and sensitivity analyses.

Statistical analyses

All measurements and graphs were made using Reviewer Manager (RevMan) v. 5.3 (Cochrane). A continuous or dichotomous technique with a fixed- or random-effect model estimated the MD, OR and 95% CIs. Estimations of the I^2 index ranged between 0% and 100%, with an I^2 index value of approx. 0% interpreted as no heterogeneity and I^2 index values of 25%, 50% and 75% interpreted as low, moderate and high heterogeneity, respectively. The random-effect model was applied if I^2 was 50% or above, and when I^2 was less than 50%, the likelihood of applying fixed influence rose. However, additional characteristics of the studies, with a high degree of similarity, were also analyzed to confirm the employment of the correct model.[8] The subgroup analysis was performed as defined before, using the stratification of the original calculation per result category.

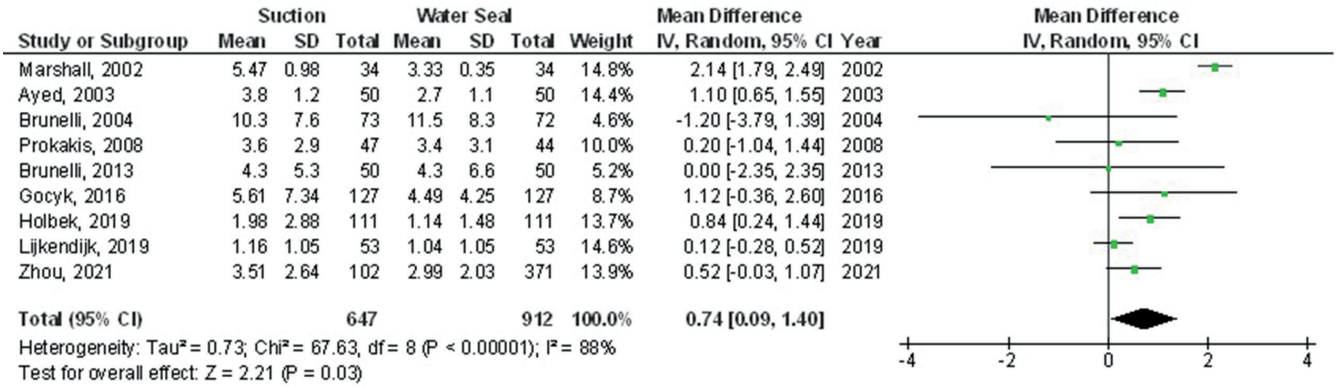

Fig. 2. Forest plot comparing chest tube duration in pulmonary surgery patients receiving suction to a simple water seal

95% CI – 95% confidence interval; df – degrees of freedom; SD – standard deviation.

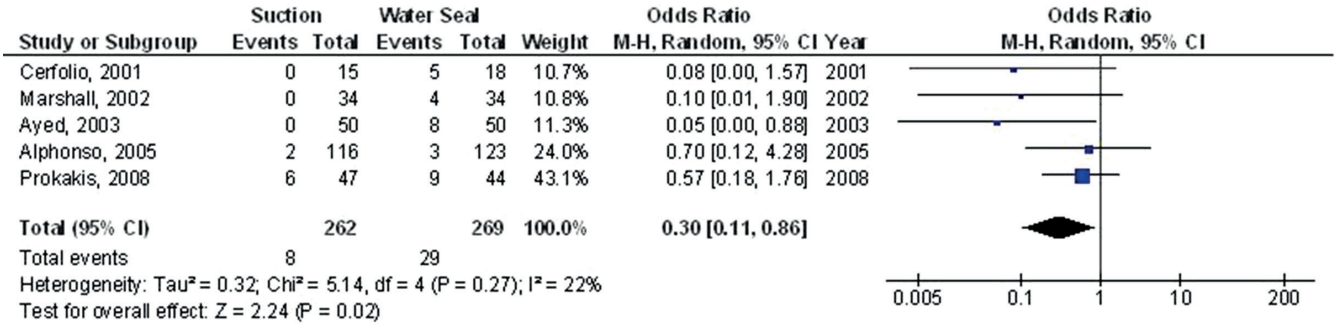

Fig. 3. Forest plot comparing postoperative pneumothorax in pulmonary surgery patients receiving suction to a simple water seal

95% CI – 95% confidence interval; df – degrees of freedom.

The Egger's regression test for bias assessment was quantitatively measured (bias was present if $p \leq 0.05$) and examined by visual inspection of funnel plots of logarithmic ORs against standard errors. All estimated p-values were two-tailed, and a value of $p < 0.05$ for discrepancies amongst subgroups reflected statistical significance.

Results

A total of 2045 studies were retrieved, with 14 studies between 2001 and 2021 meeting the inclusion criteria for integration in the meta-analysis.[5,6,9–20] The studies included 2449 participants undergoing surgical lung procedures, with 1092 receiving suction and 1357 receiving a simple water seal. All studies investigated suction addition to a basic simple water seal on lung surgery outcomes.

The sample sizes of the studies ranged from 31 to 500 participants, and the data retrieved from 14 trials are depicted in Table 2. Thirteen trials provided data on chest tube duration, 5 reported data on postoperative pneumothorax, 7 provided data on protracted air leaks, 5 reported tiered air leak duration data, and 6 reported data on length of hospital stays.

Suction resulted in significantly longer chest tube duration (MD = 0.74, 95% CI: 0.09–1.40, p = 0.03, Z = 2.21) with considerably high heterogeneity (I^2 = 88%), and a lower incidence of postoperative pneumothorax (OR = 0.27, 95% CI: 0.30 (0.11–0.86), p = 0.02, Z = 2.24) with low estimated heterogeneity (I^2 = 22%), in comparison to a simple water seal in patients undergoing lung surgery (Fig. 2,3). However, when compared to a simple water seal, suction made no significant difference in terms of prolonged air leak (OR = 1.08, 95% CI: 0.29–4.06, p = 0.91, Z = 0.12) with highly estimated heterogeneity (I^2 = 93%), air leak duration (MD = 0.58, 95% CI: −0.48–1.64, p = 0.28, Z = 1.07) with heterogeneity value of 94%, or length of hospital stay (OR = 1.30, 95% CI: −0.81–3.41, p = 0.23, Z = 1.2) with highly estimated heterogeneity (I^2 = 90%) (Fig. 4–6).

The analysis of the studies' stratification and adjustment for gender, race and age was not performed because none of the studies included adjustments or stated the influence of these variables. The Egger's regression analysis estimates (p = 0.89) revealed no publication bias based on visual and quantitative evaluation of the funnel plots. Despite this, most studies included in the meta-analysis had low procedure quality due to their limited sample size. None of the studies had selective reporting bias or inadequate outcome data.

Discussion

This meta-analysis included 14 trials with 2449 patients who had undergone lung surgery, of which 1092 participants

Table 2. Characteristics of the studies selected for the meta-analysis

Study, year, reference	Country	Total	Suction	Water seal	Participant	Disease type	Number of tubes	Initial suction	Suction pressure [mm Hg]	Drainage system type	Definition of prolonged air leak	Drainage threshold of removal [mL/day]
Cerfolio et al., 2001 [9]	USA	33	15	18	lung surgery	lung disease	1–2	no	–20	regular	>6	<200
Marshall et al., 2002 [10]	USA	68	34	34	video-assisted thoracic surgery	lung disease	1–2	no	–20	regular	>6	<200
Ayed, 2003 [11]	Kuwait	100	50	50	lobectomy	lung disease	1	no	–20	regular	>6	<200
Brunelli et al., 2004 [6]	Italy	145	73	72	lobectomy	primary spontaneous pneumothorax	2	yes	–20	regular	>5	<100
Alphonso et al., 2005 [12]	UK	239	116	123	lung surgery	lung cancer	2	yes	–20	regular	>5	<100
Daneshvar Kakhki et al., 2006 [13]	Iran	31	13	18	lung surgery	lung cancer	2	yes	–11 to –20	digital	>6	<100
Prokakis et al., 2008 [14]	Greece	91	47	44	lung surgery	lung cancer	1–2	yes	–20	digital	>6	<150
Brunelli et al., 2013 [15]	Italy	100	50	50	lung surgery	lung cancer	1–2	yes	–20	digital	>6	<300
Leo et al., 2013 [16]	Italy	500	250	250	lung surgery	lung cancer	1	no	–20	regular	>5	<300
Gocyk et al., 2016 [5]	Poland	254	127	127	lung surgery	lung cancer	2	no	–10 to –18	regular	>5	<200
Lijkendijk et al., 2019 [17]	Denmark	106	53	53	lobectomy	lung cancer	1–2	no	–15	regular	>8	<200
Holbek et al., 2019 [18]	Denmark	222	111	111	lobectomy	lung cancer	1–2	yes	–15	digital	>7	<100
Vageriya et al., 2020 [19]	India	87	51	36	lobectomy	lung cancer	2	yes	–15	regular	>7	<100
Zhou et al., 2021 [20]	China	473	102	371	lobectomy	lung cancer	2	yes	–15 to –20	regular	>7	<100
Total		2449	1092	1357	–							

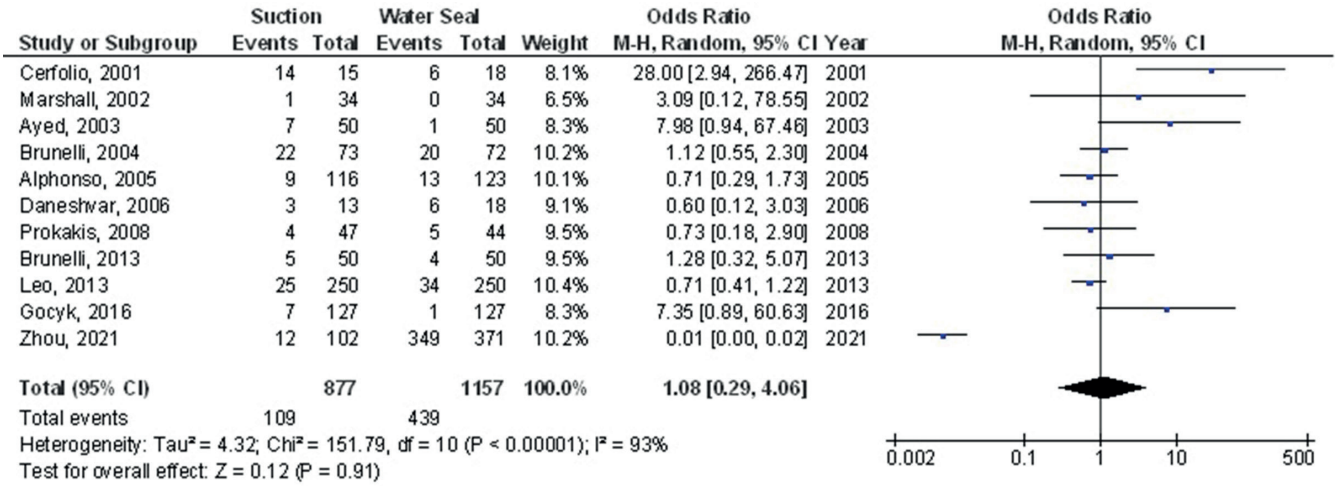

Fig. 4. Forest plot comparing prolonged air leak in pulmonary surgery patients receiving suction to a simple water seal

95% CI – 95% confidence interval; df – degrees of freedom.

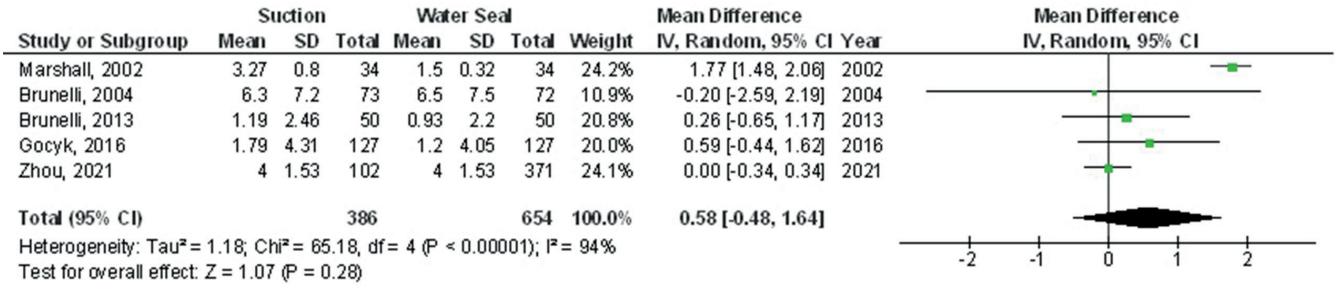

Fig. 5. Forest plot comparing air leak duration in pulmonary surgery patients receiving suction to a simple water seal

95% CI – 95% confidence interval; df – degrees of freedom; SD – standard deviation.

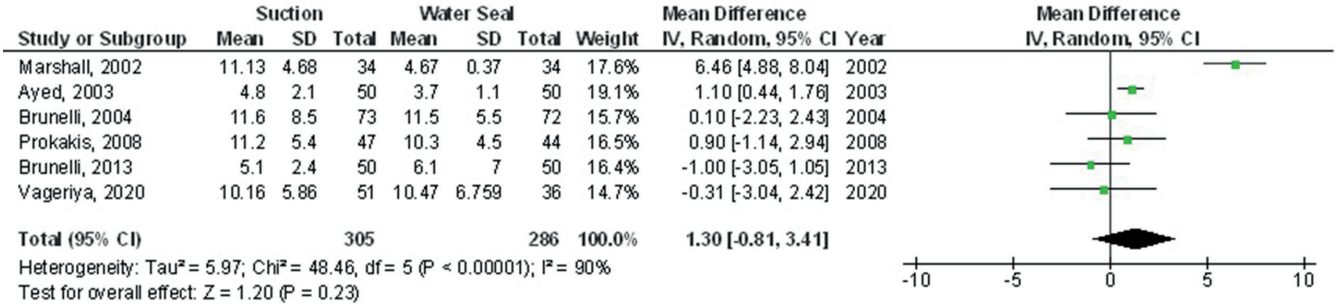

Fig. 6. Forest plot comparing hospital stay duration in pulmonary surgery patients receiving suction to a simple water seal

95% CI – 95% confidence interval; df – degrees of freedom; SD – standard deviation.

received suction drainage and 1357 a simple water-seal drainage.[5,6,9–20] In those undergoing pulmonary surgery, suction led to considerably longer chest tube duration and lower postoperative pneumothorax than a simple water seal. However, suction did not impact sustained air leak, air leakage time span or hospital stay duration. Nonetheless, caution must be taken when interpreting the results due to the relatively few studies included and the limited sample size of most of them. Indeed, 6 out of 12 trials had a sample size <100 subjects. Therefore, further studies are required to confirm these outcomes and increase confidence in the effects observed.

Meta-analysis is a methodical assessment of numerous RCTs, followed by statistical pooling of the data.[21] Randomized clinical studies showed that suction enhanced postoperative drainage volume[5] and increased chest drainage volume due to higher suction pressure.[18] However, suction can lead to concurrent negative pressure that may increase the production of fluid. Adding external suction to a water seal may speed up the drainage of fluid. In pulmonary surgery, there are 2 contradictory consequences of fluid production and removal, including equilibrium being biased towards removal with suction, resulting

in a lower drainage volume. Nonetheless, patients who received suction had far longer duration of chest tube use than those treated with a simple water seal. Chest tube removal criteria may help to clarify the situation. The withdrawal of a chest tube depends on detecting no air leakage and minimal fluid drainage. Suction addition to the water seal should accelerate the removal of chest tube. However, the development of a prolonged air leak may contribute to the postponement of chest tube removal. Given the pleura's tendency to absorb fluid, the frequency of prolonged air leaks may result in a longer chest tube duration than the output of the fluid chest drainage.

Suction-based drainage was found to be a risk factor for a higher incidence of prolonged air leakage, suggesting that the addition of external suction to the basic water seal could increase air leakage. The relationship between suction drainage and the incidence of prolonged air leaks is still debatable[16,18] as it depends on the type of surgery and target population selection. Numerous definitions of prolonged air leaks have been recommended, including postoperative air leaks that last for 3–10 days,[22,23] although a leak greater than 5 days is the most widely used and based on the average postoperative hospital stay length.[24] Once a prolonged air leak occurs, late chest tube removal and hospital discharge are expected.[25] Reducing the duration of air leakage can be achieved by applying endobronchial valves, sterilized compressed sponges, or through non-interventional supportive therapy.[26–29] Additionally, digital chest drainage, a relatively new system, can be implemented to guide chest tube removal.[30] Although the old drainage method was more widely employed in the assessed studies, the digital drainage approach was used in a limited number of studies at a considerable financial cost.[5]

This meta-analysis investigated the impact of adding suction to a water seal on postoperative outcomes following lung surgery. However, more research is warranted to confirm the possible connection between the two and to demonstrate clinically significant results. This was also suggested in previous meta-analyses that found that suction and a simple water seal had similar impact on patients undergoing different types of surgery.[30–33] The insignificant results between suction and a simple water seal in several studied outcomes require further investigation and clarification, because no obvious rationale could explain these clinical outcomes. Well-conducted prospective trials are also needed to investigate these factors and the effect of different co-factors, such as age, gender and ethnicity. Indeed, this meta-analysis did not find any impact of these variables.

In 2013, the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) Statement was established as a procedure to help improve clinical trial protocol quality.[34] The Consolidated Standards of Reporting Trials (CONSORT) Statement (2010) contains a 25-item checklist and a flowchart to help the authors depict the randomized trials with more clarity.[35] The design and report of clinical trials according to the SPIRIT and CONSORT protocols and checklists will help ensure the recording of all critical trial features, which will decrease the risk of bias and improve the quality of suction and simple water seal RCTs.[34,35] Conducting properly designed RCTs to assess the effects of suction and a simple water seal on lung surgery patients is essential, and because published data should guide clinical practice, the publication of completed research studies is fundamental.[36]

In summary, suction led to significantly longer chest tube use and decreased postoperative pneumothorax in patients undergoing lung surgery than in those receiving a simple water-seal drainage. However, suction had no significant impact on sustained air leakage, air leak duration or length of hospital stay. More research is warranted to validate these findings.

Limitations

Due to a large number of the retrieved studies initially being eliminated from the current meta-analysis, selection bias could be present in the study. However, the studies which were eliminated did not meet the inclusion criteria, with several failing to connect outcomes to characteristics such as age and ethnicity. Furthermore, some studies assessed the effects of suction and a water seal on clinical outcomes by comparing their results to those of previous studies, which may have introduced bias due to incomplete information. This meta-analysis included 14 studies, out of which 6 were relatively small, with less than 100 subjects. There was significantly high heterogeneity in some of the studies, and the sensitivity analysis indicated publication bias in favor of suction, which could explain this variability. Also, co-factors such as ethnicity, age and nutritional status were likely bias-causing factors that need further investigation. Furthermore, the pooled results may be skewed due to unpublished papers and missing data, as well as variability in management methods, doses and healthcare organization standards. Indeed, the length of suction and simple water seal management were inconsistent, and they did not adequately assess the cost burden and patient's quality of life, which are vital outcomes.

Conclusions

In participants undergoing pulmonary surgery, suction led to considerably longer chest tube duration and smaller postoperative pneumothorax than a simple water seal. However, suction was no better than water seal at preventing prolonged hospital stays, air leaks or air leak duration. Nonetheless, the small number of studies included and the relatively small sample sizes mean the results must be interpreted cautiously. Therefore, well-designed and in-depth additional studies are recommended to validate and improve confidence in these findings.

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Evaluating the effects of glucagon-like peptide-1 receptor agonists on cognitive function in Alzheimer's disease: A systematic review and meta-analysis

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

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Abstract

Background. Alzheimer's disease (AD) is the most common type of dementia. At present, some drug and non-drug therapies can be used to slow disease progression or prevent cognitive deterioration. More treatment options still need to be explored.

Objectives. A meta-analysis was performed to compile the relevant evidence for the use of glucagon-like peptide-1 (GLP-1) receptor agonists in preventing AD.

Materials and methods. We systematically searched English and Chinese databases, including Embase, PubMed, Cochrane Library, China National Knowledge Infrastructure (CNKI), Wanfang Data Knowledge Service Platform, and Weipu website (VIP), based on the PICOS (Participants, Interventions, Comparisons, Outcomes, Study design) principles. The reviewers evaluated the search results and conducted the analysis; 5 articles with a total sample size of 184 patients were included. Changes in cognitive function, body mass index (BMI), blood glucose level, and insulin content were analyzed.

Results. A low risk of bias and no publication bias were found in these studies. The following results were obtained: 1) cognitive function: mean difference (MD) = 2.16, 95% confidence interval (95% CI): 1.45–2.88; 2) BMI change: MD = –1.16, 95% CI: –1.71––0.61; and 3) blood glucose change: standard MD (SMD) = –0.64, 95% CI: –1.21––0.88. No statistically significant difference was found in insulin content.

Conclusions. In this review, we showed that GLP-1 receptor agonists can effectively change cognitive function, BMI and blood glucose levels in patients with AD. This provides relevant clues for the prevention of AD. However, more studies are needed to refine these conclusions.

Key words: Alzheimer's disease, meta-analysis, cognitive function, hypoglycemic drugs, glucagon-like peptide-1 receptor agonists

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Introduction

The nervous system is a very important component of the human body, as it mediates many life activities. The available research has shown that cognition and emotion are regulated by the prefrontal lobe of the brain,[1] so when the nervous system is damaged, related functions are also affected. Several previous studies have revealed possible mechanisms for nervous system damage, including mitochondrial damage and associated inflammation in nerve cells[2,3]; therefore, neurologically related diseases need to be studied in depth.

Alzheimer's disease (AD), which is associated with progressive neurodegeneration, affects millions of people worldwide. The prevalence of AD is 10% in people over 65 years of age and 40% in people over 85 years of age. This amounts to a tremendous global health burden. Globally, there are nearly 46.8 million people suffering from AD, and treatment costs were estimated at USD 818 billion in 2015.[4] The main pathological features of AD include inflammation in the nervous system, amyloid plaques and neurofibrillary tangles in the brain.[5,6] Previous studies have shown that emotions such as fear are closely related to the central and peripheral nervous systems.[7] Synaptic dysfunction, neurotransmitter imbalance and neuroinflammation are closely related to the progression of AD.[8] Thus, homeostasis of the nervous system is essential for maintaining cognitive integrity. Thus far, only a few drugs that have been approved for the treatment of AD are being used in clinical environment, such as the acetylcholinesterase inhibitors and the non-competitive N-methyl-D-aspartate receptor antagonists. While these drugs can provide partial symptomatic relief, they cannot alter the progression of AD.[9] Although the U.S. Food and Drug Administration (FDA) approved aducanumab as the first disease-modifying therapy (DMT) for AD in June 2021,[10] there has been considerable medical and scientific controversy regarding its curative effect.[11] Two phase III trials of aducanumab have shown opposite results[12]; thus, currently available data do not provide sufficient evidence to support the clinical efficacy of aducanumab.[13] Therefore, the identification of a safe and effective DMT is a matter of critical importance.[14]

Diabetes is an endocrine disease characterized by abnormally high blood glucose levels.[15] There is a strong correlation between type 2 diabetes mellitus (T2DM) and AD[16]; the former is gradually being recognized as a risk factor for AD. There are many clinical and pathological similarities between T2DM and AD, including damage to the insulin signaling pathway.[17] In the central nervous system, insulin contributes to synaptic maintenance, neuron growth and survival, as well as maintenance and regulation of learning and memory[18]; therefore, insulin resistance becomes a potential risk factor for AD. Furthermore, hyperglycemia is strongly associated with the occurrence of AD. Karmi et al. conducted a magnetic resonance imaging (MRI) study and found that long-term chronic hyperglycemia can mediate hippocampal dysfunction,[19] and hyperglycemia inflammatory mediators, rheological factors, and dysregulation of the hypothalamic–pituitary–adrenal axis also may exacerbate cognitive decline.[20] In addition, some clinical evidence has found that diabetes patients with higher A1C levels are associated with diminished cognitive function.[21] Thus, it is believed that increasing insulin levels in the body may slow the progression of AD.

In recent years, glucagon-like peptide-1 (GLP-1) receptor agonists have been used to control blood glucose levels in patients with diabetes. The pharmacological action of GLP-1 is to promote β-cell regeneration, growth and differentiation, and to inhibit β-cell apoptosis.[22] At the same time, GLP-1 can protect the nervous system, as it acts as a kind of growth factor.[23] A recent study found that GLP-1 improves the supportive ability of astrocytes to neurons, which explains the neuroprotective mechanism.[24] Importantly, GLP-1 can cross the blood–brain barrier, and it has also been reported that cells in the hypothalamus and hippocampus highly express GLP-1 receptors. However, abnormalities in the structure and function of the hippocampus and prefrontal cortex can cause cognitive impairment.[25] Thus, GLP-1 could help to slow the progression of AD. The GLP-1 can also induce neurite growth, thus achieving the effect of an AD intervention.[26,27] Animal studies have shown that the administration of a GLP-1 receptor agonist in rat ventricles can reduce nerve cell damage caused by neurotoxic stimulation, which may improve learning and memory function.[28] According to a mouse model of AD, treatment with the GLP-1 analog liraglutide can prevent the progression of memory decline.[29] Another recent study found that liraglutide can reduce associated brain complications when T2DM and AD occur simultaneously.[30] In 2019, Femminella et al. conducted the Evaluating Liraglutide in Alzheimer's Disease (ELAD) study to assess the effect of the novel GLP-1 analog liraglutide on AD, but they have not yet published their conclusions.[31] Therefore, relevant clinical evidence needs to be further studied.

This meta-analysis was conducted based on the P (Participants), I (Interventions), C (Comparisons), O (Outcomes), and S (Study design) (PICOS) principles. High-quality randomized controlled trials reported in both Chinese and English were included in this analysis. All patients had a clear diagnosis of AD or cognitive impairment. All the interventions in the trial were GLP-1 receptor agonist monotherapy, and the outcomes of all trials had clear data support.

Objectives

Although GLP-1 receptor agonists appear to be efficacious in treating AD, there is not yet enough clinical evidence to support this claim. Therefore, we conducted a meta-analysis to summarize what is known regarding the efficacy of these drugs in AD patients. We selected randomized controlled clinical studies on GLP-1 receptor

agonists in the treatment of AD to determine whether patients with AD can have changes in memory ability, skills and other dimensions of cognitive function after treatment, based on a scale test before and after the study. We evaluated changes in cognitive function and other indicators after GLP-1 receptor agonist interventions in patients with AD to provide a relevant basis for the prevention of the disease.

Materials and methods

Literature search and search strategy

Searches were conducted in a comprehensive manner for publications of relevant peer-reviewed papers and dissertations between 1990 and 2022 in the Embase, PubMed, Cochrane Library, China National Knowledge Infrastructure (CNKI), Wanfang Data Knowledge Service Platform, and Weipu website (VIP) databases for all clinical studies on GLP-1 interventions in AD without language restrictions, using the Medical Subject Headings (MeSH) terms such as "glucagon-like peptide 1", "Alzheimer's disease" and "cognitive function". We also added all entry terms with the same meaning under all MeSH terms, such as "GLP-1", "AD" and "cognitions" into the search strategy. Then, the MeSH terms and entry terms of the same noun were connected by "OR", and words with different meanings were connected by "AND". Finally, the selection scope was limited to randomized controlled trials. The references of the included studies were searched manually as literature supplements.

Inclusion and exclusion criteria

The following criteria were used to determine which studies were eligible based on the PICOS principles[32]: 1) participants were aged ≥18 years with a diagnosis of AD or with cognitive impairment without a diagnosis of AD; 2) the intervention consisted of administration of a GLP-1 receptor agonist; 3) the outcome of the study included at least changes in cognitive function (not limited to type of cognitive function); 4) the study type was a randomized controlled trial; and 5) the baseline data were complete and included the number of observations, source of cases, follow-up time, and other indicators.

The exclusion criteria were as follows: 1) studies involving pregnant or breastfeeding patients; 2) studies on patients with other brain diseases or brain injuries; 3) review articles and animal or cell experiments; and 4) literature for which the outcome data could not be extracted.

Data extraction

An EndNote X9 (Clarivate Analytics, London, UK) library was initially created. A review of titles and abstracts was conducted. Based on the inclusion criteria, 2 researchers (ZB and WW) consulted the guidelines on data extraction for systematic reviews and meta-analyses, independently screened the literature, and extracted and cross-checked the data.[33] The basic information of the article was recorded, including the first author, publication date, sample size, average age, and sex of the patients, type of study design, diagnostic criteria for AD, intervention methods, and assessment methods of cognitive function. The 3rd investigator (LW) was consulted to resolve disagreements.

Quality assessment

A 5-dimensional evaluation of the included studies was conducted using the Cochrane Risk of Bias Assessment Tool (RoB v. 2.0; The Cochrane Collaboration, London, UK): 1) randomization process; 2) deviations from the intended interventions; 3) missing outcome data; 4) measurement of the outcome; and 5) selection of the reported result. When all the items of a dimension were satisfied, the quality level was considered low-risk; when some of the items were satisfied, the quality level was considered medium-risk; and when the reference did not meet the key requirements, the quality level was considered high-risk.

Statistical analyses

The STATA v. 16.0 (STATA Corp., College Station, USA) and Review Manager v. 5.4 (RevMan; The Cochrane Collaboration) were used to process the data. There was a single point representing each study connected to a forest plot with a regression line. After the effect size was log-transformed and divided by standard error (SE) (z-score), it was represented on the y-axis and expressed as the reciprocal of SE on the x-axis. Statistical heterogeneity across trials was assessed using the Cochran's Q test (with $p < 0.1$ indicating significance) and quantified using the I^2 statistic ($I^2 > 50\%$ for significant heterogeneity).[34,35] Considering that clinical heterogeneity and methodological heterogeneity could exist in any trial, we used the random effects model for the meta-analysis. Publication bias was assessed using funnel plots and Begg's test. In order to investigate publication bias, we constructed a funnel plot using Egger's test.[36]

Results

Search results and study characteristics

Based on the search strategy (Fig. 1), there were 427 articles in total; CNKI yielded 14 articles, Wanfang Data Knowledge Service Platform – 39 articles, VIP – 0 articles, PubMed – 316 articles, Embase – 31 articles, and Cochrane Library – 27 articles. We excluded 43 duplicate studies using EndNote X9.[37] In accordance with the title of the article, preliminary screening was conducted.

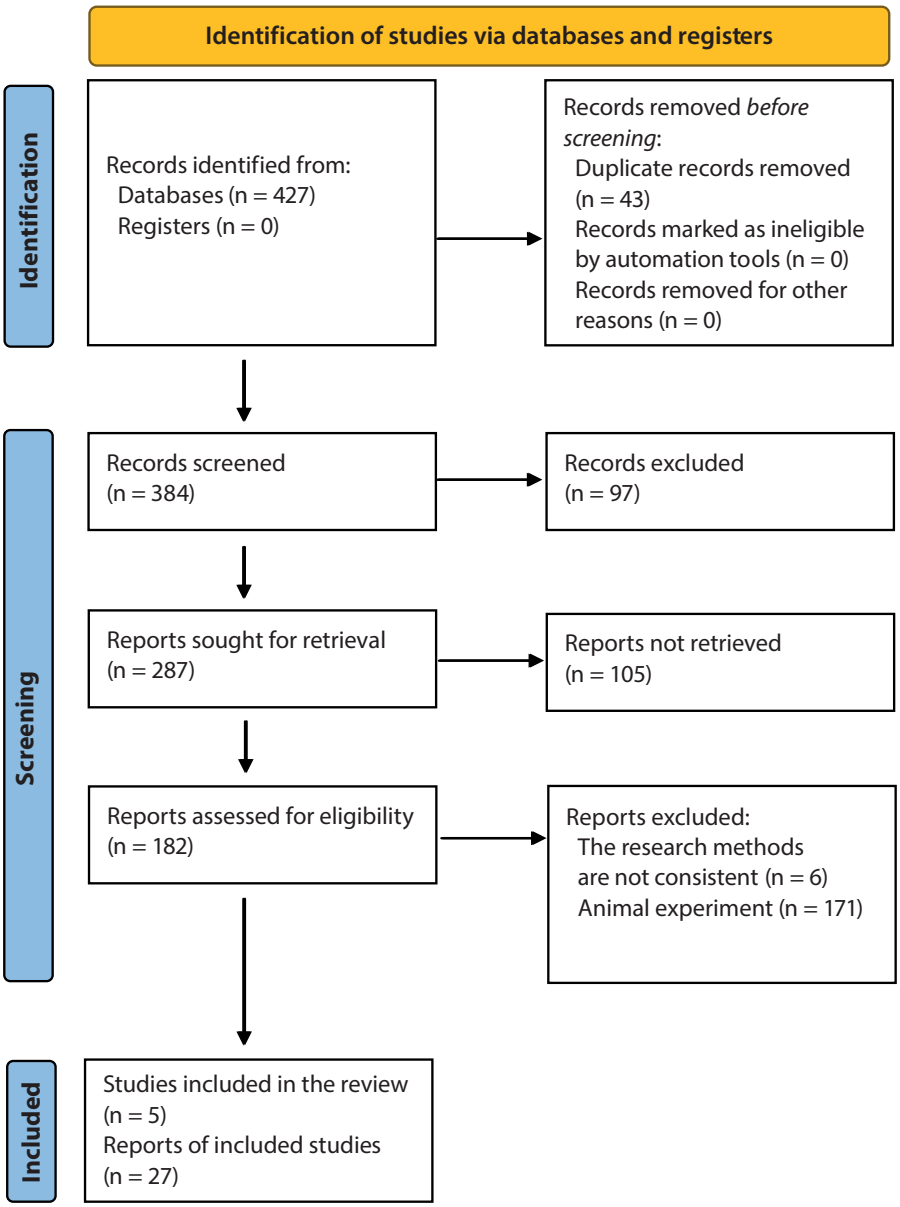

Fig. 1. Flow diagram of the literature search strategy used in this study

There were 97 exclusions after browsing the title, including 79 review articles or meta-analyses, and another 18 studies were excluded because their subjects were unrelated. Next, we reviewed the abstracts and excluded 105 studies that did not meet the study requirements. Of the remaining 182 articles, 171 were animal studies, and 6 studies had inconsistent methods, so they were excluded. Finally, 5 randomized controlled trials were included.

The inclusion and exclusion criteria were clearly stated in each of the 5 trials (Table 1). In all trials, the treated groups had similar baseline demographics, including age and gender. The total enrollment was 177. The mean patient age was 68 years. All patients were diagnosed with AD before the trial. In 3 of the studies, patients were treated with liraglutide, whereas exenatide was used in the other 2 studies. All cognitive function tests were conducted using a scale. The scales used were the Mini-Mental State Examination (MMSE) and the Wechsler Memory Scale–Fourth Edition (WMS-IV).

Study quality

The quality of all studies included in the meta-analysis was carefully evaluated using the Cochrane Risk of Bias Assessment Tool (RoB v. 2.0). The risk of bias was categorized as low, high or unclear, under the guidance of the Cochrane Handbook (https://training.cochrane.org/handbook). The risk of bias in all included studies is shown in Fig. 2,3. Overall, all 5 studies displayed a low risk of bias.

Results of GLP-1 intervention in AD

Four indicators were extracted from the 5 included studies,[38–42] all of which were randomized controlled trials. The major outcome included effects on cognitive function before and after the use of GLP-1 receptor agonists (Fig. 4). Additionally, insulin, blood glucose level and body mass index (BMI) were measured as secondary outcomes (Fig. 5–7).

Table 1. Characteristics of the selected studies

Author	Year	Randomization blinding	Study popula-tion	Diagnostic criteria for AD/MCI	Duration	Sample size, n	Cognitive measurement	Treatment	Sex (M/F)	Age (M ±SD)
Zheng[38]	2017	open-controlled	AD with DM	NINCDS-ADRDA	12 months	57	MMSE and ADL	exenatide + other	28/0	80.5 ±10.3
								placebo	29/0	
Mullins et al.[39]	2019	open-controlled	AD or MCI	CDR	6 months	21	MMSE and WMS-IV	exenatide	7/4	71.7 ±6.9
								placebo	4/6	74.0 ±6.4
Watson et al.[40]	2019	open-controlled	AD or MCI	MMSE	12 weeks	41	WMS-IV	liraglutide 1.8 mg	11/14	60.88 ±5.79
								placebo	5/11	59.56 ±5.69
Gejl et al.[41]	2016	double-blind	AD	MMSE	26 weeks	34	WMS-IV	liraglutide 1.8 mg	6/8	66.6
								placebo	15/5	63.1
Isik et al.[42]	2012	open-controlled	AD with DM	MMSE	6 months	24	MMSE	liraglutide 1.8 mg	–	71 ±6
								placebo	–	70 ±6

M – male; F – female; M ±SD – mean ± standard deviation; AD – Alzheimer's disease; DM – diabetes mellitus; MCI – mild cognitive impairment; NINCDS-ADRDA – National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association; CDR – Clinical Dementia Rating; MMSE – Mini-Mental State Examination; WMS-IV – Wechsler Memory Scale–Fourth Edition; ADL – activities of daily living.

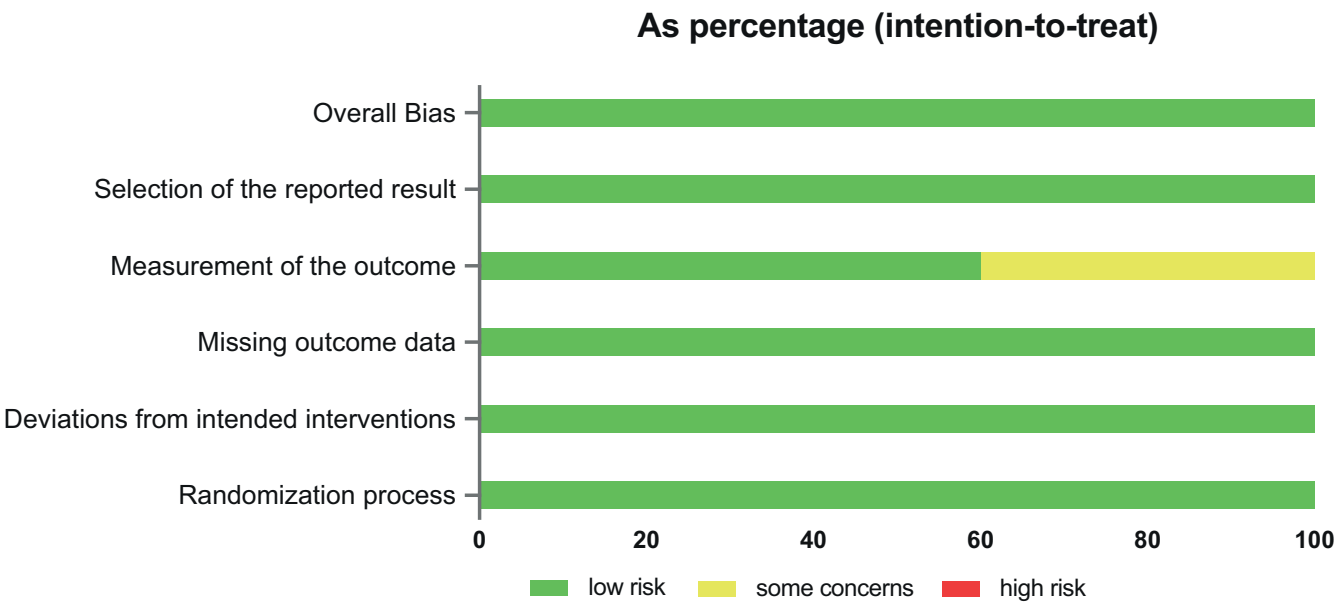

Fig. 2. Risk of bias graph

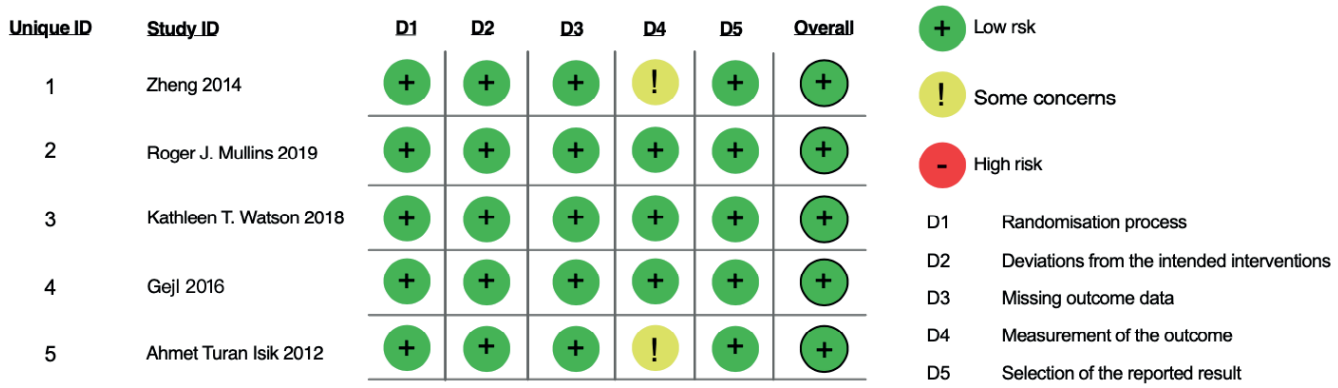

Fig. 3. Assessment of risk of bias

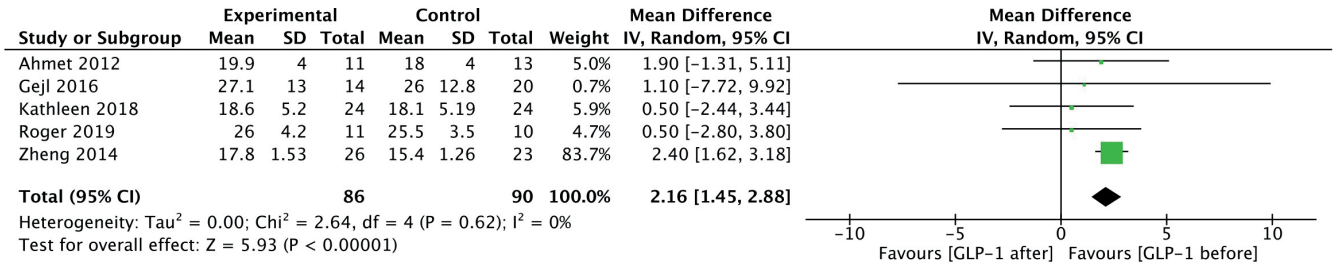

Fig. 4. Changes in cognitive function

SD – standard deviation; 95% CI – 95% confidence interval; df – degrees of freedom; GLP-1 – glucagon-like peptide-1.

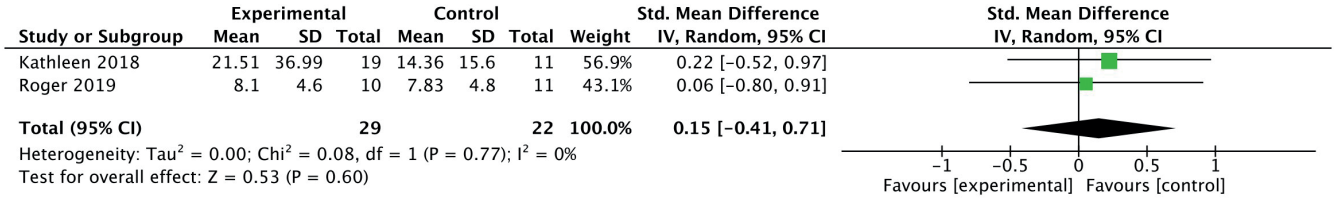

Fig. 5. Changes in insulin content

SD – standard deviation; 95% CI – 95% confidence interval; df – degrees of freedom.

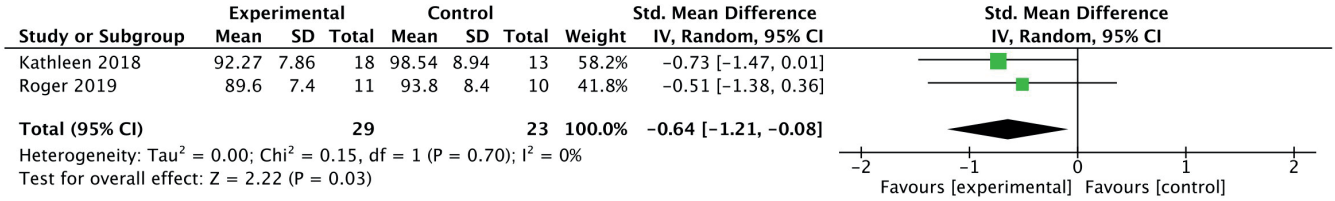

Fig. 6. Changes in blood glucose content

SD – standard deviation; 95% CI – 95% confidence interval; df – degrees of freedom.

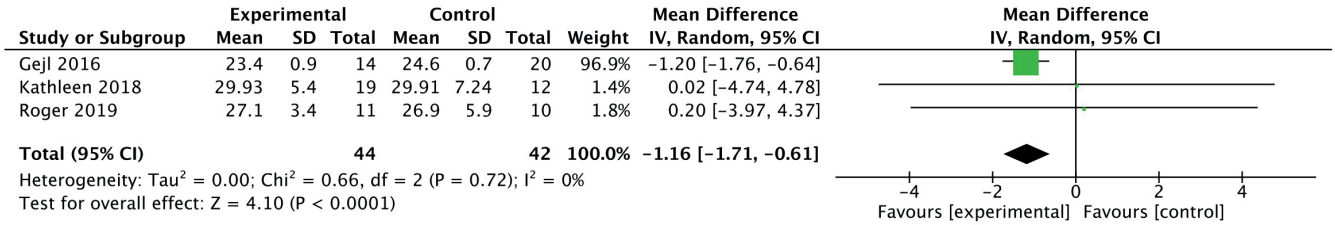

Fig. 7. Changes in body mass index (BMI)

SD – standard deviation; 95% CI – 95% confidence interval; df – degrees of freedom.

Cognitive function was assessed using a random effects model that included 177 patients. In the test of heterogeneity, $I^2 = 0\%$ and $p = 0.62$, indicating low heterogeneity. After combining effect size, the meta-analysis showed that mean difference (MD) = 2.16, 95% confidence interval (95% CI): 1.45–2.88 and $p < 0.05$, and no publication bias was found (Begg's Test = 0.806, Egger's test = 0.153) after using STATA v. 16.0 to analyze the data.

Among the secondary outcomes, the results of insulin change concerned 51 patients, in which $I^2 = 0\%$ and $p = 0.77$ after the heterogeneity test. However, after combining the effect sizes, the p-value equaled 0.60, which indicated that there was no statistical significance; thus, there was no need to discuss this result in further detail. Owing to the different units in the included articles, a fixed-effect model was used to assess changes in blood glucose content. According to the heterogeneity test, $I^2 = 0\%$ and $p = 0.7$, indicating low heterogeneity, and the standard mean difference (SMD) = −0.64, 95% CI: −1.21–−0.08, and $p = 0.03$, indicating statistical significance. The BMI changes were studied using a random effects model, and the results showed that heterogeneity was low ($I^2 = 0\%$, $p = 0.72$), with MD = −1.16, 95% CI: −1.71–−0.61 and $p < 0.05$. The results of the sensitivity analysis showed that all the studies had little influence on the total combined effect size, and the results were reliable and acceptable.

Discussion

Currently, clinical evidence regarding the efficacy of GLP-1 in treating AD patients is lacking. Therefore, we conducted a meta-analysis to summarize what is known about the effectiveness of this class of drugs in AD.

There is a strong link between cognitive dysfunction and obesity, hypertension, dyslipidemia, and T2DM.[43] There is extensive experimental evidence that patients with T2DM may suffer from cognitive decline accompanied by deterioration of memory, attention, intelligence, processing speed, and executive function. In addition, there are white matter abnormalities and brain atrophy (particularly in the cortical, subcortical and hippocampal regions).[44,45] Considering the pathologic similarities between T2DM and AD, and the characteristic effects of GLP-1 receptor agonists, treatment with enterosecretin analogs may be helpful in treating the cognitive deficits that occur in AD.[46] Therefore, a meta-analysis was conducted to address the efficacy of GLP-1 receptor agonists in patients with AD. The included studies reported changes in cognitive function before and after treatment in patients with AD, as well as BMI and levels of blood glucose and insulin after treatment. The following tools were used: MMSE, WMS-IV and Activities of Daily Living (ADL). The MMSE is a screening tool for detecting changes in cognitive skills, and WMS-IV and ADL are used to measure memory ability in patients with AD; all these tests are used to measure the cognitive function of AD patients. These results indicate that GLP-1 receptor agonist treatment can significantly improve the cognitive function of AD patients.

The treatment of T2DM with GLP-1 receptor agonists, such as liraglutide, has been approved. According to extensive animal studies, neuroprotection may be achieved with GLP-1 receptor agonists. It promotes the proliferation and differentiation of neurons, neurite outgrowth, synaptic plasticity, and memory formation, and reduces the toxicity of β-amyloid.[47] Furthermore, β-amyloid toxicity is mediated by insulin resistance.[48] Meanwhile, a recent in vitro model study found that as a result of reducing the activity of β-secretase 1 (BACE-1), an enzyme in insulin-resistant cells, liraglutide can decrease the production of β-amyloid.[49] Gejl et al. used positron emission tomography (PET) and found that glucose fluctuation levels in patients' brains can be reduced with GLP-1 receptor agonists.[50] Therefore, we hypothesized that GLP-1 receptor agonists could be used as potential drugs for preventing AD.

There is a strong association between unhealthy lifestyles, weight gain and obesity, and an increased risk of T2DM worldwide, as 60–90% of patients with T2DM are obese.[51] It has become a risk factor for AD, as a longitudinal study measured the sagittal abdominal diameter of 6583 individuals and found there is a nearly threefold risk of developing dementia for patients with the largest diameter compared to those with the smallest. Thus, they concluded that central obesity in midlife increased the risk of dementia.[52] Therefore, it is necessary to determine the effect of weight on T2DM patients. Liraglutide therapy has been reported to cause weight loss as an additional benefit. A higher dose formulation was developed specifically for obesity by the manufacturer in response to these findings. In December 2014, the FDA formally approved liraglutide 3 mg/day for this indication.[53] For BMI changes, the results of our study showed that MD = −1.16, 95% CI: −1.71−−0.61 and $p < 0.05$. This result was statistically significant and suggested that the BMI (or weight) of patients can be effectively changed after GLP-1 receptor agonist treatment, which is consistent with reports of relevant clinical trials. Ng and Wilding observed significant weight loss (1–3 kg) after 20–30 weeks of clinical liraglutide treatment in patients with T2DM.[54] Moreover, weight loss has been reported in T2DM patients treated with liraglutide (either monotherapy or combination therapy).[55] In patients with diabetes, there was a close correlation between BMI and GLP-1 receptor agonist use.[56] These results may be related to the mechanism underlying GLP-1 secretion. Kyriacou and Ahmed verified that exenatide can slow gastric emptying and reduce food intake.[57] Furthermore, the direct effect of GLP-1 on satiety signaling in the central nervous system, independent of vagal afferents, has been demonstrated to inhibit food intake.[58–60] The above studies indicate that GLP-1 can cause weight loss and reduce BMI. Meanwhile, Mansur et al. provided an explanation for the association between body weight and cognitive function, suggesting that the positive treatment effect on cognition may be partly due to the weight loss observed with liraglutide administration.[61]

We also analyzed changes in insulin and blood glucose levels after GLP-1 receptor agonist treatment in patients with AD. For the change in insulin content, the meta-analysis showed that $p = 0.60$ (>0.05), indicating no significant difference in the results. For the change in blood glucose, the meta-analysis showed that SMD = −0.64, 95% CI: −1.21−−0.08 and $p = 0.03$, which was statistically significant. The results showed that GLP-1 receptor agonists effectively reduced blood glucose levels in patients with AD. For changes in insulin level, although the result was not statistically significant, the effect size showed that GLP-1 receptor agonists could increase insulin content in vivo, which is consistent with the pharmacological effects of the drug.

Limitations

Meta-analyses review published literature to evaluate and quantitatively analyze the results of multiple studies in a comprehensive manner. This meta-analysis, however, has some limitations owing to the limited number of referenced studies. First, because of the small number of studies meeting the inclusion criteria, only 5 reports

were extracted and merged, which increased the uncertainty of the results. Second, the sample size of the study was small; therefore, verification of the results may be weak. More recent research reports should be included to supplement this meta-analysis. However, this study is the first to report the effect of GLP-1 receptor agonists during the treatment of AD, which is undoubtedly significant, and more research in similar direction should be conducted in the future.

Conclusions

Our study summarized current clinical studies and found that GLP-1 receptor agonists can effectively improve cognitive function, BMI and blood glucose levels in AD patients. There have been several previous animal studies demonstrating the effectiveness of GLP-1 receptor agonists on AD, based on neural or metabolic pathways. Therefore, we would suggest that GLP-1 receptor agonists may help slow the progression of AD. These findings provide a relevant basis for the prevention of AD. However, more clinical trials will need to be included to overcome the limited sample size and complement these findings.

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Pulmonary-specific quality of life and dyspnea among patients hospitalized for coronavirus disease 2019: Evaluation of patient groups 1, 3 and 6 months after discharge

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Abstract

Background. Short- and long-term lung damage after coronavirus disease 2019 (COVID-19) has been emphasized in many studies, but pulmonary-specific health-related quality of life (HRQOL) has been examined only in a limited capacity.

Objectives. In this study, we aimed to assess pulmonary-specific HRQOL and dyspnea among patients hospitalized for COVID-19 by applying the St George's Respiratory Questionnaire (SGRQ) to patient groups 1, 3 and 6 months following discharge (groups T1, T3 and T6).

Materials and methods. This cross-sectional study was conducted between April 2020 and December 2020 at a tertiary hospital in Turkey. A total of 345 patients with a definite diagnosis of COVID-19 were included in our research.

Results. Total SGRQ score was significantly lower in the T6 group than in the T1 group ($p < 0.001$). The SGRQ-Symptom score was similar in the T3 and T6 groups, while the T1 group had significantly higher values ($p < 0.001$). The SGRQ-Activity score was significantly lower in the T6 group than in the T1 and T3 groups ($p = 0.001$), while the SGRQ-Impact score was significantly higher in the T6 group compared to the other 2 groups ($p < 0.001$). When the patients were analyzed statistically in terms of dyspnea, the difference between the baseline and 6-month results was found to be statistically significant ($p < 0.001$).

Conclusions. Although long-term consequences are still not fully known, the SGRQ scores and dyspnea outcomes of our patients show that pulmonary-specific HRQOL and dyspnea remain at a similar level from discharge until the 6th month after discharge. Studies with extended and longitudinal follow-up are required.

Key words: COVID-19, pulmonary-specific quality of life, St George's Respiratory Questionnaire (SGRQ), dyspnea

Background

Coronavirus disease-2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), emerged in late 2019 and quickly spread to become a worldwide pandemic.[1] As of August 22, 2022, a total of 593,269,262 people were diagnosed with the virus globally, resulting in 6,446,547 deaths.[2] Previous studies have shown that COVID-19 affects multiple organs, although the lung is the main organ affected.[3,4] In addition to the effects of the disease on the pulmonary system in the acute phase, the long-term consequences on pulmonary functions and the extent to which they influence health-related quality of life (HRQOL) have been the subject of many studies.[5–7] They showed the presence of chronic symptom burden and poor quality of life in COVID-19 survivors. These studies also show that COVID-19 can affect the HRQOL of patients, as well as healthcare employees and the general population.[8–11] A previous cross-sectional study revealed that COVID-19 patients had poor HRQOL at 1-month follow-up, which was influenced by various risk factors.[12] Another study demonstrated a reduction in HRQOL 3 months after recovery from acute COVID-19.[13]

Many scales have been used to measure the impact of COVID-19 on HRQOL during active disease and follow-up periods.[5–7,14,15] However, the majority of scales used assess overall HRQOL, and the number of studies measuring pulmonary-specific HRQOL is limited. The St George's Respiratory Questionnaire (SGRQ) is a standardized, self-administered test that measures impacts on general health, daily living and perceived well-being in lung-specific chronic diseases such as chronic obstructive pulmonary disease, asthma, bronchiectasis, kyphoscoliosis, sarcoidosis, and cystic fibrosis.[16,17] There are currently very few studies in which SGRQ was applied in the follow-up of COVID-19 patients, and although they confirm that patients' lung-specific HRQOL improved over time, the number of patients was low and follow-up periods were limited.[6,7,18] Despite the emphasis on short- and long-term lung damage after COVID-19, little is known about the extent to which these sequelae affect pulmonary-specific HRQOL.

Objectives

This study aimed to determine changes observed over time regarding pulmonary-specific HRQOL and dyspnea symptoms in patients hospitalized for COVID-19 by assessing groups of patients 1, 3 and 6 months after discharge.

Materials and methods

Study design

This cross-sectional single-center study was conducted between April 2020 and December 2020 at the Sultan 2nd Abdulhamid Han Training and Research Hospital (Istanbul, Turkey), a pandemic hospital of the Health Sciences University (Istanbul, Turkey). The Ethics Committee of the Health Sciences University, Hamidiye Clinical Research Institute, approved the study (approval No. 18/7 issued on June 14, 2021). The study conformed to the ethical standards of the Declaration of Helsinki. All study participants provided written informed consent.

Study population

The study included 345 patients diagnosed with COVID-19 using positive SARS-CoV-2 polymerase chain reaction (PCR) test results from nasopharyngeal swab samples or with a conclusive diagnosis of COVID-19 according to computed tomography (CT) and clinical and laboratory findings, as recommended by the relevant guidelines of the World Health Organization (WHO). Other inclusion criteria included age >18 years and serious illness or symptoms caused by or suspected to have been caused by COVID-19 that required hospitalization (intensive care units (ICUs) or wards). Exclusion criteria were refusal to participate, advanced heart failure, deep anemia, advanced chest deformity, neuromuscular diseases that may cause respiratory distress, pregnancy, dialysis, malignancy, long-term immobility, and cognitive problems preventing scale administration.

Data collection

The hospital database identified patients who had passed the 1-month (T1), 3-month (T3) and 6-month (T6) thresholds of discharge (from the day of diagnosis). Participants were randomly selected using patient codes, and each was contacted by trained healthcare personnel via telephone to schedule appointments. A total of 1562 patients were contacted, and 345 randomly selected individuals (115 patients in each group) participated in the study. Data obtained from hospital records during the hospitalization period (inpatient wards or ICUs) included sociodemographic data, comorbidities, smoking status, day of admission after symptom onset, PCR test results, length of hospital stay, symptoms, dialysis requirement, intensive care need, type of respiratory support, and oxygen saturation.

Follow-up consultations with the patients took place in the COVID-19 outpatient clinic, which was established for the follow-up of COVID-19 patients in the Sultan 2nd Abdulhamid Han Training and Research Hospital. All participants were interviewed face-to-face by trained physicians, and provided the SGRQ themselves. Changes in the number of people who developed dyspnea were also evaluated.

The St George's Respiratory Questionnaire scale and its administration

The SGRQ consists of 76 items divided into 3 sections, including symptoms (assessing respiratory symptoms,

frequency and severity), activity (activities causing or limited due to dyspnea) and impacts (aspects associated with functioning and psychological disturbances resulting from respiratory illness). Each section and total score received scores ranging from 0 to 100 points.[16,19] The total score summarizes the overall impact of the illness on health status, with a higher score indicating worse health and 0 indicating best possible health in relation to pulmonary disease. A reduction of 4 units in the SGRQ score after a medical or non-medical intervention is generally accepted in the literature as a valid minimally important difference (MID) of beneficial therapy.[17] The patients filled out the questionnaires while seated in a quiet room and after receiving advice on how and why they should fill in the questionnaire and answer all the questions. When the patient finished, the questionnaire was checked to ensure that all questions were answered, and items without a response were shown to the patient who completed it. The 3 SGRQ component scores and the total score were calculated using a Microsoft Excel 2016 spreadsheet (Microsoft Corp., Redmond, USA) called the SGRQ Calculator.

Statistical analyses

All analyses employed IBM SPSS v. 25 (IBM Corp., Armonk, USA) or Number Cruncher Statistical System (NCSS) 2020 Statistical Software (NCSS LLC, Kaysville, USA), with $p < 0.05$ accepted as statistically significant and 95% confidence intervals (95% CIs) calculated. Quantitative variables are reported as mean ± standard deviation (M ±SD), and qualitative variables as frequency, percentage and median (Q1–Q3 percentile values). The Shapiro–Wilk test and visual inspection of box plots were employed to evaluate data distribution. A comparison of the 2 groups utilized Student's t-tests or Mann–Whitney U tests, as appropriate. Meanwhile, a one-way analysis of variance (ANOVA) was used to compare 3 groups, followed by a post hoc Bonferroni test, Kruskal–Wallis test or Dunn test, as appropriate. Wilcoxon signed rank test enabled intragroup evaluations, and the relationships between variables were evaluated using Pearson's or Spearman's correlation analysis, as appropriate. Further analysis used linear regression models. A comparison of qualitative data utilized a χ^2 test with Yates's correction for continuity or Fisher's exact test, as appropriate.

Results

The study included 196 males and 149 females, with no significant difference between the groups in terms of sex distribution ($p = 0.541$). The overall mean age was 53.02 ±16.02 years, and the group means were 60.40 ±13.78 years (T1), 53.89 ±16.10 years (T3) and 44.76 ±16.66 years (T6). There was a significant difference in age between the groups ($p < 0.001$). While the percentages of patients with dyspnea in the T1 and T3 groups were similar, there was a statistically significant decrease in the percentage of patients with dyspnea in the T6 group ($p < 0.001$). Table 1 presents total and individual values and the differences between groups for comorbidities, smoking status, day of admission after symptom onset, PCR positivity, length of hospital stay, symptoms, dialysis need, intensive care need, oxygen saturation, and type of ventilation support.

Although the SGRQ-Total score was significantly lower in the T6 group than in the T1 group ($p < 0.001$), no significant difference was observed when the T3 group was compared to the other 2 groups. Meanwhile, the SGRQ-Symptom score was similar in the T3 and T6 groups, and significantly lower in both groups relative to the T1 group ($p < 0.001$). The SGRQ-Activity score was significantly lower in the T6 group than in the T1 and T3 groups ($p = 0.001$), while values were similar in the T1 and T3 groups. Likewise, the T1 and T3 groups had similar SGRQ-Impact scores, and the T6 group had significantly higher values compared to the other 2 groups ($p < 0.001$) (Table 2 and Fig. 1).

A multiple linear regression analysis revealed that the unstandardized β coefficient for the SGRQ-Total score in the T6 group was 1.106 points lower than in the T1 group ($p = 0.001$), and in the T3 group it was 0.739 points lower than in the T1 group ($p = 0.019$) after adjusting for age, sex, chronic disease, smoking status, and PCR result. In addition, being female ($\beta = 1.074$) and having a chronic disease ($\beta = 1.008$) significantly increased the SGRQ-Total score ($p < 0.01$).

The unstandardized β coefficient for the SGRQ-Symptom score was 0.768 points lower in the T6 group than in the T1 group ($p = 0.003$), and in the T3 group it was 0.782 points lower than in the T1 group ($p = 0.001$), after adjusting for age, sex, chronic disease, smoking status, and PCR result. Also, being female ($\beta = 0.504$) significantly increased the SGRQ-Symptom score ($p < 0.05$).

The unstandardized β coefficient for the SGRQ-Activity score was 1432 points lower in the T6 group than in the T1 group ($p = 0.004$) after adjusting for age, sex, chronic disease, smoking status, and PCR result. Furthermore, being female ($\beta = 1.572$) and having a chronic disease ($\beta = 1.528$) significantly increased the SGRQ-Activity score ($p < 0.01$).

The unstandardized β coefficient for the SGRQ-Impact score was 1270 points lower in the T6 group than in the T1 group ($p = 0.001$), and in the T3, it was 0.951 points lower than in the T1 group ($p = 0.008$), after adjusting for age, sex, chronic disease, smoking status, and PCR result. Moreover, being female ($\beta = 1.062$) and having a chronic disease ($\beta = 1.144$) significantly increased the SGRQ-Impact score ($p < 0.01$) (Table 3).

Discussion

Although the primary goal of treating hospitalized COVID-19-infected patients is limiting mortality, it has

Table 1. Summary of patient characteristics and COVID-19-related characteristics with regard to evaluation time

Patient characteristics	Time				Test value	p-value
	total (n = 345)	T1 (n = 115)	T3 (n = 115)	T6 (n = 115)		
Age						
M ±SD	53.02 ±16.02	60.40 ±13.78	53.89 ±16.10	44.76 ±16.66	F = 29.264	<0.001°
Median (IQR)	54 (42–66)	60 (47–75)[a]	57 (43–66)[b]	45 (27–57)[c]		
Sex						
Male	196 (56.8%)	64 (55.7%)	62 (53.9%)	70 (60.9%)	$\chi^2 = 1.229$	0.541‡
Female	149 (43.2%)	51 (44.3%)	53 (46.1%)	45 (39.1%)		
Comorbidities	189 (54.8%)	76 (66.1%)[a]	69 (60.0%)[a]	44 (38.3%)[b]	$\chi^2 = 19.869$	<0.001‡
Diabetes mellitus	93 (27.0%)	39 (33.9%)	30 (26.1%)	24 (20.9%)	$\chi^2 = 5.035$	0.081‡
Hypertension	119 (34.5%)	50 (43.5%)[a]	42 (36.5%)[ab]	27 (23.5%)[b]	$\chi^2 = 10.493$	**0.005**‡
Coronary artery disease	38 (11.0%)	19 (16.5%)[a]	14 (12.2%)[ab]	5 (4.3%)[b]	$\chi^2 = 8.931$	**0.011**‡
Heart failure (mild–moderate)	17 (4.9%)	8 (7.0%)	7 (6.1%)	2 (1.7%)	$\chi^2 = 3.836$	0.147‡
Asthma/COPD	35 (10.1%)	15 (13.0%)	13 (11.3%)	7 (6.1%)	$\chi^2 = 3.307$	0.191‡
Rheumatic disease	13 (3.8%)	2 (1.7%)	6 (5.2%)	5 (4.3%)	F.$\chi^2 = 2.147$	0.450†
Chronic renal disease	13 (3.8%)	4 (3.5%)	7 (6.1%)	2 (1.7%)	F.$\chi^2 = 2.857$	0.251†
Hypothyroidism	14 (4.1%)	5 (4.3%)	6 (5.2%)	3 (2.6%)	F.$\chi^2 = 1.042$	0.700†
Smoking	48 (13.9%)	7 (6.1%)[a]	16 (13.9%)[ab]	25 (21.7%)[b]	$\chi^2 = 11.761$	**0.003**‡
Admission day after symptoms onset						
M ±SD	4.58 ±3.37	4.73 ±2.89	4.61 ±3.03	4.39 ±4.08	K–W = 5.049	0.080▪
Median (IQR)	4 (2–7)	4 (3–6)	4 (2–7)	3 (2–6)		
Positive PCR	270 (78.3%)	94 (81.7%)[ab]	96 (83.5%)[b]	80 (69.6%)[a]	$\chi^2 = 7.769$	**0.021**‡
Length of hospital stay						
M ±SD	9.16 ±6.12	9.12 ±5.88	8.56 ±4.99	9.80 ±7.26	K–W = 1.033	0.596▪
Median (IQR)	7 (5–13)	7 (5–12)	7 (5–11)	8 (5–14)		
Symptoms						
Fever	178 (51.6%)	60 (52.2%)	63 (54.8%)	55 (47.8%)	$\chi^2 = 1.137$	0.566‡
Cough	190 (55.1%)	56 (48.7%)[a]	58 (50.4%)[a]	76 (66.1%)[b]	$\chi^2 = 8.528$	**0.014**‡
Sore throat	58 (16.8%)	16 (13.9%)	18 (15.7%)	24 (20.9%)	$\chi^2 = 2.155$	0.340‡
Myalgia/arthralgia	110 (31.9%)	35 (30.4%)	41 (35.7%)	34 (29.6%)	$\chi^2 = 1.148$	0.563‡
Fatigue	142 (41.2%)	42 (36.5%)	53 (46.1%)	47 (40.9%)	$\chi^2 = 2.178$	0.337‡
Headache	55 (15.9%)	15 (13.0%)	20 (17.4%)	20 (17.4%)	$\chi^2 = 1.082$	0.582‡
Nausea/vomiting	44 (12.8%)	11 (9.6%)	22 (19.1%)	11 (9.6%)	$\chi^2 = 5.857$	0.051‡
Diarrhea	44 (12.8%)	4 (3.5%)[a]	17 (14.8%)[b]	23 (20.0%)[b]	$\chi^2 = 14.744$	**0.001**‡
Dyspnea	141 (40.9%)	67 (58.3%)[a]	51 (44.3%)[a]	23 (20.0%)[b]	$\chi^2 = 1.222$	**<0.001**‡
Stay in intensive care unit	27 (7.8%)	11 (9.6%)	9 (7.8%)	7 (6.1%)	$\chi^2 = 0.964$	0.617‡
Ventilation support						
Mechanical ventilation	5 (1.4%)	4 (3.5%)	1 (0.9%)	0 (0.0%)	$\chi^2 = 4.245$	0.133†
Non-invasive mechanical ventilation	14 (4.1%)	5 (4.3%)	6 (5.2%)	3 (2.6%)	F.$\chi^2 = 1.080$	0.700†
High-flow	16 (4.6%)	6 (5.2%)	6 (5.2%)	4 (3.5%)	$\chi^2 = 0.524$	0.769‡
Nasal	156 (45.2%)	86 (74.8%)[a]	42 (36.5%)[b]	28 (24.3%)[b]	$\chi^2 = 64.310$	**<0.001**‡
Mask	84 (24.3%)	40 (34.8%)[a]	26 (22.6%)[ab]	18 (15.7%)[b]	$\chi^2 = 11.708$	**0.003**‡
Prone position	18 (5.2%)	4 (3.5%)	10 (8.7%)	4 (3.5%)	$\chi^2 = 4.220$	0.121‡
Oxygen saturation						
M ±SD	94.43 ±4.21	92.82 ±4.57	94.29 ±4.55	96.20 ±2.47	F = 20.795	<0.001°
Median (IQR)	96 (93–97)	94 (91–96)[a]	96 (93–97)[b]	97 (95–98)[c]		

T1 – group assessed after 1 month; T3 – group assessed after 3 months; T6 – group assessed after 6 months; M – mean; SD – standard deviation; IQR – interquartile range; ANOVA – analysis of variance; COPD – chronic obstructive pulmonary disease; PCR – polymerase chain reaction. Data are given as M ±SD, median (1st quartile–3rd quartile) for continuous variables according to normality of distribution and as frequency (percentage) for categorical variables. °one-way ANOVA test and post hoc Bonferroni test (F); ‡ χ^2 test; ▪Kruskal–Wallis test and post hoc Dunn test (K–W); † Fisher–Freeman–Halton test (Fχ^2). Values in bold are statistically significant. The presence of the same superscripted letters in different columns indicates that the values for those time points were similar according to Bonferroni correction. If 2 columns do not have the same superscripted letter, this means that those time points demonstrated a significant difference from each other according to Bonferroni correction.

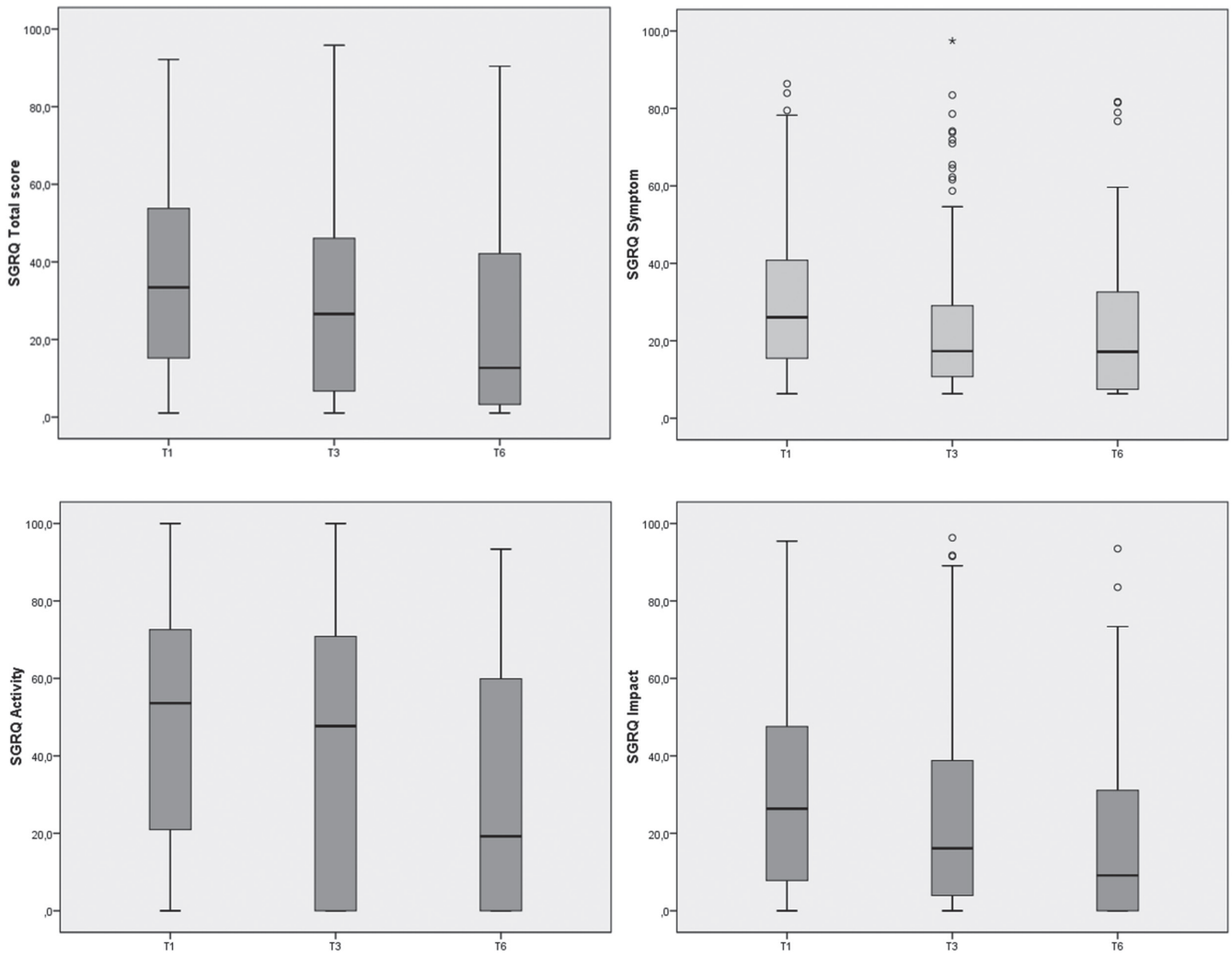

Fig. 1. Relationship between time and St George's Respiratory Questionnaire (SGRQ) scores

Table 2. Summary of St George's Respiratory Questionnaire (SGRQ) scores and dyspnea status with regard to evaluation time

SGRQ total scores		Time			Test value
SGRQ	total (n = 345)	T1 (n = 115)	T3 (n = 115)	T6 (n = 115)	▪p-value
		Total			<0.001
Median (IQR)	26.15 (6.97–46.76)	33.43 (15.09–54.66)[a]	26.57 (6.13–46.18)[ab]	12.68 (3.23–42.31)[b]	K–W:15.548
		Symptom			<0.001
Median (IQR)	19.80 (10.88–34.38)	26.07 (15.44–41.03)[a]	17.34 (10.75–29.87)[b]	17.16 (6.32–33.09)[b]	K–W:16.940
		Activity			0.001
Median (IQR)	42.34 (0.00–67.67)	53.62 (18.65–72.82)[a]	47.69 (0.00–72.29)[a]	19.24 (0.00–60.03)[b]	K–W:13.830
		Impact			<0.001
Median (IQR)	22.26 (3.97–50.12)	30.61 (8.28–52.18)[a]	23.56 (4.51–62.84)[a]	9.12 (0.00–31.16)[b]	K–W:16.714

T1 – group assessed after 1 month; T3 – group assessed after 3 months; T6 – group assessed after 6 months; IQR – interquartile range. Data are given as median (1st quartile–3rd quartile) for continuous variables according to normality of distribution and as frequency (percentage) for categorical variables. ▪Kruskal–Wallis test and post hoc Dunn test (K–W). The presence of the same superscripted letters in different columns indicates that the values for those time points were similar according to Bonferroni correction. If 2 columns do not have the same superscripted letter, this means that those time points demonstrated a significant difference from each other according to Bonferroni correction.

become clear that these infections can have significant long-term effects. Thus, healthcare systems have begun to open clinics dedicated to diagnosing and treating symptoms that persist following COVID-19.[20] The primary aim of this study was to show how the pulmonary-specific HRQOL of COVID-19 patients changed in the 1st, 3rd and 6th months after discharge. The secondary aim was to assess changes in the number of patients with dyspnea during

Table 3. Relationship between time and St George's Respiratory Questionnaire (SGRQ) scores

Variables	SGRQ-Total score			SGRQ-Symptoms score			SGRQ-Activity score			SGRQ-Impact score		
	unstandardized β	t	p-value	unstandardized β	t	p-value	unstandardized β	t	p-value	unstandardized β	t	p-value
Constant	4.570	7.277	<0.001	4.685	9.730	<0.001	5.155	5.561	<0.001	3.701	5.155	0.009
Time = T3† (+)	–0.739	2.366	0.019	–0.782	–3.267	0.001	–0.653	–1.417	0.157	–0.951	–2.666	0.008
Time = T6† (+)	–1.106	–3.282	0.001	–0.768	–2.970	0.003	–1.432	–2.879	0.004	–1.270	–3.295	<0.001
Age	–0.002	–0.255	0.799	0.002	0.209	0.835	–0.009	–0.624	0.533	–0.003	–0.287	0.774
Sex, female	1.074	4.048	<0.001	0.504	2.477	0.014	1.572	4.012	<0.001	1.062	3.501	0.001
Chronic disease (+)	1.008	3.343	0.001	0.317	1.369	0.172	1.528	3.432	0.001	1.144	3.318	0.001
Smoking (+)	0.277	0.711	0.477	0.541	1.816	0.070	–0.295	–0.515	0.607	0.484	1.089	0.277
Positive PCR (+)	0.008	0.027	0.979	0.084	0.353	0.724	–0.252	–0.551	0.582	0.197	0.557	0.578
Dependent variables	Dependent variable: SGRQ-Total score; $R^2 = 0.144$; F = 8.105; p < 0.001			Dependent variable: SGRQ-Symptoms score; $R^2 = 0.078$; F = 4.051; p = 0.001			Dependent variable: SGRQ-Activity score; $R^2 = 0.139$; F = 7.447; p < 0.001			Dependent variable: SGRQ-Impact score; $R^2 = 0.133$; F = 7.372; p < 0.001		

T1 – group assessed after 1 month; T3 – group assessed after 3 months; T6 – group assessed after 6 months; PCR – polymerase chain reaction. † reference category – group assessed after 1 month.

this period. According to the results, the SGQR-Total, SGQR-Activity and SGQR-Impact domains of the SGRQ score decreased gradually from the 1st month to the 6th month. The SGRQ-Syptom score was similar in the T3 and T6 groups, while the T1 group had significantly higher values. A higher score means worse health and a score of 0 indicates the best possible health status regarding lung disease. The multiple regression analysis also confirmed these significant relationships. In addition, the number of patients with dyspnea decreased significantly after 6 months.

The rate and severity of long-term pulmonary complications after COVID-19 are currently unknown. Nonetheless, current research shows that there can be a variety of persistent respiratory symptoms several months after recovery from SARS-CoV-2 infection.[21] The long-term effects of COVID-19 on HRQOL and mental wellbeing have been evaluated in several studies using various scales,[4–6,9,15,21–23] such as the SGRQ, which is a pulmonary-specific HRQOL scale used for the evaluation of diseases such as chronic obstructive pulmonary disease, asthma, bronchiectasis, kyphoscoliosis, sarcoidosis, and cystic fibrosis.[16,17] However, studies using pulmonary-specific HRQOL rating scales for COVID-19 are scarce. One such study used the SGRQ to assess patients with pneumonia and severe respiratory failure due to COVID-19 on the day they were discharged and on the 15th day after discharge, and found a significant decrease in all 3 domain scores and total scores when comparing results from day 15 to those from the day of discharge.[6] Zhou et al. also used the SGRQ in a prospective cohort study in which they divided COVID-19 patients into severe/critical, mild/moderate, asymptomatic, and healthy control groups. The SGRQ evaluation performed 3 months after recovery showed that the impact score, symptom score, activity score, and total score increased as the severity of the disease increased. However, the study did not measure how the score changed over time.[24] Likewise, another study showed that the adapted SGRQ (aSGRQ) improved in hospitalized patients 6–8 weeks after discharge compared to baseline, though scores were still worse than in the general population. The same study reported a significant association between the male sex and hospitalization with a reduced quality of life.[25]

In a study of healthcare workers in Wuhan, China, that presented results from patients 1 year after discharge, the median SGRQ-Total score was higher than that of healthy controls, and the SGRQ-Total score and all 3 subscale scores were significantly higher in the critical/severe disease group than in the mild/moderate disease group.[26] The results of the current study support the findings of similar studies using SGRQ. Indeed, the total score, symptom score, activity score, and impact score in the T6 group were significantly better than the T1 group. Furthermore, there were no differences between the T3 and T1 groups for total, activity and impact scores. Also, symptom scores were lower in T3 than in T1 but similar

to T6, which was confirmed using the multiple regression analysis. Considering the follow-ups for improvement in the SGRQ score, it is thought that the evaluations to be made at the 6th month will be the most appropriate response time to treatment, though these data should be supported by more comprehensive studies with more homogeneous patient groups and longitudinal assessments.

Dyspnea is a subjective symptom of respiratory distress, usually developing 7–8 days after the onset of COVID-19 symptoms, and is more common in patients with severe illness.[6] Even 2–3 months after discharge, approx. 50% of patients who recover from COVID-19 may continue to complain of dyspnea at rest and during exercise or daily activities.[6] Dyspnea is associated with reduced functional capacity and a lower HRQOL.[27] Several studies reported dyspnea as the most common symptom observed during follow-up after COVID-19.[28] In a prospective study, persistent dyspnea was common and reported by 58.4% of patients 1 year after discharge from ICU.[28] In another prospective study, dyspnea was reported in 54% of the participants 3 months after discharge, although there was no difference between patients admitted to the ICU and those who were not.[14] In our study, dyspnea was the most common initial symptom. However, there was no significant change in the number of patients with dyspnea in the T3 group compared to the T1 group. Although complaints of dyspnea did not return to a normal level for a given population, there were significant decreases in this regard after 6 months. Therefore, longer follow-up studies are required to understand the mechanisms of persistent dyspnea in survivors and to improve patient management after COVID-19.

Limitations of the study

Patients included in the three-time periods were not the same individuals, but different, which was considered a limitation of our study. The most important reason for this was the necessity of face-to-face interviews with the patients since the validity of administering the SGRQ questionnaire via other means has not been confirmed.[16] Another limitation was the inevitable consequence of heterogeneity between patient groups. Although many differences between the groups, including age, seem to be a limitation, this disadvantage was minimized by the multiple regression analysis adjusted for parameters such as age, sex, chronic disease, smoking status, and PCR result. In addition, there was no difference between the groups in terms of pulmonary comorbidity.

A control group was not included due to the difficulty in conducting the questionnaire and the nature of the study. Furthermore, a control group was deemed unnecessary based on the evident differences between controls and patients with COVID-19. Although the follow-up period was relatively longer than in previous studies, the reversibility of pulmonary injury in the longer term is a matter of debate, and further follow-up could be necessary to elucidate possible improvements.

The laboratory and radiological findings and treatment characteristics that may affect the outcome were not specified in the groups. Indeed, viral load may be an important marker for the prognosis of COVID-19, but it was not examined in this study.

Advantages of this study over similar studies include a 3-stage follow-up period evaluated, which allowed for the assessment of gradual changes in pulmonary-specific HRQOL. Furthermore, the number of participants was higher than in most of similar studies. Nonetheless, studies with a larger patient count, longer follow-up periods, more homogeneous patient groups, and more detailed data are needed to confirm these results.

Conclusions

In conclusion, this study found that the SGRQ-Total, -Activity and -Impact scores of patients discharged after COVID-19 were lower in the T6 group compared to the T1 and T3 groups. Of note, the T6 and T3 groups demonstrated significantly better results compared to the T1 group in terms of SGRQ-Symptom scores. There was also a significant improvement in pulmonary-specific HRQOL and dyspnea complaints in the T6 group. Studies on the effects of COVID-19 on pulmonary-specific HRQOL are important to allow for a better preparation for future outbreaks caused by new variants of SARS-CoV-2 or other microorganisms. Furthermore, studies with a more homogeneous distribution of patient characteristics that employ long-term follow-up are required to assess and appropriately manage persistent lung injury in COVID-19.

Supplementary materials

The supplementary files are available at https://doi.org/10.5281/zenodo.7742320. The package contains the following files:

Supplementary Table 1. Normal distribution.

Supplementary Table 2. Predicting SGRQ-Total score.

Supplementary Table 3. Predicting SGRQ-Activity score.

Supplementary Table 4. Predicting SGRQ-Symptom score.

Supplementary Table 5. Predicting SGRQ-Impact score.

Supplementary Fig. 1. Distribution of SGRQ-Total scores.

Supplementary Fig. 2. Distribution of SGRQ-Symptom scores.

Supplementary Fig. 3. Distribution of SGRQ-Activity scores.

Supplementary Fig. 4. Distribution of SGRQ-Impact scores.

Supplementary Fig. 5. SGRQ-Total score distribution Q-Q plots.

Supplementary Fig. 6. SGRQ-Symptom score distribution Q-Q plots.

Supplementary Fig. 7. SGRQ-Activity score distribution Q-Q plots.

Supplementary Fig. 8. SGRQ-Impact score distribution Q-Q plots.

Supplementary Fig. 9. SGRQ-Total score detrended normal Q-Q plots.

Supplementary Fig. 10. SGRQ-Symptom score detrended normal Q-Q plots.

Supplementary Fig. 11. SGRQ-Activity score detrended normal Q-Q plots.

Supplementary Fig. 12. SGRQ-Impact score detrended normal Q-Q plots.

Supplementary Fig. 13. Box plot of SGRQ-Total score and sub-factors.

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In vitro effect of beer, red and white wine on the morphology and surface roughness of human enamel

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Abstract

Background. Beer, red and white wine are acidic drinks whose frequent consumption can increase the risk of dental erosion.

Objectives. To establish the effect of beer, red and white wine on the morphology and surface roughness (SR) of human enamel using different exposure times in a cyclic de- and remineralization model in vitro.

Materials and methods. The experiment included 33 surgically extracted impacted human third molars from patients aged 18–25 years. Enamel samples obtained by cutting crowns (n = 132) were submitted to alternate cycles of demineralization in (1) beer, (2) red wine, (3) white wine, (PC) positive control (orange juice), and remineralization in artificial saliva, which also represented a medium for negative control (NC). The experiment included cycles with different exposure times in alcoholic beverages and orange juice of 15, 30 and 60 min. Thus, 12 groups were formed (for each drink and each exposure time) containing 10 samples each, while the NC group consisted of 12 samples. Experiments were repeated 3x/day for 10 days. Enamel surface alterations were determined by stylus profilometry (average surface roughness (R_a)) and scanning electron microscopy (SEM). The Shapiro–Wilk test, independent samples Kruskal–Wallis test and multiple comparisons (all pairwise) were performed.

Results. With increasing exposure time, there was a positive correlation with R_a for white wine- and orange juice-immersed samples (60 min compared to 15 min), which was also observed using SEM. There was no significant difference in the R_a between the other experimental samples for the same exposure time.

Conclusions. This study confirms a certain erosive potential of beer, red and white wine, and a significant relationship with pH, titratable acidity (TA) and SR, but not with the exposure time for all tested alcoholic beverages. Moreover, differences among the ultrastructural patterns caused by alcoholic beverages over the enamel surface were observed.

Key words: alcoholic beverages, dental erosion, SEM, stylus profilometry

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Background

The most cited definition of tooth erosion is Imfeld's, which implies the loss of tooth substance by chemical processes without bacterial involvement.[1] The etiology of dental erosion is multifactorial and can arise from extrinsic acidic substances (acidic beverages/food or medications)[2,3] or intrinsic factors that involve the migration of gastric juice into the oral cavity (reflux disease, laryngopharyngeal reflux, eating disorders, chronic alcoholism, pregnancy, etc.).[4]

While the erosive potential of soft drinks is well documented, there is insufficient data on the impact of alcoholic beverages on dental tissues. High and frequent use of alcoholic beverages can be seen as both an internal and external factor of dental erosion. People who often and excessively consume alcohol with, for example, the habit of keeping drinks in their mouths, prolong the contact of alcoholic beverages with the tooth surface and increase the risk of erosion.[5] Alcoholics also have poor dietary control and tend to consume more acidic foods and drinks. Additionally, the chemical properties of alcohol can cause vomiting, resulting in frequent contact of gastric acid with the tooth surface.[4]

The most studied alcoholic beverage in terms of erosion is wine, mainly in special occupational groups. Wine tasters who consume over 20 types of this drink a day have a higher risk of dental tissue erosion than people who enjoy alcohol occasionally.[6,7] Previous data on the prolonged action of wines show their high erosive potential. Moreover, continuous exposure of enamel samples to white wines for 24 h may lead to severe dental erosion, a conclusion established based on surface roughness (SR) and the amount of released calcium.[6] On the other hand, some types of red wine have been reported to significantly reduce enamel microhardness when in contact for at least 120 s.[8]

There is little data on the analysis of the erosive potential of beer, and most evidence is based on the detection of released calcium and phosphate.[9] Other researchers have examined the effect of beer on the enamel surface hardness and concluded that some brands of beer have a potential dental effect that is much less pronounced compared to soft drinks.[2,10]

Interestingly, the previously mentioned studies used single, shorter or longer exposures of enamel samples to alcoholic beverages. In contrast, cyclic erosion experiments better reflect the challenges faced by dentition by alternately exposing samples to de- and re-mineralizing solutions.[11–13] To the best of our knowledge, only 1 study used such a model to verify the erosion kinetics of an alcoholic beverage (red wine) on enamel. The cyclic procedure caused the polyphenols from red wine to modify the acquired enamel pellicle, reducing the erosive potential of the beverage.[14]

In the present research, the null hypothesis was that in the multiple exposure model, beer, red and white wine do not affect the increase in SR and ultrastructure changes of the enamel surface in relation to the exposure time.

Objectives

This study aimed to determine the erosive effect of beer, red and white wine of well-known Serbian brands on human enamel in a cyclic de- and remineralization model in vitro. Effects were assessed based on the analysis of average SR and scanning electron microscopy (SEM) observations using different exposure times.

Materials and methods

Tested alcoholic beverages

Three alcoholic beverages were tested: beer, red wine and white wine, which could be found in the free sale. Orange juice was used as the positive control. Table 1 shows the compositions of the experimental beverages as listed on their packaging.

Sample preparation and group divisions

This study was approved by the Research Ethics Committee of Faculty of Medicine, University of Niš, Serbia (approval No. 12- 14250-2/5-2018). The experiment included 33 impacted human third molars, which had been surgically extracted for medical reasons from patients aged 18–25 years.

After extraction and the usual cleaning procedure (storage in 1% sodium hypochlorite for 24 h and organic debris

Table 1. Compositions of the tested drinks as listed on their packaging

Drink	Manufacturer	Composition
Life Premium 100% orange juice	NECTAR Group, Bačka Palanka, Serbia	water, concentrated orange fruit juice, citric acid
Zaječar beer	Heineken, Zaječar, Serbia	water, barley malt, corn grits, hop extract
Rubin Vranac red wine	Rubin A.D., Kruševac, Serbia	water, alcohol 12%, glycerol, organic acids, tannins, phenols, anthocyanins, flavan-3-ols
Royal Riesling white wine	Levač Winery, Rekovac, Serbia	water, alcohol 10.5%, lactic acid, malic acid, tartaric acid, citric acid, succinic acid, acetic acid, sulphates

removal), the roots were removed, and the crowns were cut into quarters (distal, mesial, buccal, and lingual), using a diamond saw under water irrigation.[3] In this way, 104 samples were obtained for enamel SR analysis and 28 for SEM observations. If any sample was damaged during cutting, it was replaced with a new one, which was prepared from a newly extracted impacted molar.

Circular molds were made and filled with self-cured resin for samples that were being tested for SR. Each sample was immersed in resin so that the enamel surface was accessible for average surface roughness (R_a) measurement. Before the erosive challenge, the samples were cleaned with non-fluoridated pumice, rinsed with water and air-dried. After preparation, the samples planned for SEM observation were immediately placed in an ultrasonic water bath to remove cutting debris, washed with water and air-dried.

The samples were randomly assigned to 3 experimental groups: 1) beer, 2) red wine and 3) white wine; and 2 control groups: (positive control (PC)) orange juice and (negative control (NC)) artificial saliva, taking into account the planned number of samples with/without circular molds. Experimental groups, including the PC, consisted of 30 samples (24 for SR analysis and 6 for SEM observation), 10 (8+2) for each planned beverage exposure time: 15 min, 30 min and 60 min, while the NC group consisted of 12 (8+4) samples.

Artificial saliva (1.5 mM $Ca(NO_3)_2$, 0.90 mM KH_2PO_4, 130 mM KCl, and 60 mM Tris buffer, pH = 7.4)[15] was used as a medium for the NC, as well as a medium for experimental and PC samples between demineralization cycles.

pH and titratable acidity measurement

The pH of beverages was measured immediately after opening at 25°C using a previously calibrated multifunctional electronic device CONSORT C830 (Consort BVBA, Turnhout, Belgium). A total of 50 mL of the beverage was placed in a beaker and stirred using a non-heating magnetic stirrer until a stable reading was reached. Titratable acidity (TA) was calculated as the volume of 0.9613 M NaOH solution required to increase the pH of each beverage to 5.5 and 7.0. The solution was added in aliquots of 0.3 mL until a stable pH reading was achieved. The pH and TA of the beverages were measured in triplicate, and an average value was calculated (Table 2).

Table 2. Average of initial pH values and TA for pHs 5.5 and 7.0 of analyzed drinks

Analyzed drinks	Initial pH	TA	
		pH 5.5	pH 7.0
Orange juice	3.82 ±0.04	4.28 ±0.03	5.83 ±0.05
Beer	3.96 ±0.05	0.64 ±0.05	1.59 ±0.07
Red wine	3.49 ±0.05	1.82 ±0.04	2.34 ±0.03
White wine	3.02 ±0.06	2.69 ±0.03	3.18 ±0.05

TA – titratable acidity: amount of base (mL of 0.9613 M NaOH) needed to raise the pH to 5.5 and 7.0. Data are presented as mean ± standard deviation (M ±SD).

Erosive challenge

The experimental samples and the PC group had the following treatment: 1) immersion in 50 mL of alcoholic beverage at room temperature for 15 min, 30 min and 60 min, with occasional shaking; 2) rinsing with 5 mL of distilled water; 3) storage in artificial saliva until the next immersion.[11] This daily cycle was performed with 3 immersions for 10 consecutive days. Experimental solutions, including the PC, were changed every 24 h. At the end of the experiment, the samples were washed with distilled water, dried and prepared for the SR analysis/SEM observation.

Determination of surface roughness

The R_a was assessed using a stylus profilometer (Surftest SJ-301; Mitutoyo, Kawasaki, Japan).[3] The points of roughness measurement were randomly selected on the sample surface. Measurements were carried out at right angles to the samples. Three measurements were performed for each sample, and the mean value was calculated. For each reading, the device needle ran 0.25 mm/s, the length of the measuring line was 0.5 mm and the cutoff was 2.5 mm. To exclude possible errors, the measurement of SR was performed by only 1 investigator.

SEM observation

Scanning electron microscopy was used as an additional method to observe the enamel surface at each step. After preparation (mounting on stubs, fixing and sputter coating with gold/palladium), the samples were examined using a scanning electron microscope (JEOL-JSM-5300; JEOL, Akishima, Japan). Photomicrographs of representative areas were taken at ×2000 magnification.

Statistical analyses

The data obtained by this research were statistically analyzed using SPSS v. 15.0 (SPSS Inc., Chicago, USA). Continuous variables are presented as mean ± standard deviation (M ±SD) (for normal distribution) or by median (Me, i.e., 2nd quartile (Q2), 1st quartile (Q1)–3rd quartile (Q3)) and 95% confidence interval (95% CI), if the data distribution deviated from normal. Data normality was tested using a Shapiro–Wilk test. Because some variables presented distribution that deviated from normal, an independent samples Kruskal–Wallis test with multiple comparisons (all pairwise) was performed. An estimation error level of less than 5% ($p < 0.05$) was used as the threshold of statistical significance.

Results

pH results and TA measurement

The initial pH values were below critical (5.5) for the evaluated acidic beverages. White wine had the lowest average pH value (3.02 ±0.06), while beer had the highest average pH value (3.96 ±0.05), greater than orange juice selected for PC.

Furthermore, white wine gave the highest TA, requiring 2.69 mL of NaOH to reach a pH value of 5.5 (and 3.18 mL to reach a pH of 7.0). Beer showed a rapid response when NaOH was added, requiring only 0.64 mL of NaOH to reach a pH value of 5.5 (and 1.59 mL to reach a pH of 7.0). Orange juice had the greatest TA (4.28 or 5.83 mL) of NaOH to reach the equivalent pH values.

The initial pH values of the analyzed drinks and TA were expressed as mean values of triple measurement ±SD (Table 2).

Results of enamel roughness measurement

The R_a values obtained after immersing the samples in different beverages for different exposure times are shown in Table 3.

By comparing independent samples defined in relation to the beverage exposure time, the Kruskal–Wallis test allowed for establishing a statistically significant difference in the R_a of samples immersed in orange juice ($p = 0.008$) and white wine ($p = 0.041$) (Table 4). The subsequent multiple comparisons revealed a statistically significant difference in the R_a of samples exposed for 15 min compared to 60 min to orange juice ($p = 0.006$) and 15 min compared to 60 min to white wine ($p = 0.044$) (Table 5).

By comparing independent samples defined in relation to the beverages used for the same exposure time, the Kruskal–Wallis test allowed for establishing a statistically significant difference at all exposure times ($p < 0.001$) (Table 6). The subsequent multiple

Table 6. Independent samples Kruskal–Wallis test average surface roughness (R_a) in relation to beverage type

Statistical parameter	Exposure time 15 min	Exposure time 30 min	Exposure time 60 min
Test statistics	24.681[a]	32.075[a]	27.748[a]
df	4	4	4
p-value	0.000	0.000	0.000

df – degrees of freedom; [a] the test statistics is adjusted for ties.

Table 3. Average surface roughness (R_a) by groups (control and experimental with different exposure times)

Exposure time [min]	Negative control (artificial saliva)	Beer	Red wine	White wine	Positive control (orange juice)
15	1.67 (1.60–1.82)	1.96 (1.68–3.03)	2.40 (1.81–3.68)	2.54 (2.29–3.13)	3.23 (3.05–3.62)
30		2.29 (1.80–3.32)	2.48 (1.86–3.67)	3.03 (2.31–3.78)	5.22 (4.55–6.12)
60		2.63 (1.61–3.68)	2.82 (1.78–4.10)	3.56 (3.29–3.73)	6.58 (5.43–6.99)

Data are given as medians: 2nd quartile (Q2) (1st quartile (Q1)–3rd quartile (Q3)).

Table 4. Independent samples Kruskal–Wallis test average surface roughness (R_a) in relation to exposure time

Statistical parameter	Negative control	Beer	Red wine	White wine	Positive control
Test statistics	0[ab]	0.665[ab]	0.180[ab]	6.405[a]	9.765[a]
df	2	2	2	2	2
p-value	1.000	0.717	0.914	**0.041**	**0.008**

df – degrees of freedom; [a] test statistics is adjusted for ties; [b] multiple comparisons were not performed because the overall test did not show significant differences across samples. Values in bold indicate statistically significant results.

Table 5. Pairwise comparisons of average surface roughness (R_a) in relation to exposure time [min] to orange juice (positive control) and white wine

Group	Exposure time [min]	Test statistic	SE	Standardized test statistic	Sig.	Adj. Sig.[a]
Positive control (orange juice)	15–30	–7.125	3.536	–2.015	0.044	0.132
	15–60	–10.875	3.536	–3.076	0.002	**0.006**
	30–60	–3.750	3.536	–1.061	0.289	0.867
White wine	15–30	–2.250	3.536	–0.638	0.525	1.000
	15–60	–8.625	3.536	–2.440	0.015	**0.044**
	30–60	–6.375	3.536	–1.803	0.071	0.214

SE – standard error; Sig. – significance; Adj. – adjusted; [a] significance values have been adjusted by the Bonferroni correction for multiple tests. Values in bold indicate statistically significant results.

comparisons revealed that at all beverage exposure times, R_a was significantly different between the NC and PC samples ($p < 0.001$), and NC and white wine ($p = 0.006$ – exposure time 15 min, $p = 0.010$ – exposure time 30 min, and $p = 0.005$ – exposure time 60 min). Furthermore, the 60-minute exposure displayed differences between samples immersed in orange juice and beer ($p = 0.039$; Table 7). There was no significant difference in the R_a between the experimental samples for the same exposure time.

Results of SEM observations

Photomicrographs of the enamel surface after immersion in artificial saliva show an unchanged surface with perikymata, weak roughness and developing pores. After the erosive challenge with orange juice, a generalized irregularity with atypical etching, as well as the presence of wrinkles and cracks that deepen with increased exposure time were manifested (Fig. 1). Differences in the quality of erosive changes were observed between samples immersed in beer and red wine, but the degree of erosive damage did not increase with exposure time. In contrast, differences in the erosive ultrastructural pattern after 30 min and 60 min of cycled exposure to white wine were observed (Fig. 2).

Discussion

Although in vitro models provide limited information on intraoral erosion, significant conclusions based on this type of research have been drawn. A large number of experiments used single exposures of samples to acidic substances, mainly to predict the erosive potential. This includes different exposure times from 10 s to 60 min,[10,16,17]

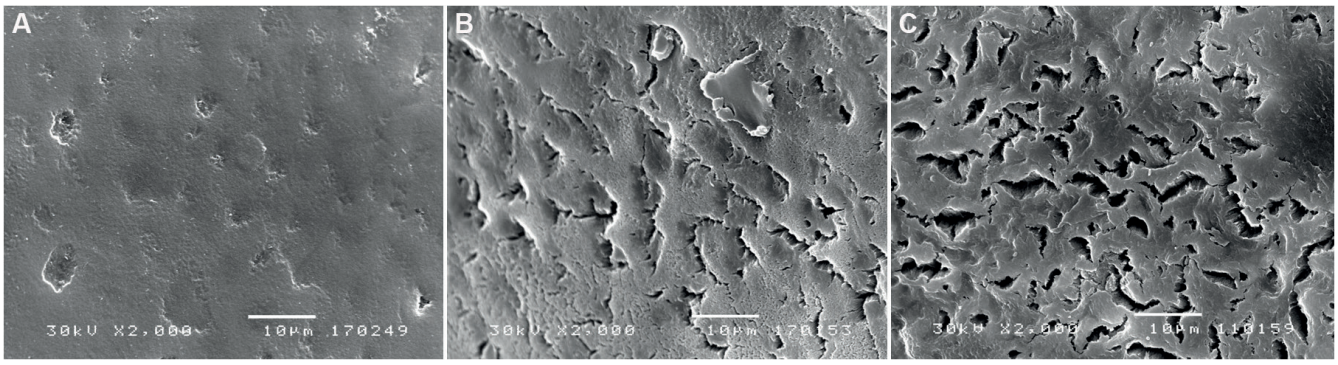

Fig. 1. Scanning electron microscopy (SEM) image of the control samples (×2000 magnification). A. Artificial saliva: unaltered surface, slight rugosity and development pores; B. Orange juice: 30 min of cyclic exposure, atypical etching of the enamel surface with deep creases and furrows, partially covered with granular crystals; C. Orange juice – 60 min of cyclic exposure, densely wrinkled areas with loss of enamel morphology

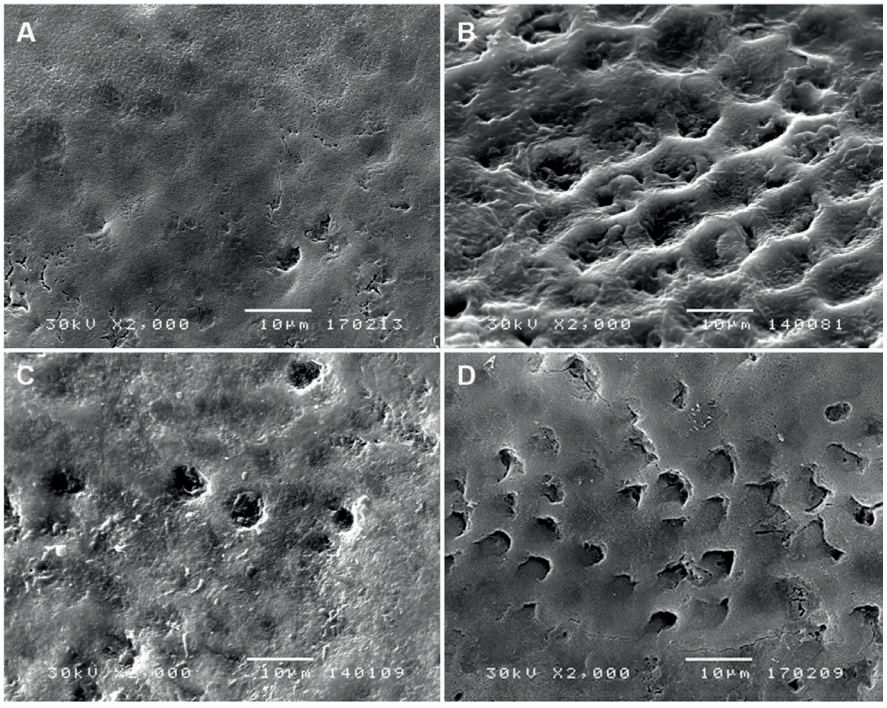

Fig. 2. Scanning electron microscopy (SEM) image of the experimental samples after cyclic exposure (×2000 magnification). A. 30 min to beer: shallow depressions, slight porosity and 2 smaller fields of atypical etching (lower part of the picture, left and right corner); B. 60 min to red wine: shallow indentations with pronounced "honeycomb" structure; C. 30 min to white wine: a greater number of increased diameter pores, rare wrinkled fields, demineralization of some enamel rods; D. 60 min to white wine: irregular areas with accentuated rod contours, rod demineralization, the visible "hoof-like" form of the rods

Table 7. Pairwise comparisons of average surface roughness (R_a) in relation to beverage type for the same exposure time

Exposure time	Groups compared	Test statistic	SE	Standardized test statistic	Sig.	Adj. Sig.[a]
15 min	NC, white wine	−20.304	5.948	−3.413	0.001	**0.006**
	NC, PC	−27.179	5.948	−4.569	0.000	**0.000**
	beer, red wine	−3.250	6.711	−0.484	0.628	1.000
	beer, white wine	−8.500	6.711	−1.267	0.205	1.000
	red wine, white wine	−5.250	6.711	−0.782	0.434	1.000
30 min	NC, white wine	−19.589	5.949	−3.293	0.001	**0.010**
	NC, PC	−32.589	5.949	−5.479	0.000	**0.000**
	beer, red wine	−1.750	6.711	−0.261	0.794	1.000
	beer, white wine	−5.687	6.711	−0.848	0.397	1.000
	red wine, white wine	−3.937	6.711	−0.587	0.557	1.000
60 min	NC, white wine	−20.643	5.948	−3.470	0.001	**0.005**
	NC, PC	−29.143	5.948	−4.899	0.000	**0.000**
	beer, PC	19.375	6.710	2.887	0.004	**0.039**
	beer, red wine	−1.937	6.710	−0.289	0.773	1.000
	beer, white wine	−10.875	6.710	−1.621	0.105	1.000
	red wine, white wine	−8.937	6.710	−1.332	0.183	1.000

PC – positive control; NC – negative control; SE – standard error; Sig. – significance; Adj. – adjusted; [a] significance values have been adjusted by the Bonferroni correction for multiple tests. Only pairs with statistical significance, as well as pairs that include experimental drinks (beer, red and white wine) are included. Values in bold indicate statistically significant results.

ending with 24 h.[6] The present research used the cyclic de- and remineralization model, i.e., samples immersion in acidic (alcoholic) beverages, including occasional agitation, followed by exposure of samples to artificial (or natural) saliva, then repeating the challenge several times.

To achieve better comparability between the tested substances, enamel samples from impacted third molars were used. Their surfaces were completely intact (they were not exposed to chewing forces), without any scratches or notches that are otherwise characteristic of teeth in function. Furthermore, they came from individuals of approximately the same age and with a similar degree of tooth mineralization.

As a medium for remineralization, we used artificial saliva with electrolytes of the same or similar formulation in previous, related studies.[13,18] Also, gentle agitation of the solutions was applied to "imitate" the usual way of drinking (no shaking or retention).

To assess the erosive damage, a stylus profilometer was used, which can read all surface irregularities along the length of the object. Although this method is flawed in that it does not register the amount of enamel loss,[19] it is applied in a large number of studies to assess the impact of erosive substances on hard dental tissues.[3,12,19,20] Of the 4 parameters registered using the stylus profilometer, R_a was singled out, which shows the average roughness value. While this parameter does not provide information about the characteristics of surface irregularities, it is a common analytical tool in the investigation of the surface of dental tissues and materials after erosive challenges (acidic beverages, bleaches, etc.).[6,21,22]

The absolute R_a values were higher compared to those observed in previously published studies.[3] A possible reason is our use of a 0.75 mN low-pressure detector with a 2-µm stylus radius. This allowed for a more precise measurement to be taken due to the recording of narrower and deeper irregularities without fear of damaging the sample surface.[20]

The enamel samples analyzed for SR were not flattened and polished before immersion in the experimental and control solutions. This methodology is justified by the fact that polishing removes significant amounts of enamel, probably a complete aprismatic layer, which leads to faster lesion progression[23]; since natural enamel surfaces require longer periods of erosion, we found that cyclic exposure to erosive solution of 15 min, 30 min or 60 min during 10 days is long enough for measurable change (such as SR) to be quantified[16]; measurement of 1 central cluster roughness of unpolished enamel represents the total SR of enamel, before and after erosion, the same as in the polished sample.[24]

In studies of the erosive potential of acidic substances, the determination of the initial pH and TA (and/or buffering capacity) is mandatory. Erosion occurs at low pH, but there is no fixed "critical" pH for tooth erosion. This value is calculated from the calcium and phosphate concentrations in the erosive solution itself.[5] From the critical values (pHc) published by Lussi and Carvalho, we singled out those that are important for this study, namely orange juice (3.6), beer (5.0) and red and white wine (5.1).[5]

Wine derives its acidity mostly from weak mono- and di-basic acids, since white wine contains malic acid and a certain amount of lactic acid, while the share of citric acid

is almost negligible. Lactic, and to a lesser extent tartaric, acid dominate in red wine.[25]

Chemical analysis indicates that beer contains phenolic acids whose presence affects its pH (around 4.0).[26] In contrast, orange juice contains citric acid, whose strong erosive effect comes from hydrogen ions, and acid anions (citrates) that build complexes with calcium, as well as undissociated acid molecules.[25]

Of the possible buffer properties, this study focused on the determination of TA, which has a "closer" relationship with the concentration of undissociated acid than the buffering capacity. Unsaturated substances with low pH and high TA have a higher erosive potential.[5,25] In the present study, we demonstrated that white wine has higher TA values than red wine and beer, which is generally consistent with other research.[2] Moreover, the high values of TA for orange juice ($4.28_{5.5}$ and $5.83_{7.0}$) are in line with the findings of other studies.[2,14,18] Cyclic exposure of 30 min and 60 min was long enough to show significantly stronger erosive potential of orange juice compared to the shorter exposure (15 min).

It seems that beer is not a strong erosive substance. Although Zaječar beer has a relatively low pH (3.9) and $TK_{5.5}$ = 0.64, the R_a values were significantly lower than the R_a for orange juice samples. Zanatta et al. examined the microhardness of bovine enamel after immersing samples in 3 different beer brands for 5 min, 30 min and 60 min.[10] Only Heineken beer showed a decrease in microhardness after exposure for 30 min, although its pH was slightly higher (pH = 4.35) than the other 2 beers tested. They assumed the reason was the larger amount of citrate, which was not completely consumed during the brewing process.[10] Similarly, Lussi et al. found that Carlsberg beer and Montagne red wine did not produce any significant changes in enamel surface hardness.[2] The present results are comparable to those of the mentioned authors, although they used a different method (microhardness) for erosion assessment in examining the erosive potential of several types and brands of beer[2,10] and wine.[2]

Willershausen et al. examined the impact of white wine and red wine on human enamel for a continuous period of 24 h. In addition to the R_a parameter analysis, the amount of released calcium was calculated. Riesling white wine was observed to have the lowest pH and highest TA, as well as significantly higher Ca release from the eroded samples. In the current study, white wine of the same type had a lower pH (3.02 compared to 3.49) and a higher TA (2.69 compared to 1.82) than red wine (Vranac). Although the absolute R_a values for white wine were higher, no statistical significance was found. Most of the previous studies have found that white wine is more erosive than red white,[2,6] and explained this by the higher amount of polyphenols in red wine.[2,14] Polyphenol molecules can react with salivary proteins to form protein–polyphenol complexes that bind to proteins of the acquired enamel pellicle. Exposing the acquired pellicle to liquids rich in polyphenols facilitates further adhesion of these complexes to the pellicle and increases its thickness and resistance to removal.[14] The present research used artificial saliva that does not contain proteins, but some studies have shown that spontaneous formation of thin polyphenolic coatings is possible on polymeric, metallic and native-oxide surfaces that are exposed to liquids rich in polyphenols.[27]

Although quantitative analyses of hard dental tissues altered by erosion provide far more objective results, SEM with grading (scoring) of the alterations can be applied for qualitative assessment of tissue surface morphology.[28,29] Acid attacks lead to a surface etching pattern with more or less exposure of enamel rods (prisms), which depends on the severity of the erosive challenge. Beyer et al. studied the ultrastructure of the enamel surface after immersing the samples in different acids for 60 s. The SEM micrographs of lactic, phosphoric and ascorbic acid-treated samples showed "cobblestone" type enamel etching with a rough surface and tiny crystals, unlike samples exposed to tartaric, malic and citric acid, which had smooth and less eroded areas.[30] Apart from the analogy regarding the acids that are an integral part of alcoholic beverages, there is no other data on the enamel surface SEM examination after exposure to beer, red and white wine. Only in the case of Bordeaux red wine, slight signs of erosion were found after a single immersion for 90 s.[8] Considering our experimental setup (cyclic model), comparisons with the results of other authors were not possible.

In contrast, our results showing the atypical erosion of the enamel surface treated with orange juice are in accordance with the results of Braga et al., who compared enamel morphology after an erosive challenge with gastric and orange juice in a cyclic procedure.[11]

In the present study, cyclic exposure to alcoholic beverages led to an increase in SR along with exposure time, but only in samples immersed in white wine (60 min compared to 15 min). However, no such result was observed with samples immersed in beer and red wine. The SEM observation showed the same result, so the null hypothesis was partially accepted.

Limitations

Erosion is a complex condition that depends on numerous factors and their interaction. Due to the limited effect of in vitro studies, several types of analyses should be conducted to allow both qualitative and quantitative assessment of tooth tissue loss. In the present study, SEM observation contributed to the qualitative analysis of enamel surfaces, but due to the small number of samples, it could not be supported by scores that would indicate the degree of erosive damage.

Conclusions

This study confirms the limited erosive potential of beer, red and white wine, and a significant relationship with pH, TA and SR, but not with the exposure time for all tested

alcoholic beverages. It also provides information on morphological differences in the intensity of erosive changes with time of exposure to white wine, as well as qualitative differences among the ultrastructural patterns caused by beer, red and white wine on the enamel surface.

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Employment of the Evolution RL sheath as a first-choice device shortens transvenous lead extraction time without affecting procedural safety and efficacy compared to its auxiliary use: Insights from the prospective multicenter EVO registry

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Abstract

Background. Transvenous lead extraction (TLE) is recommended in cases of local and systemic infections related to cardiac implantable electronic devices (CIEDs). Additionally, TLE is indicated in the event of lead damage or CIED malfunction. The extraction procedure is associated with a risk of life-threatening complications.

Objectives. The aim of the EVO registry was to assess the safety and efficacy of birotational Evolution tool usage.

Materials and methods. This registry study was prospectively conducted in 8 high-volume implantation centers in Poland. The study included 133 patients aged 63.5 ±15.1 years, and 76.69% were male. Indications for the procedure were: local or systemic infection (33.1%) and lead dysfunction (66.9%). The number of leads extracted varied from 1 (39.84%) to 3 (9.77%).

Results. Clinical procedural success was achieved in 99.1% of cases. A total of 226 leads were extracted, and 206 used the Evolution system. Two procedural strategies were identified while using the Evolution system: (1) usage of locking stylet, propylene sheaths and the Evolution system (118 leads, 52%) – group A; (2) usage of locking stylet and Evolution (88 leads, 39%) – group B. There were no differences in the number of complications between these 2 groups. The extraction time was significantly shorter ($p = 0.02$) in group B than in group A. Major complications occurred in 5.2% of cases with 2 intraprocedural deaths. Minor complications occurred in 1.5% of patients.

Conclusions. The registry confirmed the efficacy and relative safety of the birotational Evolution sheath. Using the rotational sheath as a first attempt significantly reduces extraction time without compromising its safety.

Key words: transvenous lead extraction, rotational sheaths, pacing leads, defibrillation leads, CIED complications

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Conflict of interest
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Background

Treatment of complications related to cardiac implantable electronic devices (CIEDs) remains challenging for the medical personnel involved in this method of therapy. Complete device and lead extraction is recommended in cases of local and systemic infections related to previously implanted systems.[1–3] Moreover, lead extraction is indicated in cases of lead damage or malfunction. Progression of the underlying heart disease requires upgrading the previously implanted system in a growing number of patients. In many cases, it is necessary to extract the lead in order to regain vascular access for the placement of new leads. Thus, the number of indications for transvenous lead extraction (TLE) is steadily growing. Despite technological progress and the release of new, advanced tools dedicated to TLE, the procedure is still associated with the risk of life-threatening complications, particularly cardiac tamponade or superior vena cava injury.[4,5] Superior vena cava injury occurred more frequently with the use of laser or powered sheaths, which seemed to discourage some physicians from their broad use despite higher effectiveness.[5] In some countries, reimbursement concerns may also influence the choice of the TLE method.[6] The bidirectional, rotational Evolution mechanical sheath (Cook Medical, Bloomington, USA) is a relatively new extraction tool. Its efficacy and safety have been shown in several retrospective studies and 1 multicenter prospective study.[7–11] However, the data are still limited, and several controversies concerning Evolution tool utilization and an optimal TLE strategy remain unresolved. One issue concerns the use of Evolution tool either as a first choice during the TLE procedure or when the initial attempt with nonpowered sheaths has failed in terms of efficacy and safety, with potential disadvantages related to the cutting properties of the Evolution tool.

Objectives

The aim of this prospective multicenter registry (EVO registry) study was to assess the safety and efficacy of the rotational Evolution tool usage, and evaluate the 2 different strategies of TLE procedures.

Materials and methods

This registry study was prospectively conducted in 8 high-volume centers (>30 TLE procedures per year) in Poland, in the years 2019–2021. The patients (≥18 years old) were included in the study if at least 1 lead was extracted using the Evolution system. The Evolution system was applied at the discretion of the operating physician. The use of additional tools and techniques was based on the decision of the operating team. The procedures were performed according to the current European Heart Rhythm Association (EHRA) and Heart Rhythm Society (HRS) guidelines.[2,3] All patients signed informed consent for lead extraction and emergency cardiosurgical procedure, if needed. Data were recorded during the procedure and placed in the case report forms immediately after the procedure, and thereafter were supplemented with clinical and demographic preoperative data. Postoperative and follow-up data were obtained prospectively. The study was approved by the ethics committee of the National Institute of Cardiology, Warsaw, Poland (approval No. IK-NPIA-0021-103/1755/18 issued on November 17, 2018).

Lead extraction procedure

As mentioned above, all procedures were performed according to the EHRA and HRS recommendations.[2,3] An arterial line for continuous blood pressure monitoring was placed in all cases. A cardiosurgical team and an extracorporeal circulation machine were on standby in the operating room. A temporary pacing wire was introduced by either the femoral or jugular vein contralateral to the CIED insertion site in pacemaker-dependent patients. The superior approach was used as a first choice in all cases. After the skin incision, the leads were dissected free and the fixation sutures were removed. The order of extracted leads was decided by the operating physicians. First, an attempt to place the standard wire into the lead lumen was made in order to check its patency and unscrew the fixation mechanism in active fixation leads (if possible). Second, gentle manual traction was performed. If this approach was unsuccessful, the Liberator Locking Stylet (Cook Medical) was inserted into the lead lumen under fluoroscopic control and its locking mechanism

was activated. In the cases of severe extravascular calcifications and adhesions in the subclavicular region, either steel sheaths or Evolution Shortie tools (Cook Medical) were applied. To grasp lead insulation and obtain greater lead stability, silk sutures were used. A Bulldog™ Lead Extender (Cook Medical) tool was used for the removal of lumenless cardiac leads. Then, leads were extracted using either polypropylene Byrd dilator sheaths (Cook Medical) or the bidirectional Evolution mechanical system. The use of the nonmechanical or mechanical tool as the first approach depended on the decision of the operating team. In cases of unsuccessful superior approach or in order to remove remnant fragments, the femoral approach with dedicated tools such as the Needle's Eye Snare® (Cook Medical) was used. In patients with noninfective indications for lead extraction, a new device was implanted during the same session, while it was postponed in patients with CIED-related infections, in accordance with the EHRA recommendations. In all the cases, the indications for reimplantation were reassessed.[3] Alternative approaches, i.e., leadless pacemakers or subcutaneous implantable cardioverter-defibrillators (ICDs), were applied if clinically justified.

Definitions

Clinical and procedural success, as well as complications, were determined according to the 2017 HRS Expert Consensus.

Complete procedural success was defined as the removal of all targeted leads and all lead materials from the vascular space without the occurrence of any permanently disabling complication or procedure-related death.

Clinical success was defined as the removal of all targeted leads and lead materials from the vascular space or retention of a small portion of the lead (≤4 cm) in the absence of complications.

Major complications were defined as outcomes that were life-threatening and/or resulted in significant or permanent disability or death, or required surgical intervention.

Minor complications were defined as events related to the procedure that required medical intervention or minor procedural intervention.

Procedure time was defined as the time from the first skin incision to the final wound closure.

Extraction time was defined as the time from lead release from pocket adhesion to complete or incomplete lead extraction.

Statistical analyses

Statistical analyses were conducted using Statistica Tibco v. 13.3 software (StatSoft Inc., Tulsa, USA). Categorical variables were presented as numbers (percentages), and differences between patient groups were compared using the χ^2 test. The Shapiro–Wilk test evaluated the normality of data distribution. The continuous variables were provided as medians with interquartile range (IQR) or mean with 95% confidence interval (95% CI), and compared using the Mann–Whitney U test, Kruskal–Wallis test or unpaired Student's t-test, where appropriate. A one-way analysis of covariance (ANCOVA) was performed to adjust for potential confounders. A Pearson's or Spearman's correlation coefficient was used to analyze the associations between continuous variables. To calculate the odds ratio (OR) with a 95% CI, the cutoff point was calculated based on receiver operating characteristic (ROC) curves. Results were considered statistically significant when the value of $p < 0.05$.

Results

The study group consisted of 133 patients who met the inclusion criteria, i.e., patients who had at least 1 lead extracted using the Evolution device. The mean age was 63.5 ±15.1 years and 102 patients were males (76.7%). The mean body mass index (BMI) was 27.3 ±5.9. Twenty-six patients (19.5%) underwent previous cardiosurgical procedures, including 2 patients operated on for congenital heart diseases and 1 with an implanted left ventricular assist device. Characteristics of the patients are presented in Table 1. The number of leads in the vascular system ranged from 1 to 4. The mean indwell time was 133.3 ±87.1 months (ranging from 1 month to 399 months) (Table 2). The most common CIED was a single-chamber ICD (48 patients, 36%). Three patients had either pacing or abandoned defibrillation leads after a previous system upgrade. Two patients were implanted with subcutaneous leads, including 1 implanted with a subcutaneous ICD, while the other had an additional defibrillation coil connected to a transvenous ICD.

Lead extraction indications

Local or systemic infection was an indication for TLE in 44 patients (33.1%). Transvenous lead extraction was performed due to noninfectious indications, mainly lead dysfunction, in the remaining 89 patients (66.9%). In this group, regaining venous access for new lead implantation or system upgrade was an additional indication in 35 cases (26.3%).

Procedural data

In total, 226 leads were extracted, of which 206 were removed using the Evolution system. The remaining 20 leads were removed with manual traction only (n = 5) or with Liberator Locking Stylet and polypropylene Byrd dilator sheaths (n = 15) (Fig. 1). For all leads removed with manual traction, the indwell time was shorter than 6 months. Lead characteristics are presented in Table 2.

Table 1. Study group demographics

Patient characteristics		Values
Age [years], M ±SD (n, min–max)		63.54 ±15.10 (133, 20–89)
Male, %		76.69 (102/133)
Coronary artery disease, %		48.12 (64/133)
Previous MI, %		38.35 (51/133)
DCM, %		30.83 (41/133)
Valvular heart defect, %		22.56 (30/133)
NYHA class 3 or 4, %		14.29 (19/133)
AFIB, %		44.36 (59/133)
AVB, %		24.06 (32/133)
SSS, %		20.30 (27/133)
DM, %		32.33 (43/133)
Renal failure, %		19.55 (26/133)
Hypertension, %		66.17 (88/133)
Previous heart surgery, %		19.55 (26/133)
ASA, %		30.83 (41/133)
VKA, %		11.28 (15/133)
NOAC, %		28.57 (38/133)
LMWH, %		13.53 (18/133)
Previous CIEDs	VVI pacemaker, %	27.82 (37/133)
	DDD pacemaker, %	10.52 (14/133)
	ICD-VR, %	33.83 (45/133)
	ICD-DR, %	11.27 (15/133)
	CRT-D, %	14.28 (19/133)
	CRT-P, %	1.50 (2/133)
	PM/ICD, %	1.50 (2/133)
Indications	infectious indications, %	33.08 (44/133)
	noninfectious indications, %	66.17 (88/133)
	regaining vascular access, %	26.32 (35/133)
Number of leads in the vascular system	1, %	31.58 (42/133)
	2, %	50.38 (67/133)
	3, %	17.29 (23/133)
	4, %	0.75 (1/133)
Number of leads planned for removal	1, %	42.11 (56/133)
	2, %	47.37 (63/133)
	3, %	10.53 (14/133)
Implantation side	left, %	86.47 (115/133)
Number of previous CIED-related procedures	1, %	37.12 (49/133)
	2, %	37.12 (49/133)
	3, %	17.42 (23/133)
	4, %	3.79 (5/133)
	5, %	4.55 (6/133)

MI – myocardial infarction; DCM – dilated cardiomyopathy; NYHA – New York Heart Association; AFIB – atrial fibrillation; AVB – atrioventricular block; SSS – sick sinus syndrome; DM – diabetes mellitus; ASA – acetylsalicylic acid; VKA – vitamin K antagonists; NOAC – novel oral anticoagulants; LMWH – low molecular weight heparin; CIEDs – cardiac implantable electronic devices; VVI – single chamber ventricular pacing system; DDD – dual chamber pacing system; ICD-VR – single-chamber implantable cardioverter defibrillator; ICD-DR – dual-chamber implantable cardioverter defibrillator; CRT-D – cardiac resynchronization therapy with defibrillator; CRT-P – cardiac resynchronization therapy pacemaker; PM – pacemaker; M ±SD – mean ± standard deviation. Continuous variables are expressed as M ±SD and categorical variables are expressed as values and percentages.

Table 2. Lead characteristics

Number of leads		226
Dwell time [months], M ±SD (n, min–max)		133.3 ±87.1 (226, 1–339)
Lead placement	RA, %	34.07 (77/226)
	RV, %	58.41 (132/226)
	LV, %	6.64 (15/226)
	other, %	0.88 (2/226)
Lead type	defibrillation, %	19.91 (45/226)
	single coil leads, %	73.33 (33/45)
	dual coil leads, %	22.22 (10/45)
	NA, %	4.44 (2/45)
	pacing, %	80.09 (181/226)
Lead fixation	active, %	81.42 (184/226)
	passive, %	18.14 (42/226)
	NA, %	0.44 (4/226)
Number of leads extracted per procedure	1, %	39.84 (53/133)
	2, %	50.37 (67/133)
	3, %	9.77 (13/133)

RA – right atrium; RV – right ventricle; LV – left ventricle; NA – not available; M ±SD – mean ± standard deviation

The femoral approach with the Needle Eye Snare® was applied in 2 cases of incomplete extraction with the superior approach for the removal of remnant parts. Steel sheaths for the dissection of extravascular adhesions in the subclavicular region were used in 64 cases (28.3%).

The number of leads extracted during a single procedure varied from 1 (53 cases, 39.84%) to 3 (13 procedures, 9.77%) (Table 2). Among the 80 procedures where more than 1 lead was removed, the right ventricular lead was extracted first in 59 cases (73.75%), whereas the right atrial was extracted first in the remaining 21 (26.25%) cases.

Complete procedural and clinical success rate

Clinical success was achieved for 224 leads (99.1%), with 214 leads completely removed (complete procedural success = 94.7%). Eight leads were partially removed with retention of a small portion of the lead (<4 cm), which did not negatively impact the outcome goals of the procedure. One lead was not removed and the remaining lead was removed during a rescue cardiosurgical procedure (Fig. 2).

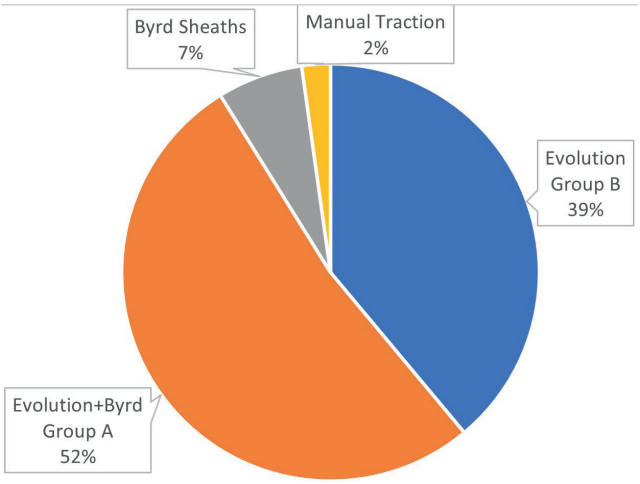

Fig. 1. Techniques and tools used for lead extraction

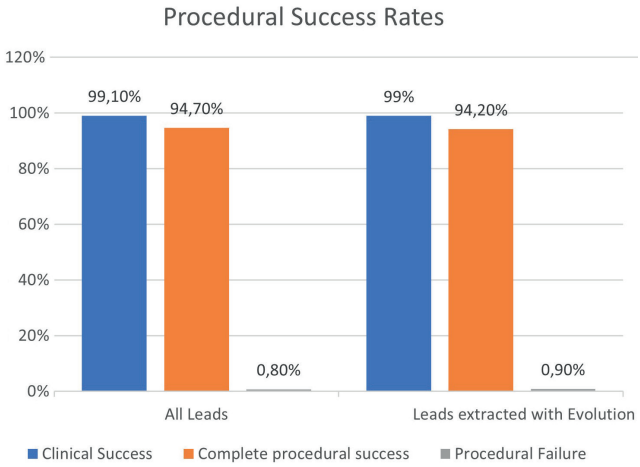

Fig. 2. Results of lead extraction procedures. The procedural success rate for all leads (panel on the left) and for leads extracted with the use of Evolution system (panel on the right)

Complications

Major complications occurred in 7 patients (5.2%). There were 2 intraprocedural deaths during the procedure. A 71-year-old male with severely impaired left ventricular function (left ventricular ejection fraction (LVEF), 12%) died during the electromechanical dissociation after a partial defibrillation lead extraction (<4 cm residual left). The patient was implanted with an ICD 36 months before the index procedure. Transvenous lead extraction was performed due to a system infection. The 2nd patient with complications was a 71-year-old male with post-myocardial heart failure (ejection fraction (EF), 20%) and a history of coronary artery bypass surgery. He was referred for TLE due to the dysfunction of the defibrillation lead implanted 9 years before the index procedure. During the procedure, cardiac tamponade occurred and the patient died despite immediate pericardiocentesis and cardiosurgical intervention.

The remaining complications included cardiac tamponade requiring emergency cardiosurgical repair (1 case), pericardiocentesis (1 case), bleeding from the subclavian vein requiring immediate surgical repair (1 case), asystole (1 case), and ventricular fibrillation (1 case). Minor complications (2 patients, 1.5%) included pneumothorax (1 patient) and worsening tricuspid valve insufficiency (1 patient). Pneumothorax occurred in patients who required subclavian vein puncture for new lead implantation. Thus, the complication might be related to this procedure and not necessarily to TLE. Detailed information on patients with complications is presented in Table 3.

Two patients died after the procedure during index hospitalization – 1 due to septic shock and 1 due to cardiogenic shock.

TLE strategies

Among leads extracted with the use of the Evolution system, 2 procedural strategies were identified (Fig. 1):

1) use of locking stylet, propylene sheaths and Evolution (118 leads, 52%) – group A;

2) use of locking stylet and Evolution (88 leads, 39%) – group B.

Comparison of groups A and B

The use of the Evolution sheath as a first choice was more frequent in the case of right atrial leads (OR – 1.54), pacing leads (OR – 1.54) and leads with dwell time ≤160 months (OR – 1.63) (Fig. 3).

The extraction time was significantly shorter (p = 0.02) in group B than in group A (Table 4).

There were no differences in the number of complications between those 2 groups, although the overall small number of complications did not allow us to reach valid conclusions. There was 1 procedure-related death in group A and 1 in group B. However, the death in group B was not caused by mechanical complications related to the TLE procedure but was rather related to the patient's underlying heart disease. Other major complications occurred in 2 patients in group A and 1 patient in group B.

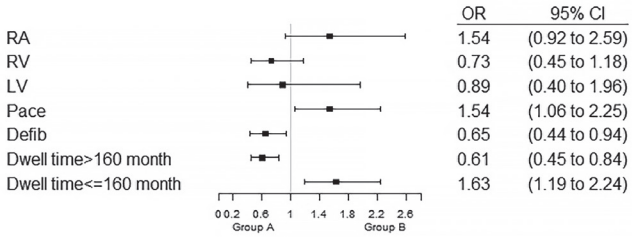

Fig. 3. Predictors for using the Evolution sheath as a first choice

RA – right atrium; RV – right ventricle; LV – left ventricle; OR – odds ratio; 95% CI – 95% confidence interval.

Table 3. Detailed description of major and minor complications

Patient number	Complication		Intervention and outcome	Age [years]	Previous CIEDs	Indications for TLE	Number of leads	Dwell time	Evolution	Byrd
1	major complications	vena subclavia injury	surgical repair, recovery	28	PM DDD	noninfectious	2	120	+	+
2		cardiac rupture	cardiosurgical repair, recovery	65	ICD-DR	noninfectious	2	168	+	+
3		cardiac rupture	cardiosurgical repair, death	71	ICD-VR	noninfectious	1	108	+	–
4		electromechanical dissociation	death	71	ICD-VR	infectious	1	36	+	+
5		hemopericardium	pericardiocentesis, recovery	84	PM DDD	noninfectious	2	32	+	–
6		ventricular fibrillation	successful defibrillation	74	PM DDD	noninfectious	2	216	+	–
7		asystole	successful resuscitation, temporary lead placement	76	CRT-D	infectious	3	180	+	+
1	minor complications	pneumothorax	drainage, recovery	85	PM DDD	infectious	2	132	+	–
2		tricuspid regurgitation	pharmacological treatment, observation	73	PM DDD	noninfectious	2	100	+	+

PM – pacemaker; DDD – dual chamber pacing system; ICD-VR – single-chamber implantable cardioverter defibrillator; ICD-DR – dual-chamber implantable cardioverter defibrillator; CRT-D – cardiac resynchronization therapy with defibrillator; CIEDs – cardiac implantable electronic devices; TLE – transvenous lead extraction.

Table 4. Comparison of extraction time between group A (Byrd sheaths and Evolution) and group B (Evolution as a first approach)

Group	n	Extraction time [min]									
		mean	lower 95% CI	upper 95% CI	median	minimum	maximum	Q1	Q3	IQR	SD
Group A	118	27.79	22.50	33.08	18.04	1.00	180.00	10.00	35.00	25.00	29.02
Group B	88	21.38	16.12	26.65	10.00	0.57	120.00	5.00	25.00	20.00	24.41

95% CI – 95% confidence interval; IQR – interquartile range; SD – standard deviation; Q1 – 1st quartile; Q3 – 3rd quartile.

Discussion

The design of our study aimed to investigate the everyday practice related to TLE and the use of the birotational Evolution mechanical sheath. Due to the complexity of the TLE procedure and heterogeneity of both patients and clinical scenarios, our knowledge on the efficacy of TLE is based mainly on retrospective and observational studies with the exception of the prospectively conducted Transvenous Lead Removal Using the Cook Evolution LEAd Extraction SystEm (RELEASE) trial.[4,7–11] Thus, our prospective multicenter registry seems to add valuable data to the current knowledge regarding the safety and efficacy of lead extraction. The data were obtained prospectively from 8 high-volume centers (defined as >30 TLE procedures per year). The studied population did not differ from other studies investigating lead extraction, although it should be noted that in terms of indications and predominance of noninfectious indications, our registry was more similar to the RELEASE study population than to older studies, thereby reflecting the current trend in clinical practice.

The leads' mean dwell time was longer in our study than in the RELEASE or European Lead Extraction ConTRolled (ELECTRA) studies. In our study, the mean dwell time was 11.56 years, whereas, in the RELEASE and ELECTRA studies, the dwell time was 7.4 and 5.3 years, respectively. Taking into account the longer dwell time, the efficacy of the TLE procedures was very high, with a complete success rate of 99.1% and a clinical success rate of 94.7% for all extracted leads, and 99.1% and 94.1% for leads extracted with the Evolution sheaths. The results are similar to those reported in the RELEASE study and current observational studies with birotational Evolution sheaths.[4,7–11]

The number of major complications was similar to those reported in the ELECTRA registry, and the intraprocedural complication rate was slightly higher than in the RELEASE trial (3.8% compared to 2.6%).[11,12] However, it should be noted that 1 major complication and periprocedural death was mainly related to the patient's underlying cardiological status and was not caused by the mechanical complications of the elaborated procedure. The 2nd death occurred as a result of cardiac rupture despite immediate

cardiosurgical intervention. The ELECTRA registry reported 20% mortality among patients with cardiac avulsion or tear despite immediate pericardiocentesis and/or cardiosurgical repair.[12] Moreover, it should be noted that according to Manufacturer and User Facility Device Experience (MAUDE) analysis, the number of lethal complications associated with rotational sheaths may be underestimated and underreported.[5]

In our cohort of patients, there were no superior vena cave injuries, which are considered the most life-threatening TLE complication.

Among minor complications, pneumothorax was probably a complication of subclavian vein puncture for the new lead placement and not the TLE procedure itself. It is possible to reduce the risk of this complication by avoiding the attempt of subclavian vein puncture and regaining vascular access by sheaths used for lead removal.[13] Similarly, ventricular fibrillation and asystole may occur during each intervention within cardiac cavities; thus, these complications are not specific to lead extraction only. However, it should be stressed that every possible effort should be made to maintain efficient pacing during the whole TLE procedure.

In contrast to the RELEASE study and the observational studies, in our study, the decision to use a mechanical sheath was made during the procedure, whereas in the RELEASE study, patients were found eligible for the study if the investigated device was intended to be used during the procedure, although only patients who had at least 1 lead removed with the Evolution system were enrolled.[11] Also, contrary to the aforementioned study, the use of nonmechanical sheaths as the first attempt was allowed, which seems to better reflect everyday clinical practice in our country as well as in other European countries. According to the data from the ELECTRA registry, nonpowered mechanical sheaths were used for lead extraction in 36.34% of cases.[4,12] In Poland, mainly due to reimbursement issues,[6] mechanical sheaths are used even more frequently.[14,15] Greater experience with mechanical sheaths and the fear of vascular complications reported when using more advanced tools might play a significant role in the selection of procedural strategy.[5,13] Thus, our study, which showed a high efficacy and a low number of complications with rotational mechanical sheaths, seems to play an important role in the ongoing discussion concerning an optimal TLE strategy. Of note, in 52% of cases in our study, the Evolution sheath was introduced when the initial attempt with Byrd dilator sheaths was unsuccessful. In all but 2 cases, this approach allowed us to achieve complete or clinical success without compromising the safety of the procedure. In comparison to this approach, the use of Evolution as a first choice strategy shortened the extraction time and thereby could lower the rate of late complications. Due to the registry design, we could not provide any evidence for the hypothesis. However, the relationship between procedure duration and infection rate shown in many studies and meta-analyses was recognized in EHRA guidelines for CIED infections as one of the most important risk factors.[16,17] Importantly, any effort aimed at shortening the procedural time is important due to the limited availability of hybrid operation rooms and cardiosurgical and anesthesia personnel.

Based on our study, it is difficult to point out the factors that influenced the operators' choice, that is to say, the use of Evolution sheath as a 1st or 2nd attempt. The fact that Evolution was used as a first choice, more frequently in the case of atrial and pacing leads, could be explained by the operation strategy, i.e., by choosing to extract the right ventricle lead before other leads in 75% of cases. If the Evolution tool was used for right ventricle lead extraction, it was also used for the extraction of the remaining, primarily right atrial, leads. The same reasoning explains the utilization of Evolution as the first choice for pacing leads removal. It remains unclear why Evolution was more willingly used for the extraction of leads with relatively shorter dwelling time (Fig. 3). It should not be ruled out that other factors like pocket adhesions might influence the operating physician's decision on TLE strategy, although this is only speculation because such data were not collected in the registry.[18]

Limitations

The registry is an observational, nonrandomized study with all its imperfections, especially the lack of a reference group. As discussed above, such a trial design would be very challenging within the context of lead extraction procedures. The patients were included in the registry based on the intraprocedural decision to introduce the Evolution tool either as a first or subsequent approach, and no data concerning patients treated with other methods in the same hospitals over the same time period were collected. Moreover, the study involved only 8 hospitals in 1 country and may not be representative of other populations.

The procedural time was not analyzed on purpose because it depends on reimplantation time in the case of noninfective complications. Thus, reimplantation time may be prolonged mainly due to the obstacles related to new lead placement, which has been reported, especially in the context of new left ventricular lead introduction.[19,20]

Conclusions

The results of the prospective, multicenter registry confirmed the efficacy and relative safety of the birotational Evolution sheath used for TLE. The strategy of using the rotational sheath as a first attempt significantly reduces extraction time without compromising safety.

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Diagnosis, management and knowledge of halitosis among Polish and Lebanese dentists: Questionnaire-based survey

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Abstract

Background. Halitosis (fetor ex ore, malodor, bad breath) is defined as an unpleasant odor coming from the oral cavity, regardless of the cause: local or systemic. It affects 22–50% of the population worldwide, leading to a significant decrease in the overall quality of life, and can have oral and extra-oral etiologies. There is an increased interest in the management of halitosis.

Objectives. This study aims to evaluate the patient–dentist communication on halitosis, the dentists' knowledge about the management and etiology of halitosis, and the treatment options used by dentists who practice in Poland and Lebanon.

Materials and methods. An online questionnaire was sent to both Lebanese and Polish dentists using Google Forms (Google LLC, Mountain View, USA). In total, 205 dentists completed the questionnaire, of which 100 practiced in Poland (group P) and 105 practiced in Lebanon (group L). A multivariate analysis was conducted to determine differences between both groups and to identify parameters that could influence a dentist's management of halitosis.

Results. According to the questionnaire, 86% of group P members and 65.7% of group L members reported communicating with patients about halitosis. Regarding the knowledge of halitosis, 78% of dentists in group P and 85.7% of dentists in group L reported that there is a classification for halitosis. A significant majority of dentists in both groups revealed not having any tool to measure halitosis (67.6% and 68% from group P and group L, respectively).

Conclusions. This study confirms the need for improved communication skills in Polish and Lebanese dentists, as well as for education on the subject among dentists in both countries, and for standardization in diagnosis, treatment modalities and management of halitosis.

Key words: questionnaire, laser, fetor ex ore, malodor, oral bad breath

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Background

Halitosis, or bad breath, is a common condition affecting 22–50% of the population worldwide, leading to a significant decrease in the overall quality of life. It can have oral and extra-oral etiologies. Halitosis (fetor ex ore, malodor, bad breath) is defined as an unpleasant odor coming from the oral cavity, regardless of the cause (local or systemic). This malodor is essentially due to the presence of chemical compounds in the exhaled air, mainly volatile sulfur compounds (VSCs) in oral pathologies and volatile organic compounds (VOCs) in the majority of extraoral causes.[1]

In the literature, reported prevalence of halitosis varies largely from study to study. This variation is due to many factors, such as its perception, definition, classification, and lack of a standard assessment method and diagnosis. For instance, the American Dental Association reports that 25% of the US population suffers from halitosis. Bornstein et al. stated that in Switzerland, 32% of the examined population suffers from halitosis, while in Japan, it is estimated to affect 23% of the population.[2] Conversely, Seemann et al. found that only 2.4% of Finnish population suffers from halitosis.[3] As for the classification, halitosis can be classified as 1) genuine halitosis, including pathological halitosis and physiological halitosis; and 2) psychological halitosis. Physiological halitosis occurs when the odor has a physiological origin and not pathological (from a putrefactive process), and is caused by a disease. Pathological halitosis presents as a pathological etiology that can be oral (such as periodontal diseases, tongue coating, bad oral hygiene, and dental caries) or extraoral (such as pneumonic disease, uncontrolled diabetes and others). Another classification based on the etiology of halitosis has also been proposed. This classification divides halitosis into physiological halitosis (type 0) and pathological halitosis (types 1–4). Type 1 has an oral etiology, type 2 – airway-related etiology, type 3 – gastroesophageal etiology, and type 4 –hematogenous etiology. According to this classification, type 5 includes subjective halitosis.[4–6]

Studies suggest that in 70% of cases, genuine halitosis originates from the oral cavity.[1,7] The most common reason is tongue coating (around 43% of all cases).[7–9] In addition, it was shown that when VSCs are accompanied by VOCs, the origin of the halitosis will most likely be extraoral (10–20% of cases). Among extraoral causes, chronic sinusitis, purulent tonsillitis, deviated septum, neoplastic changes, esophageal diverticula, severe gastroesophageal reflux disease, *Helicobacter pylori* infections, bronchitis, bronchiectasis, lung cancer, diabetic ketoacidosis, and uremia are well documented.[4,7–10]

There has always been a high demand internationally for the proper management of halitosis, and since 70% of halitosis cases originate from the oral cavity, the role of oral and dental healthcare providers seems to be intuitive.[11] However, halitosis-related education is limited among the dental community. Harmouche et al. established that halitosis-related knowledge is insufficient in the French and Lebanese dentist populations and highlighted the need for professional education in both countries, targeting proper diagnosis and treatment strategies for halitosis.[12]

Objectives

This study aimed to assess halitosis-related knowledge among dentists in Poland and Lebanon to determine the need for halitosis-related professional education.

Materials and methods

Study sample

An online questionnaire was sent to Polish (group P) practicing dentists and Lebanese (group L) practicing dentists via WhatsApp and e-mail using a sheet from Google Forms (Google LLC, Mountain View, USA) . The questionnaire sent to group P was in Polish, while group L received an English translation. The contacted dentists were informed regarding the scope and content of the study. The e-mail addresses and WhatsApp numbers were obtained from the Lebanese Dental Association and Polish Dental Association. One hundred and thirty dentists from each group were chosen arbitrarily from the master list of dentists, and the survey was sent to them with an explanation of the type and aim of the study. This study was conducted from September 2021 to September 2022. The questionnaire is the first part of our research on the treatment of halitosis with lasers. This study was approved by the local Medical Chamber (approval No. 12/148/2021).

Questionnaire design

This study was carried out using self-administered, structured questionnaires. The questionnaire used in this study was based on a previous study that evaluated French and Lebanese dentists regarding their knowledge and management of halitosis.[12] However, some modifications were made in our questionnaire.

Statistical analyses

For statistical analysis purposes, the data collected in the study were recorded, processed and analyzed using Statistica v. 13.3 (TIBCO Software Inc., Palo Alto, USA). All statistical tests were two-sided. A p-value of less than 0.05 was considered statistically significant. Categorical variables are presented in the contingency tables as percentages. Comparisons of the proportions of the Polish and Lebanese groups were made using the Pearson's χ^2 test.

Fisher's exact test was used due to a low expected frequency in the contingency table. Fisher's exact test was used

to determine if there is a significant difference between the responses of the two groups. To conduct Fisher's exact test, survey responses from the two groups were obtained and sorted into different categories. A contingency table was then created to display the frequencies of each category for both groups. Next, Fisher's exact test was applied to calculate the probability of obtaining the observed frequencies under the null hypothesis that there is no difference between the two groups. If the p-value obtained from the test is less than the predetermined significance level, it suggests that there is a significant difference between the two groups, which would allow to draw conclusions about the potential reasons for these differences.

Results

Demographic characteristics of the populations

A total of 205 participants were enrolled in our study (100 Polish and 105 Lebanese dentists), including 60% of women (71 Polish and 52 Lebanese female dentists). The mean value of age was 40 years old for group P and 49 years old for group P dentists), with an average professional experience of more than 20 years for Polish and <5 years for Lebanese respondents. The demographic characteristics of the 2 groups were significantly different in terms of age ($p < 0.001$), professional experience ($p < 0.001$), type of practice ($p < 0.01$), and specialization ($p < 0.01$) (Table 1).

Diagnosis and communication with patients

Concerning the diagnosis of halitosis, no significant difference was observed between both groups. A total of 32.7% of dentists, of which 31% were from Poland and 34.3% from Lebanon, received more than 10 patients per year seeking treatment for halitosis. According to 58% of dentists in group P and 50.5% of dentists in group L, less than 10 patients per year sought care for halitosis. Additionally, 11% of group P and 15.2% of group L members reported not receiving any patients per year complaining of halitosis (Table 2). Multivariate analysis showed that members of group P were more prone to inform patients about halitosis if they detected it during a check-up (odds ratio (OR) = 3.47, $p < 0.01$). However, interestingly, 68% of the Polish and 67.6% of the Lebanese dentists revealed not having any instrument for the diagnosis of halitosis (Table 3).

Table 1. Demographic characteristics of the studied population

Variable	Detailed information	Poland n (%)	Lebanon n (%)	χ^2/df	p-value
Gender	male	29 (29.0)	51 (50.5)	9.54/2	0.008[a]
	female	71 (71.0)	53 (49.5)		
	prefer not to say	0 (0.0)	1 (1.0)		
Age [years]	<30	12 (12.0)	84 (80.0)	97.4/4	<0.001[a]
	30–39	31 (31.0)	10 (9.5)		
	40–49	34 (34.0)	7 (6.7)		
	50–59	20 (20.0)	2 (1.9)		
	>59	3 (3.0)	2 (1.9)		
Professional experience [years]	<5	16 (16.0)	71 (67.6)	72.2/3	<0.001[a]
	5–10	14 (14.0)	20 (19.0)		
	11–20	34 (34.0)	5 (4.8)		
	>20	36 (36.0)	9 (8.6)		
Type of practice	private practice	96 (96.0)	76 (72.4)	–	<0.001[b]
	dental hospital	6 (6.0)	6 (5.7)	–	1.000[b]
	academics	32 (32.0)	50 (47.6)	5.21/1	0.022[a]
Dental specialization	general practice	43 (43.0)	76 (74.2)	18.2/1	<0.001[a]
	specialized in periodontology	9 (9.0)	5 (4.8)	–	0.275[b]
	specialized in oral surgery	13 (13.0)	4 (3.8)	–	0.022[b]
	specialized in endodontics	10 (10.0)	6 (5.7)	–	0.303[b]
	specialized in orthodontics	7 (7.0)	2 (1.9)	–	0.095[b]
	specialized in pediatric dentistry	6 (6.0)	3 (2.9)	–	0.323[b]
	specialized in prosthodontics	12 (12.0)	7 (6.7)	1.73/1	0.188[a]

[a] – Pearson's χ^2 test; [b] – Fisher's exact test; df – degrees of freedom.

Table 2. Results of the diagnosis and communication with patients among the population

Question	Answer	Group P n (%)	Group L n (%)	χ^2/df	p-value
Did you come across people with bad breath at your daily practice?	Yes, frequently	46 (46.0)	79 (75.2)	18.7/2	<0.001[a]
	Yes, rarely	53 (53.0)	25 (23.8)		
	No, never	1 (1.0)	1 (1.0)		
Do you receive patients that are aware of their bad breath and want to treat it?	Yes, more than 10 per year	31 (31.0)	36 (34.3)	1.40/2	0.496[a]
	Yes, but less than 10 per year	58 (58.0)	53 (50.5)		
	No	11 (11.0)	16 (15.2)		
Do you receive patients seeking advice for someone else's bad breath (wife/husband, friend, family member...)?	More often patients	85 (85.0)	84 (80.0)	0.38/1	0.538[a]
	More often doctors	15 (15.0)	20 (19)		
Do you diagnose or comfort your patients about their bad breath in your daily practice?	Yes	57 (57.0)	43 (41.0)	6.88/2	0.032[a]
	Only if patient brings the subject up	37 (37.0)	58 (55.2)		
	No	6 (6.0)	4 (3.8)		
If you detect a patient's halitosis, do you inform them about it?	Yes	86 (86.0)	69 (65.7)	10.4/1	0.001[a]
	No	14 (14.0)	36 (34.3)		
Do you try to educate your patients about halitosis?	Yes	76 (76.0)	91 (86.7)	3.18/1	0.074[a]
	No	24 (24.0)	14 (13.3)		
Do you feel uncomfortable when talking about halitosis with a patient?	Yes, often	23 (23.0)	26 (24.8)	0.09/2	0.957[a]
	Yes, rarely	31 (31.0)	32 (30.5)		
	No, never	46 (46.0)	47 (44.8)		

[a] – Pearson's χ^2 test; df – degrees of freedom.

Knowledge among dentists

In group P, 78% of dentists responded that there is a classification for halitosis, 69% stated that they do not know the classification, and 22% claimed there is no classification for halitosis. In addition, 1% of group P members claimed that halitosis was made up by pharmaceutical companies, and 61% had not heard of pseudo-halitosis.

As for group L, 85.7% of dentists responded that there is a classification for halitosis, 50.5% stated that they do not know the classification, and 14.3% claimed there is no classification for halitosis. In addition, 18.1% of group L members claimed that halitosis was made up by pharmaceutical companies and 44.8% have not heard of pseudo-halitosis. Moreover, the majority of participants reported halitosis to have an oral etiology (84% in group P and 86.7% in group L) (Fig. 1, Table 3).

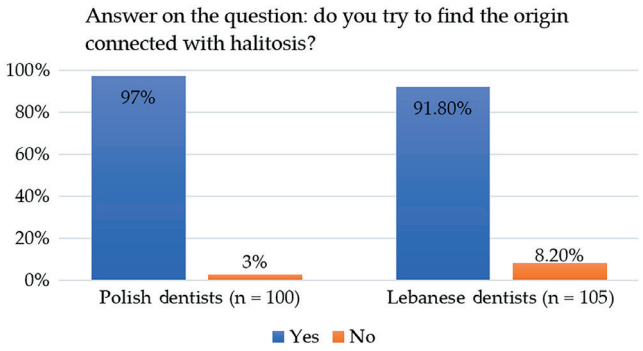

Fig. 1. Distribution of answers to the question: "Do you try to find the origin connected with halitosis?" in both groups

Skills for halitosis management

Most of the surveyed dentists revealed that they do not frequently treat halitosis in their clinical practice (87% for group P and 89.6% for group L), while only 13% of respondents in group P and 10.4% in group L reported treating halitosis frequently. Specific toothpastes, mouthwashes and tongue scrapers were prescribed, but infrequently. In addition, the use of lasers for the management of halitosis was not a known treatment for the majority of dentists (81% in group P and 85.7% in group L) (Fig. 2). Overall, respondents reveal that halitosis treatments can be successful, and 57% of dentists in group P and 58.1% in group L considered the treatment they applied effective. The need for professional education focusing on halitosis appears to be of importance for dentists in both Poland and Lebanon. No significant differences in terms of treatment modalities were observed between group P and group L ($p > 0.05$) (Table 4).

Discussion

In this study, the level of knowledge about halitosis and competence in the diagnosis and management of halitosis among Polish and Lebanese dentists (a total of 205 respondents) was evaluated using self-administered questionnaires.

Table 3. Knowledge of halitosis among dentists

Question	Answer	Group P n (%)	Group L n (%)	χ^2/df	p-value
Do you think that there is a classification of halitosis?	Yes	78 (78.0)	90 (85.7)	1.57/1	0.210[a]
	No	22 (22.0)	15 (14.3)		
Do you know any classification of halitosis?	Yes	31 (31.0)	52 (49.5)	6.54/1	0.011[a]
	No	69 (69.0)	53 (50.5)		
Do you think that halitosis was made up by pharmaceutical concerns?	Yes	1 (1.0)	19 (18.1)	74.5/3	<0.001[a]
	No	83 (83.0)	31 (29.5)		
	Maybe	0 (0.0)	34 (32.4)		
	I don't have an opinion	16 (16.0)	21 (20.0)		
Have you heard about pseudo-halitosis?	Yes	39 (39.0)	58 (55.2)	4.79/1	0.029[a]
	No	61 (61.0)	47 (44.8)		
Have you heard about halitophobia?	Yes	39 (39.0)	76 (72.4)	21.8/1	<0.001[a]
	No	61 (61.0)	29 (27.6)		
Do you have any instruments to help you diagnose halitosis? If yes, what? (e.g., Halimeter, oral chroma)	Only my own senses	31 (31.0)	33 (31.4)	0.01/2	0.997[a]
	No	68 (68.0)	71 (67.6)		
	Halimeter	1 (1.0)	1 (1.0)		
What do you think is the main etiology of halitosis?	Gastro-intestinal conditions	59 (59.0)	15 (14.3)	42.4/1	<0.001[a]
	Oral hygiene	82 (82.0)	74 (70.5)	3.12/1	0.077[a]
	Systematic diseases	82 (82.0)	14 (13.3)	94.4/1	<0.001[a]
	Others	57 (57.0)	2 (1.9)	–	<0.001[b]

[a] – Pearson's χ^2 test; [b] – Fisher's exact test; df – degrees of freedom.

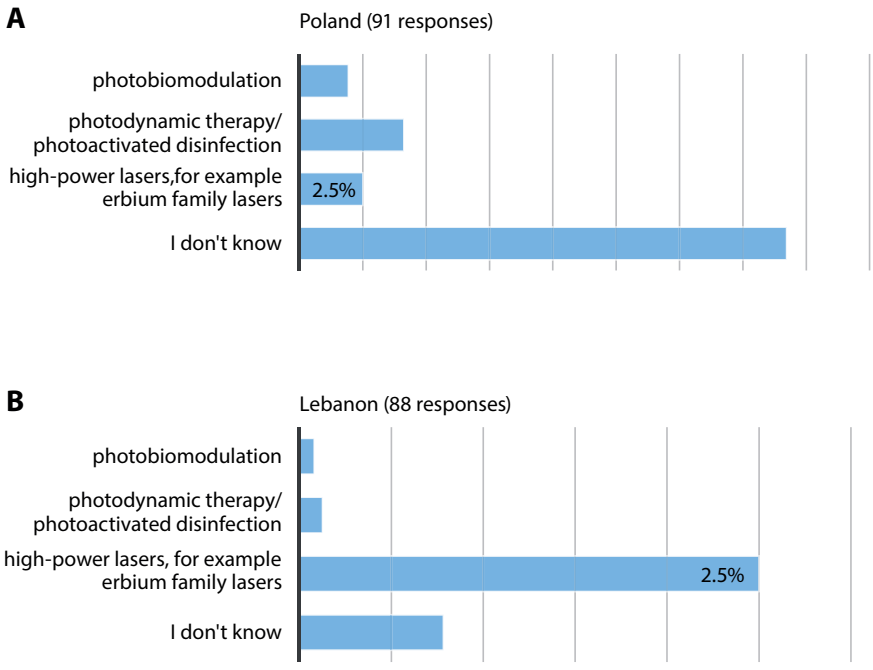

Fig. 2. Laser treatment modalities for the management of halitosis as reported by dentists practicing in Poland (n = 19) and in Lebanon (n = 15). Please note that the participants could choose only 1 answer from a given list

To the best of our knowledge, this was the first questionnaire-based study on halitosis among dentists practicing in Poland, but not in Lebanon. The diagnosis and management of halitosis are of great importance in regard to the social and health impact as well as negative influence of this symptom on a patient's quality of life. Yet, it was demonstrated in this survey that it is still uncomfortable for patients and healthcare providers to discuss the problem openly. In this study, dentists admitted to having a relatively insufficient level of knowledge about halitosis. In fact, 84% of respondents (n = 84) in group P and 65.7% (n = 69) in group L reported a lack of knowledge about halitosis. Harmouche et al. showed that only 36.5% of French dentists are aware of physiological halitosis, and 61% of them overestimated

Table 4. Results of the skills for halitosis management

Question	Answer	Group P n (%)	Group L n (%)	χ^2/df	p-value
How many patients have you treated in the last 6 months for halitosis?	None	59 (59.0)	43 (41.0)	9.21/3	0.026[a]
	<5	28 (28.0)	51 (48.6)		
	5–15	11 (11.0)	9 (8.6)		
	>15	2 (2.0)	2 (1.9)		
Do you think that halitosis treatments are successful?	Yes	57 (57.0)	61 (58.1)	1.86/2	0.394[a]
	No	1 (1.0)	4 (3.8)		
	Maybe	42 (42.0)	40 (38.1)		
In your opinion, is it necessary to repeat the treatment?	Yes	69 (69.0)	70 (66.7)	2.96/2	0.228[a]
	No	3 (3.0)	9 (8.6)		
	I don't have an opinion	28 (28.0)	26 (24.8)		
Do you have any instruments for halitosis treatment?	Yes	30 (30.0)	20 (19.0)	2.76/1	0.096[a]
	No	70 (70.0)	85 (81.0)		
Do you recommend mouthwashes to patients with halitosis?	Yes	74 (74.0)	95 (90.5)	8.50/1	0.004[a]
	No	26 (26.0)	10 (9.5)		
Do you recommend dedicated toothpastes to patients with halitosis?	Yes	61 (61.0)	58 (55.2)	0.48/1	0.488[a]
	No	39 (39.0)	47 (44.8)		
Do you recommend tongue scrapers to patients with halitosis?	Yes, more than 3 times a week	34 (34.0)	33 (31.4)	5.65/2	0.056[a]
	Yes, 1–3 times a week	37 (37.0)	54 (51.4)		
	No	29 (29.0)	18 (17.1)		
Have you heard about using lasers to treat halitosis?	Yes	19 (19.0)	15 (14.3)	0.52/1	0.472[a]
	No	81 (81.0)	90 (85.7)		

[a] – Pearson's χ^2 test; df – degrees of freedom.

the involvement of extraoral causes.[12] It was clearly stated in the results that regardless of age, experience or country, dentists have a massive problem discussing and analyzing malodor during dental appointments. Therefore, respondents did not present appropriate knowledge and methodical preparation (in total, 72%). It is worth emphasizing that dental offices are the best places to gain information and receive proper treatment. Moreover, this study revealed that 67.6% and 68% of interviewees from group P and group L, respectively, admitted not using any tools or instruments to diagnose halitosis. The findings are again in accordance with the study by Harmouche et al., who stated that only a very limited number of dentists use any tools to monitor VSCs or use the organoleptic method to manage halitosis.[12] In addition, the majority of the studied population revealed that when halitosis is detected or diagnosed during routine treatment, dentists do not discuss it with patients (57% in group P and 41% in group L). Moreover, the respondents showed poor knowledge about psychosomatic and real halitosis, including the classification and treatment options. For instance, to properly examine halitosis, dental surgeons should have at least an organoleptic measurement (sensory test), chromatography with a flame photometric detector for VSCs in the breath, or apparatus for sulfide monitoring (like a Halimeter) available.

On the other hand, it can also be concluded that in both countries, the undergraduate curriculums do not cover properly the subjects related to halitosis diagnosis, management and etiology. In fact, the Internet is the main source of knowledge about halitosis in 57% of dentists in group P and 51.4% in group L dentists. Books or scientific papers were pointed out as knowledge sources by 58% of respondents in group P and 44.8% in group L. While 54% of dentists in group P and 45.7% in group L reported that the knowledge about halitosis provided during their studies was insufficient, 33% of group P members and 2.9% of group L members answered they did not receive any instruction at the university/medical school about halitosis. Interestingly, despite insufficient knowledge about halitosis, dentists are generally not seeking any extra courses to deepen their knowledge (94% and 73.3% of respondents in group P and group L, respectively).

The mean values of age were 40 years for group P and 49 years for group L, revealing that there is no significant difference between both groups. Our study highlights that regardless of age and/or professional experience, the quality of halitosis knowledge acquired at universities/medical schools has not changed much over the past 20 years. Combining the outcomes, more than 67% of respondents are dissatisfied with the level of teaching about halitosis at their universities/medical schools. This can be considered a relatively powerful information showing how an undergraduate student's curriculum could be improved.

The management of halitosis should be assigned regarding the origin of the disease. Patients with oral halitosis should be treated by dentists, while extraoral halitosis should be treated by general medical practitioners with the help of appropriate specialists. On the other hand, patients with halitophobia must be referred and encouraged to consult a psychologist and/or psychiatrist.[13] The gold standard treatment for halitosis remains a detailed interview with the patient to exclude any extraoral sources, like diet and chronic infection of the liver or respiratory tract. If these causes are eliminated, the next step is to exclude intraoral sources, and educate and instruct the patient about proper oral hygiene. Harmouche et al. claimed that despite the uncomplicated procedures of dealing with halitosis, the overall satisfaction with treatment outcomes was very low, as only 39.7% of those interviewed in France and 28% in Lebanon thought their treatment was effective.[12] Our survey found that 59% and 41% of Lebanese and Polish interviewees, respectively, declared that in the last 6 months, they did not treat any patients for halitosis, and 28% and 48%, respectively, treated halitosis in less than 5 patients in the last 6 months. Hence, this questionnaire confirmed a poor level of awareness about laser therapy – 81% of group P and 85.7% of group L dentists have not heard about using lasers to treat halitosis. Additionally, 76.9% of group P and 78.4% of group L did not know the protocol for the use of lasers in the treatment of oral malodor.

A study by AlSadhan showed that in Riyadh, 46.6% of interviewees claimed that halitosis affects their social life, mostly (over half of the answers) by alienation.[14] In a 2010 study by Settineri et al.,[15] 19.39% of examined Italians had self-reported halitosis, and 22.8% of students from Saudi Arabia had self-perceived halitosis.[16] These numbers may increase due to the use of face masks as protection from COVID-19 infections. This is a factor contributing to oral malodor.[17]

The diagnosis and management of halitosis should be systematically performed by dentists in their routine practice. The diagnosis, discussion with patients, patient education, and treatment of halitosis should be included in the standard care provided by oral health practitioners.[5,6,18] In this context, the use of lasers for the management of halitosis proved to be an interesting and promising approach.[19–26] For instance, lasers can effectively eliminate microorganisms found in the deep periodontal pockets,[26] tongue, uvula, and tonsils which produce VSCs. For instance, the literature contains a relatively large number of studies showing a significant reduction in the bacteria involved in halitosis when lasers were used in photodynamic therapy, also referred to as photoactivated disinfection (the use of a photosensitizer with a low power laser). More specifically, the populations of *Fusobacterium nucleatum*, *Capnocytophaga gingivalis*, *Solobacterium moorei*, *Treponema denticola*, *Prevotella intermedia*, *Prevotella veroralis*, *Peptostreptococcus micros*, *Veillonella parvula*, *Treponema denticola*, and *Porphyromonas gingivalis* have been reported to be significantly reduced in examined patients after treatment.[19–25] In 2008, Kara et al. investigated the success rate of oral malodor treatments in 60 patients with periodontitis using Nd:YAG laser treatment in a randomized controlled clinical study. They noted that the treatment of the periodontal pocket with 5–7 mm can significantly decrease the rate of VSCs measured using a Halimeter ($p < 0.05$).[19] Moreover, neodymium lasers have been used to reduce the microbial population during periodontal pocket treatment by numerous researchers.[22,24–28] Therefore, it seems reasonable to include the use of lasers for the management of halitosis associated with periodontitis into the general treatment protocol. Krespi et al. reported in a randomized controlled prospective study that using a single 10-minute Er,Cr:YSGG laser treatment on the dorsum of the tongue with a power of 4 W, a frequency of 40 Hz and a non-contact swiping motion lead to a significant reduction of VSCs measured using a Halimeter, when compared to tongue scraping alone.[25] Moreover, diode lasers and antibacterial photodynamic therapy (aPDT) are also effective and promising approaches to reducing microorganisms. The aPDT can be defined as the therapeutic use of light to stimulate a photo-activated agent that has a bactericidal effect. This therapeutic modality proved to be safe and predictable,[23–29] and a promising treatment for halitosis. Studies evaluated the effects of aPDT on halitosis in older adults with complete dentures and demonstrated elimination of halitosis for longer than 1 month in comparison to mouth disinfection using tongue scrapping.[30–33] For example, Patil et al. treated severe malodor patients with a single dorsal tongue session involving aPDT and methylene blue (6 points for 90 s each). Using real-time polymerase chain reaction (PCR) analysis, Patil et al. reported that such protocol resulted in a significant reduction in Halimeter scores on the 3rd and 7th day, and that the populations of *P. gingivalis* and *F. nucleatum* bacteria significantly decreased.[32]

Although the sample size in this study was enough to compare 2 groups and perform statistical analysis, we invite researchers to use the suggested questionnaire on a larger sample size and different dentists in different countries. This will reflect a more accurate account of the general halitosis-related knowledge among dentists worldwide.

Compared to the conventional treatment of halitosis, laser therapy is considered a minimally invasive and promising approach. The present survey confirms that there is a lack of standardization of procedures related to the treatment of halitosis, and the knowledge of dentists should be broadened, as should be the availability of information about treatment and diagnosis options. Furthermore, studies should be conducted to examine the best procedure to manage halitosis and reduce VSCs.

Limitations of the study

This study was made on a relatively small sample population and in only 2 countries.

Conclusions

The treatment of halitosis is still considered challenging for Polish and Lebanese practicing dentists. Moreover, there is still a lack of professional knowledge and training about halitosis among this population. Hence, there is a need for further education and training about halitosis.

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mTOR/ULK1 signaling axis mediated ultrashort wave regulation of autophagy to alleviate diabetic kidney disease

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Abstract

Background. Diabetic kidney disease (DKD) is closely related to autophagy and inflammation. The mTOR/unc-51 like autophagy activating kinase 1 (ULK1) signaling axis is involved in the regulation of autophagy. Ultrashort wave (USW) therapy has been extensively studied in inflammatory diseases. However, the therapeutic effect of USW on DKD and the role of the mTOR/ULK1 signaling axis in USW interventions remain uncertain.

Objectives. This study aimed to explore the therapeutic effects of USW on DKD rats and the role of the mTOR/ULK1 signaling axis in USW interventions.

Materials and methods. A DKD rat model was established using a high-fat diet (HFD)/sugar diet and streptozocin (STZ) induction. The optimal duration of USW intervention was determined using different USW treatments. The levels of metabolism, inflammation and fibrosis associated with kidney injury in rats were measured. Western blot analysis was performed on the related indexes of autophagy and the mTOR/ULK1 signaling axis.

Results. In DKD rats, microalbuminuria (MAU), glucose (GLU), creatinine (CRE), and blood urea nitrogen (BUN) levels decreased after the USW intervention. Levels of interleukin (IL)-1β, inducible nitric oxide synthase (iNOS), immunoglobulin M (IgM), immunoglobulin G (IgG), IL-18, tumor necrosis factor alpha (TNF-α), and IL-6 decreased in the USW group compared to the model group. The IL-10 and arginase (Arg-1) levels were increased in the USW group. The content of fibrosis-related indexes (vascular endothelial growth factor (VEGF), fibronectin (FN), type IV collagen, and type I collagen) decreased in the urine of the DKD rats. After USW treatment, LC3B and Beclin1 levels increased, while the level of p62 decreased. The levels of nephrin, podocin and synaptopodin increased. Ultrashort wave could reduce p-mTOR/mTOR ratios and increase ULK1 expression. After the overexpression of ULK1, the levels of LC3B and Beclin1 were higher in the overexpression (oe)-ULK1 group than in the oe-negative control (NC) group, while the level of p62 decreased. After mTOR activation, LC3B and ULK1 expression decreased, while CRE, BUN, MAU, and GLU levels increased.

Conclusions. Ultrashort wave alleviated kidney injury induced by the HFD/sugar diet and STZ. The USW intervention reversed the decreased autophagy levels in the DKD rats. The mTOR/ULK1 signaling axis mediated USW to promote autophagy.

Key words: inflammation, autophagy, ultrashort wave (USW), diabetic kidney disease (DKD), mTOR/ULK1 signaling axis

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Background

Diabetic kidney disease (DKD) is a common complication of diabetes and the main cause of end-stage renal disease (ESRD).[1] The main features of DKD include glomerular basement membrane thickening, podocyte apoptosis, interstitial inflammatory infiltration, and renal interstitial fibrosis.[2] The pathogenesis of DKD involves blood glucose (GLU),[3] inflammation[3] and autophagy.[4] At present, the treatment of DKD mainly focuses on renal replacement therapy in terms of kidney dialysis and kidney transplantation, as well as drug therapy related to the control of blood GLU, blood pressure and the renin–angiotensin system.[5,6] While these treatments can alleviate patients' symptoms, they place a tremendous economic burden on them.[7] Therefore, there is still a need for new treatments.

Ultrashort wave (USW) therapy is a method in which ultrahigh-frequency alternating current is applied to the body to exert therapeutic effects. Low-dose USW has been shown to effectively treat various types of acute inflammation.[8,9] A previous study showed that USW could improve spinal cord injuries in a spinal cord injury model of rats established using the Allen method.[9] Ultrashort wave also reduced neuroinflammation in spinal cord injury rat models.[10] In rabbit models of compression injury, USW accelerated peripheral nerve regeneration.[11] In a study of chronic knee osteoarthritis, USW reduced the level of inflammatory factors and improved knee function.[12] It alleviated lipopolysaccharide-induced acute lung injury by reducing immune cell infiltration and inflammatory cytokine levels.[13] Our previous study showed that USW has a protective effect on cerebral ischemia–reperfusion injury in rats.[14] However, whether USW has a therapeutic effect on DKD remains unclear.

Autophagy is essential in maintaining the integrity of podocytes, and the induction of autophagy can serve as the main protective mechanism against glomerular injury.[15–17] One study showed that the pathogenesis of DKD is related to impaired autophagy.[4] Yang et al. found that activating autophagy attenuates DKD development in mice.[18] Therefore, autophagy can be used as an important indicator in the development of DKD. Therapeutic strategies targeting autophagy may serve as a potential developmental direction for alleviating DKD. The mTOR, a serine/threonine-protein kinase in the PI3K-related protein kinase family, constitutes the mTOR complex 1 (mTORC1) that affects autophagy.[19,20] The mTOR is involved in the regulation of multiple functional mechanisms within cells, including mitochondrial energy production,[21,22] inflammation,[23,24] autophagy,[25–27] and immune responses.[28] The mTOR signaling affects the development of a variety of diseases through its involvement in autophagy, including neurocognitive impairment,[29] chronic heart failure,[30] spinal cord injury,[31] and osteoarthritis.[32] In DKD, attenuating mTOR signaling to activate autophagy may play a protective role.[18] One study showed that mTOR could affect autophagy by regulating the phosphorylation level of unc-51 like autophagy activating kinase 1 (ULK1).[33] Other studies have shown that the mTOR/ULK1 signaling axis is involved in the process of autophagy in cells.[34–36] Thus, we focused on the mTOR/ULK1 signaling axis in DKD.

Objectives

This study aimed to explore the therapeutic effects of USW on DKD rats and the role of the mTOR/ULK1 signaling axis in USW interventions.

Materials and methods

Ethics statement

All experimental protocols were reviewed and received approval from the Institutional Animal Care and Use Committee (IACUC) of the Second Xiangya Hospital of Central South University (Changsha, China; approval No. 2021612). The experimental process strictly followed the approved protocol. We made significant efforts to minimize animal suffering.

Animal experiments

Fifty-five male Sprague Dawley rats with a body weight of 235 ±15 g were purchased from Human SJA Laboratory Animal Co., Ltd. (Changsha, China). The rats were housed under conditions of 22–24°C and a 12-hour light/dark cycle with a normal diet and water for 7 days. The model rats received a high-fat diet (HFD)/sugar diet, including 29.4% sugar, 20% protein, 45% fat, and 35% carbohydrate, that delivered 22.0 MJ/kg of energy.[37] After 4 weeks, 50 mg/kg streptozocin (STZ; Sigma-Aldrich, St. Louis, USA) in 0.05 mol/L citrate buffer (pH = 4.5) was injected intraperitoneally in rats.[38] The model rats were fasted the night before STZ induction. The blood GLU concentrations were measured after STZ injection for 3 days. Rats with blood GLU levels higher than 16.7 mmoL/L and with significant microalbuminuria (MAU) were considered successful DKD modeling. The control rats received a normal diet and were injected with citrate buffer.

To study the optimal treatment time for USW, the rats were randomly divided into 7 groups (n = 5 in each group): a control group, a model group, a USW3 group, a USW5 group, a USW10 group, a USW20 group, and a metformin (MET) group. An Ultrashort Wave Electrotherapy Machine (DL-CII) was purchased from Shantou Medical Equipment Factory Co., Ltd. (Shantou, China). The rats were anesthetized with pentobarbital sodium 50 mg/kg intravenously. After stabilization, the 2 electrodes of the USW therapeutic instrument were placed on opposite sides of the rat's abdomen, approx. 0.5–1 cm away from the skin. The diameter

of the circular electrodes was 12 cm. The first gear output was selected, and the power was 10 W. Rats in the USW3 group were treated for 3 min, USW5 for 5 min, USW10 for 10 min, and USW20 for 20 min. Ultrashort wave treatment was performed once daily for 3 weeks. The MET group was used as the positive control group. Metformin (Yuanye Bio-Technology, Shanghai, China) was dissolved in distilled water, and the rats were administered 250 mg/kg/day of MET intragastrically for 21 days.[39] The rats in the control, model and MET groups were given sham applications of electrotherapy under general anesthesia. The MAU and blood GLU levels were determined at 0, 7, 14, and 21 days. The creatinine (CRE) and blood urea nitrogen (BUN) levels were evaluated to determine the optimal USW intervention time.

To investigate the regulatory pathway of USW in alleviating DKD, 20 more rats were randomly divided into 4 groups (n = 5 in each group): a control group, a model group, a USW group, and a USW+L-leucine group. The control, model and USW groups were treated as described above. L-leucine (0.45 g/kg/day; Yuanye Bio-Technology), an activator of mTOR, was injected intraperitoneally into the rats,[40] and USW treatment was performed in the USW+L-leucine group rats.

After 21 days of USW treatment, all rats were euthanized with an intraperitoneal injection of 150 mg/kg pentobarbital sodium. Kidney, urine and blood samples were collected for further experiments.

Enzyme-linked immunosorbent assay

Fresh urine and blood samples were collected. Rat enzyme-linked immunosorbent assay (ELISA) kits for GLU (A154-1-1), CRE (C011-2-1) and BUN (C013-2-1) were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The ELISA kits for MAU (CSB-E12991r), interleukin (IL)-1β (CSB-E08055r), IL-10 (CSB-E04595r), inducible nitric oxide synthase (iNOS; CSB-E08325r), immunoglobulin G (IgG; CSB-E07981r), immunoglobulin M (IgM; CSB-E07978r), type I collagen (CSB-E08084r), type IV collagen (CSB-E08883r), fibronectin (FN; CSB-E04553r), and vascular endothelial growth factor (VEGF; CSB-E04757r) were purchased from CUSABIO (Wuhan, China). The MAU, IgG, IgM, type I collagen, type IV collagen, FN, and VEGF levels were determined in the rats' urine. The GLU, CRE, BUN, IL-1β, IL-10, and iNOS levels were measured in the rats' serum, according to the manufacturer 's instructions.

Western blot

Proteins from renal tissue, rat renal proximal tubular epithelial cells (RRPTEpiC) and podocyte cells were extracted using radioimmunoprecipitation assay (RIPA) lysate and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto nitrocellulose membranes. Phosphate-buffered saline with Tween (PBST) was used to prepare 5% skim milk. The membranes were blocked with skim milk at 4°C overnight. The primary and secondary antibodies were incubated for 90 min each. Electrochemiluminescence (ECL) working fluid was added to the nitrocellulose membrane. The blots were visualized using a ChemiScope 6000 chemiluminescence imaging system (CLiNX, Shanghai, China). The primary antibodies were as follows: IL-1β (1:1000, ab254360), IL-18 (1:1000, ab243091), tumor necrosis factor alpha (TNF-α; 1:1000, ab205587), IL-6 (0.5 μg/mL, ab9324), IL-10 (0.2 μg/mL, ab271261), arginase (Arg-1; 1:1000, ab91279), LC3 (1:2500, 14600-1-AP), Beclin1 (1:1000, 11306-1-AP), p62 (1:1000, 18420-1-AP), nephrin (1:1000, ab216341), podocin (1:1000, 20384-1-AP), synaptopodin (1:1000, 21064-1-AP), p-mTOR (1:1000, 67778-1-Ig), mTOR (1:25000, 66888-1-Ig), ULK1 (1:1000, 20986-1-AP), β-actin (1:5000, 60008-1-Ig), horseradish peroxidase (HRP) goat anti-mouse IgG (1:5000, SA00001-1), and HRP goat anti-rabbit IgG (1:6000, SA00001-2). The IL-1β, IL-18, TNF-α, IL-6, IL-10, Arg-1, and nephrin were purchased from Abcam (Cambridge, UK), while LC3, Beclin1, p62, podocin, synaptopodin, p-mTOR, mTOR, ULK1, β-actin, HRP goat anti-mouse IgG, and HRP goat anti-rabbit IgG were obtained from Proteintech (Wuhan, China). The expression of β-actin was used as a loading control.

Quantitative real-time polymerase chain reaction

The RNA was extracted from the rat renal tissues and cells using the Trizol method. The RNA was reversely transcribed into cDNA using a HiFiScript cDNA Synthesis Kit (CW2569M; CWBIO, Beijing, China). Polymerase chain reaction (PCR) amplification was performed using SYBR-Green PCR Master Mix (CW2601S; CWBIO). The $2^{-\Delta\Delta Ct}$ was performed to calculate the RNA levels. The primers are listed in Table 1. The expression of β-actin was applied as an endogenous control.

Hematoxylin and eosin staining

The rats were sacrificed and renal tissues were immediately collected. The kidney tissues were fixed, embedded and cut into 5-μm sections. The sections were deparaffinized with xylene. Ethanol gradients were used for rehydration. After washing the sections, hematoxylin solution was used to stain for 5 min. The eosin solution was applied to counterstain for 1 s. Ethanol gradients (95–100%) were applied to dehydrate, and xylene was used to treat the sections. The sections were sealed and observed under a light microscope (model BA210T; Motic, Xiamen, China).

Masson staining

After fixation and embedding, rat renal tissue was cut into 5-μm sections. Masson staining was performed

Table 1. Primer sequences used in the study

Name	Sequence (5'-3')
β-actin	F-ACATCCGTAAAGACCTCTATGCC
	R-TACTCCTGCTTGCTGATCCAC
IL-1β	F-CAGCAGCATCTCGACAAGAG
	R-AAAGAAGGTGCTTGGGTCCT
IL-18	F-ACCGAACAGCCAACGAA
	R-TGTCCTGGCACACGTTT
TNF-α	F-CCCCTCTATTTATAATTGCACCT
	R-CTGGTAGTTTAGCTCCGTTT
IL-6	F-TCACTATGAGGTCTACTCGG
	R-CATATTGCCAGTTCTTCGTA
IL-10	F-AATAAGCTCCAAGACAAAGGT
	R-TCACGTAGGCTTCTATGCAG
Arg-1	F-CATATCTGCCAAGGACATCGT
	R-TCCATCACTTTGCCAATTCCC
LC3B	F-AACACAGCCACCTCTCGACCT
	R-ACACAACCCACACACGGCAG
Beclin-1	F-GTGGCGGCTCCTATTCCATC
	R-GACACCCAAGCAAGACCCCA
p62	F-AGCATACAGAGAGCACCCAT
	R-ACATACAGAAGCCAGAATGCAG
ULK1	F-ACACACCCTCTCCCCAAGTG
	R-TGGGACGAACGACATGGAAG

IL – interleukin; TNF-α – tumor necrosis factor alpha; Arg-1 – arginase; ULK1 – unc-51 like autophagy activating kinase 1.

according to the instructions of the Masson staining kit (Wellbio, Changsha, China).The kidney sections were stained with Weigert's iron hematoxylin staining solution for 5 min. Ponceau S working solution was applied to the sections for 2 min. The sections were differentiated with phosphomolybdic acid n-hydrate solution for 30 s and stained with blue aniline solution for 8 min. After the sections were rinsed and mounted, the rat kidney tissues were examined with a light microscope (model BA210T).

Flow cytometry

The rat kidney tissues were isolated, and a cell suspension was prepared. Cells were stained with 5 µL annexin V-FITC (KGA1030-100; KeyGEN BioTECH, Nanjing, China) and 5 µL propidium iodide (PI) for 10 min in the dark. Cell apoptosis was detected using a flow cytometer (Beckman Coulter, Brea, USA).

Cell culture and treatment

Rat renal tubular epithelial cells (Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd., Shanghai, China) were cultured using the iCell Primary Epithelial Cell Culture System (iCell Bioscience, Shanghai, China). Rat podocyte cells (Procell, Wuhan, China) were cultured using the iCell Primary Renal Podocyte Culture System (iCell Bioscience). The cells were cultured at 37°C and 5% CO_2.

To explore the effect of ULK1 on autophagy in RRPT-EpiC and podocyte cells, 4 groups were set up (n = 5 in each group): a control group, a model group, an overexpression (oe)-ULK1 group, and an oe-negative control (NC) group. The oe-NC and oe-ULK1 plasmids were purchased from Abiowell Biotechnology Co., Ltd. (Changsha, China). A DKD cell model induced by high glucose (HG) was constructed as previously described.[41] The oe-NC and oe-ULK1 plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). After 24 h, the cells were treated with 30 mmol/L glucose in the oe-NC and oe-ULK1 groups. The cells in the control group did not undergo any intervention.

Statistical analyses

Data were presented as mean ± standard deviation (M ±SD). Statistical analyses were carried out using SPSS v. 18.0 (SPSS Inc., Chicago, USA) and GraphPad Prism v. 8.0.1 (GraphPad Software, San Diego, USA) software. The Shapiro–Wilk test and exploratory descriptive statistics test were adopted to analyze data distribution for normality and homogeneity of variance (Supplementary Tables 1–6). One-way analysis of variance (ANOVA) and two-way ANOVA were performed to compare data between multiple groups. Tukey's post hoc test was applied. The value of $p < 0.05$ was considered statistically significant.

Results

USW intervention improved DKD metabolism

To explore whether USW has therapeutic effects on DKD rats, we treated them with USW. The concentrations of MAU in urine and GLU in serum were measured at 0, 7, 14, and 21 days. The ELISA results showed that urinary MAU (treatment: $p < 0.05$, ANOVA, $F = 609.6$; time: $p < 0.05$, ANOVA, $F = 507.2$) and blood GLU (treatment: $p < 0.05$, ANOVA, $F = 496.3$; time: $p < 0.05$, ANOVA, $F = 1106.0$) concentration decreased in the USW group compared to the model group (Fig. 1A,B). Serum CRE ($p < 0.05$, ANOVA, $F = 26.47$) and BUN ($p < 0.05$, ANOVA, $F = 144.8$) concentrations decreased after USW treatment (Fig. 1C). There was no obvious difference between the USW10, USW20 and MET groups, but a clear therapeutic effect was observed. Therefore, USW10 was used for the following experiments.

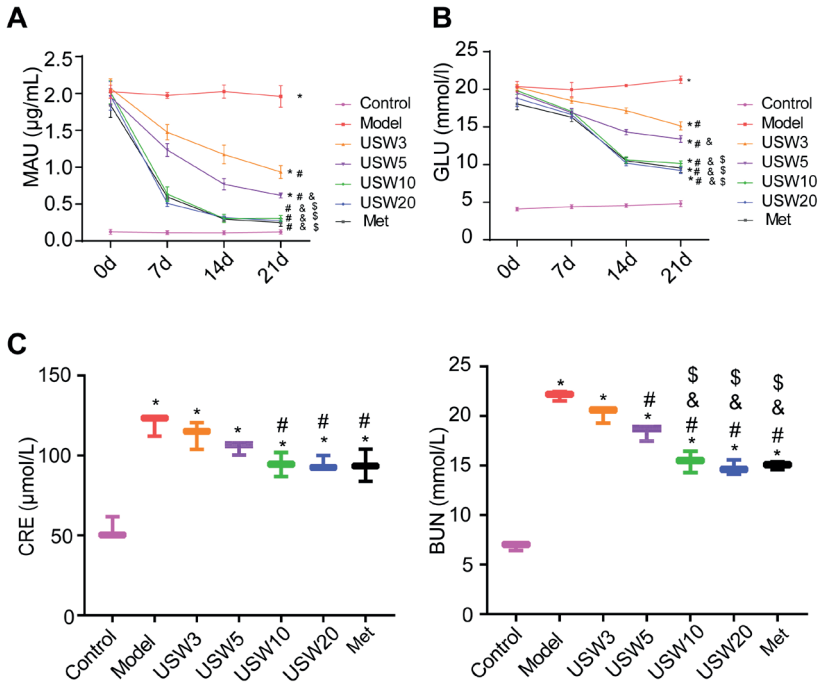

Fig. 1. Ultrashort wave (USW) treatment affected the metabolism of diabetic kidney disease (DKD) rats. Urinary microalbuminuria (MAU) (A) and blood glucose (GLU) (B) concentration of DKD rats were measured at 0, 7, 14, and 21 days using enzyme-linked immunosorbent assay (ELISA). Statistical analysis was performed using two-way analysis of variance (ANOVA) and Tukey's post hoc test; C. After the USW intervention, ELISA was performed to determine the serum creatinine (CRE) and blood urea nitrogen (BUN) concentrations. Statistical analysis was performed using one-way ANOVA and Tukey's post hoc test

*$p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the model group; $^{\&}p < 0.05$ compared to the USW3 group; $^{\$}p < 0.05$ compared to the USW5 group.

USW inhibited the inflammatory response in DKD rats

Next, we measured the level of inflammation in the DKD rats. The concentrations of IL-1β, IL-10 and iNOS in the serum changed (Fig. 2A). The IL-1β ($p < 0.05$, ANOVA, $F = 59.88$) and iNOS ($p < 0.05$, ANOVA, $F = 841.3$) decreased after USW treatment compared to the model group. The IL-10 concentration ($p < 0.05$, ANOVA, $F = 201.3$) increased in the USW group. After USW intervention, the IgM ($p < 0.05$, ANOVA, $F = 63.33$) and IgG ($p < 0.05$, ANOVA, $F = 55.0$) levels in the urine decreased. (Fig. 2B). These results suggest that USW could inhibit the development of inflammation in DKD rats to some extent. At the gene and protein levels, IL-1β ($p < 0.05$, ANOVA, $F = 174.0$), IL-18 ($p < 0.05$, ANOVA, $F = 128.3$), TNF-α ($p < 0.05$, ANOVA, $F = 120.8$), and IL-6 ($p < 0.05$, ANOVA, $F = 158.6$) levels were decreased in the USW group compared with the model group (Fig. 2C,D). The IL-10 ($p < 0.05$, ANOVA, $F = 111.4$) and Arg-1 ($p < 0.05$, ANOVA, $F = 113.0$) levels were increased in the DKD rats. This further suggests that USW could relieve kidney inflammation in DKD rats.

USW alleviated kidney injury in DKD rats

Pathological examination was performed on the renal tissues of the DKD rats. The hematoxylin and eosin (H&E) staining results showed that the renal tissue of the DKD rats had obvious glomerular hypertrophy, basement membrane thickening and renal tubule dilatation. Ultrashort wave alleviated these abnormalities to some extent (Fig. 3A). In the DKD rats, renal structural lesions led to abnormal protein content of VEGF, FN, type IV collagen, and type I collagen in the urine.[42] The USW intervention decreased VEGF ($p < 0.05$, ANOVA, $F = 168.2$), FN ($p < 0.05$, ANOVA, $F = 179.7$), type IV collagen ($p < 0.05$, ANOVA, $F = 173.2$), and type I collagen ($p < 0.05$, ANOVA, $F = 30.0$) levels in the urine (Fig. 3B). Renal interstitial fibrosis is a typical pathological feature of DKD. Masson staining showed that USW could reduce renal interstitial fibrosis in the DKD rats (Fig. 3C). The renal tissue apoptosis rate of the DKD rats was also significantly reduced after USW treatment ($p < 0.05$, ANOVA, $F = 8207$; Fig. 4A). Nephrin and podocin are key glomerular proteins. Nephrin, podocin and synaptopodin levels were detected using the western blot method (Fig. 4B). The results showed that nephrin ($p < 0.05$, ANOVA, $F = 117.4$), podocin ($p < 0.05$, ANOVA, $F = 71.47$) and synaptopodin ($p < 0.05$, ANOVA, $F = 175.9$) were increased in the USW group compared to the model group. Considering the above experimental results, USW alleviated kidney injury in the DKD rats to a certain extent.

USW alleviated the autophagy decrease induced by HFD/STZ and affected the mTOR/ULK1 signaling axis

Next, we verified the expression of the autophagy-related indicators (LC3, Beclin1 and p62) using quantitative real-time polymerase chain reaction (qPCR) and western blot (Fig. 5A, B). The LC3 (mRNA: $p < 0.05$, ANOVA, $F = 150.9$; protein: $p < 0.05$, ANOVA, $F = 233.0$)

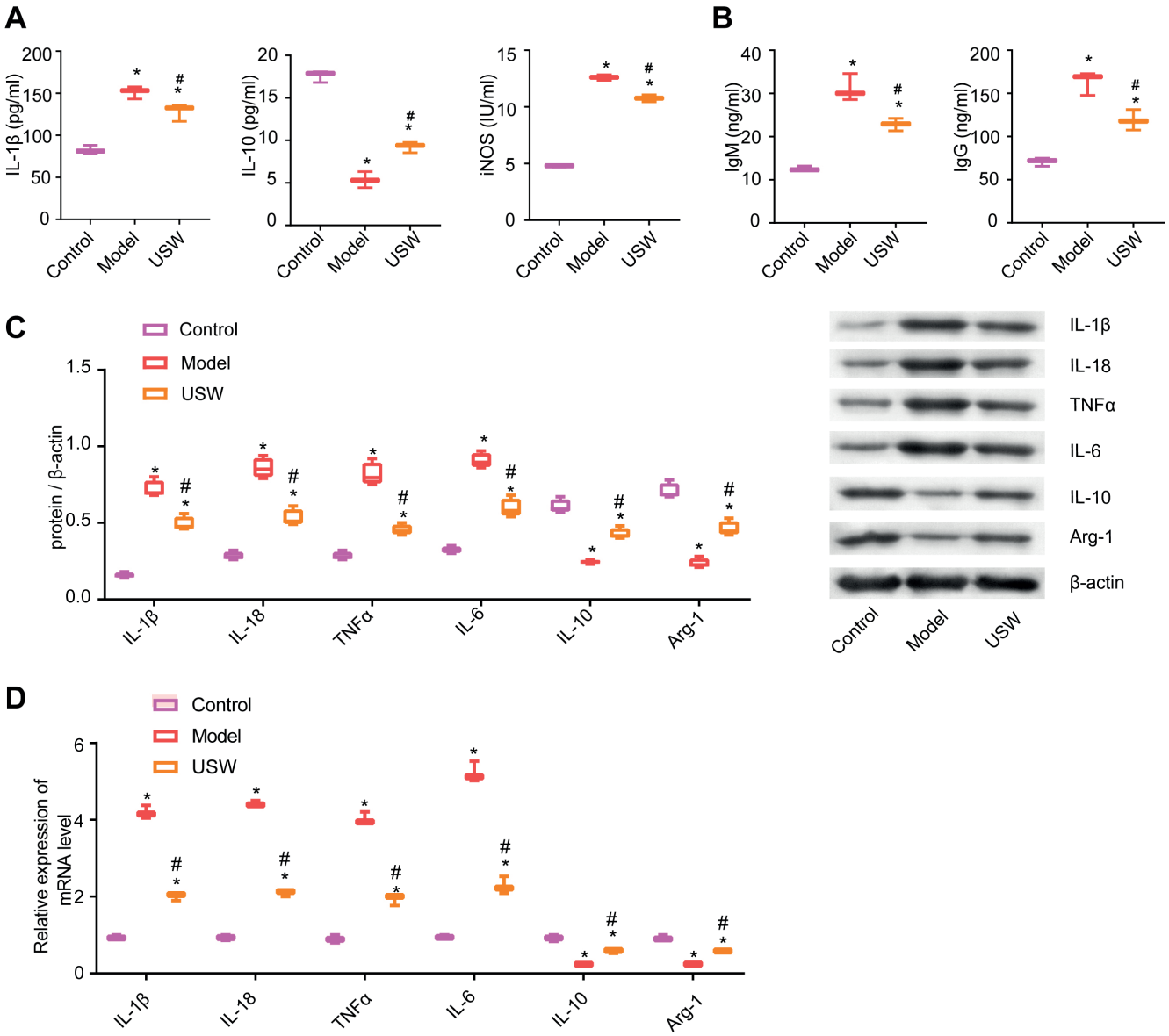

Fig. 2. Ultrashort wave (USW) inhibited the development of inflammation in diabetic kidney disease (DKD) rats. A,B. After USW treatment, the serum interleukin (IL)-1β, IL-10, and inducible nitric oxide synthase (iNOS) levels of DKD rats were measured with enzyme-linked immunosorbent assay (ELISA). Immunoglobulin M (IgM) and immunoglobulin G (IgG) levels in the urine of DKD rats were determined. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test; C,D. The protein and gene expression of IL-1β, IL-18, tumor necrosis factor alpha (TNF-α), IL-6, IL-10, and arginase (Arg-1) were analyzed in the kidney tissue of DKD rats. Statistical analysis was performed using one-way ANOVA and Tukey's post hoc test

*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the model group.

and Beclin1 (mRNA: $p < 0.05$, ANOVA, $F = 181.3$; protein: $p < 0.05$, ANOVA, $F = 124.8$) levels were increased in the USW group compared to the model group. The p62 (mRNA: $p < 0.05$, ANOVA, $F = 373.0$; protein: $p < 0.05$, ANOVA, $F = 271.7$) levels were reduced in the USW group. Protein expressions of p-mTOR, mTOR and ULK1 were measured using western blot (Fig. 5C). Compared with the model group, the ratio of p-mTOR/mTOR ($p < 0.05$, ANOVA, $F = 331.9$) decreased in the USW group, and the ULK1 level ($p < 0.05$, ANOVA, $F = 28.5$) increased. This hinted that USW treatment could restore the level of renal autophagy and affect the mTOR/ULK1 signaling axis in DKD rats.

ULK1 signaling affected autophagy injury induced by HG

To explore the effect of ULK1 on autophagy in DKD cells, we overexpressed ULK1 in RRPTEpiC and podocyte cells. At the gene and protein level, the expression of ULK1 was identified (Fig. 6A,B). Compared with the control group, ULK1 protein levels decreased in the model group (RRPTEpiC: $p < 0.05$, ANOVA, $F = 20.53$; podocyte: $p < 0.05$, ANOVA, $F = 31.74$). The LC3, Beclin1 and p62 levels were altered after the overexpression of ULK1 (Fig. 6C). The LC3 (RRPTEpiC: $p < 0.05$, ANOVA, $F = 179.7$; podocyte: $p < 0.05$, ANOVA, $F = 351.1$) and Beclin1 (RRPTEpiC:

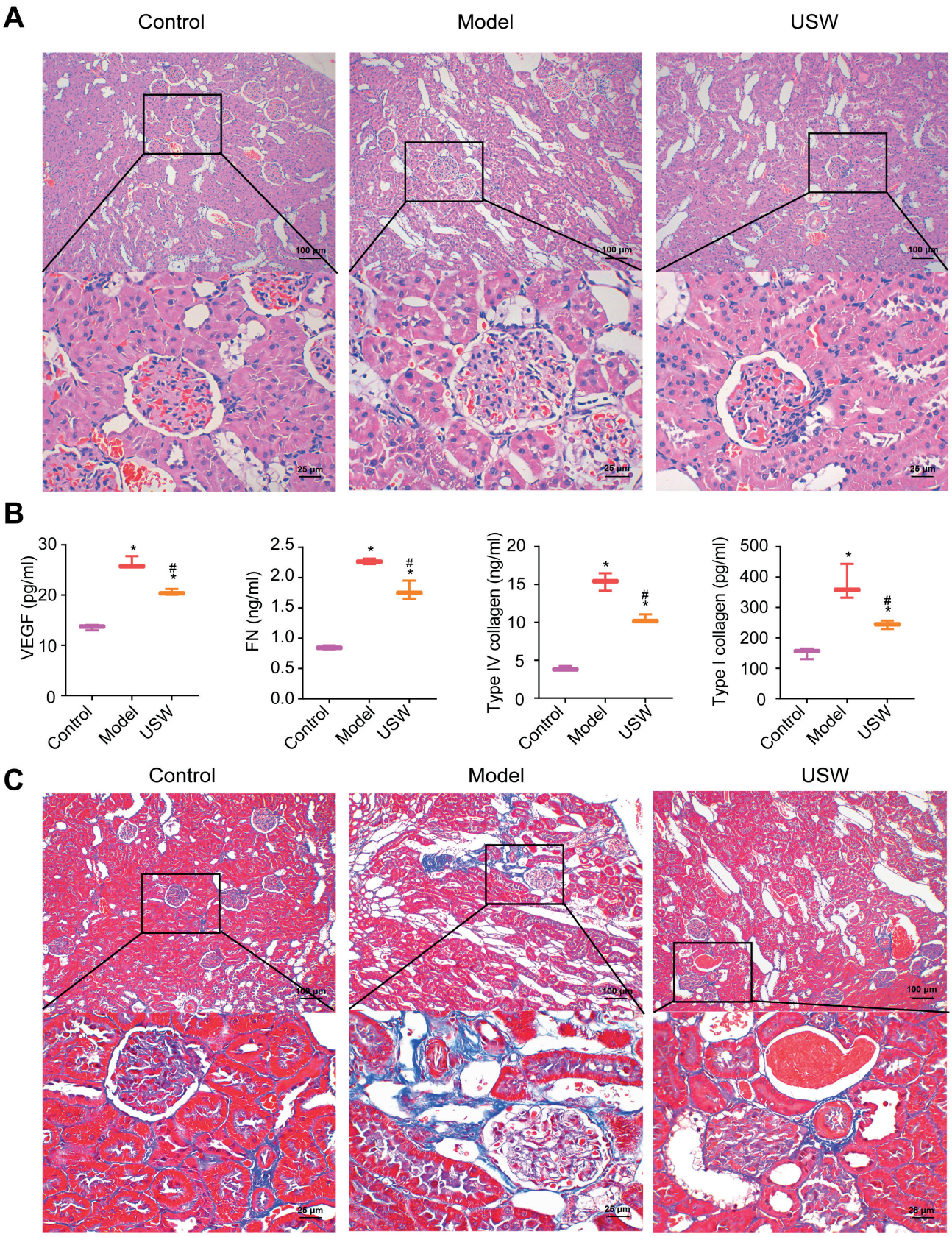

Fig. 3. Ultrashort wave (USW) relieved kidney injury in diabetic kidney disease (DKD) rats. A. Hematoxylin and eosin (H&E) staining analysis was used to observe the pathological morphology of rat renal tissue. Scale bars represent 100 µm (above) and 25 µm (below); B. The concentrations of vascular endothelial growth factor (VEGF), fibronectin (FN), type IV collagen, and type I collagen in the urine were detected using enzyme-linked immunosorbent assay (ELISA). Statistical analysis was performed with one-way analysis of variance (ANOVA) and Tukey's post hoc test; C. Masson staining was performed to analyze the degree of renal tissue fibrosis. Scale bars represent 100 µm (above) and 25 µm (below)

*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the model group.

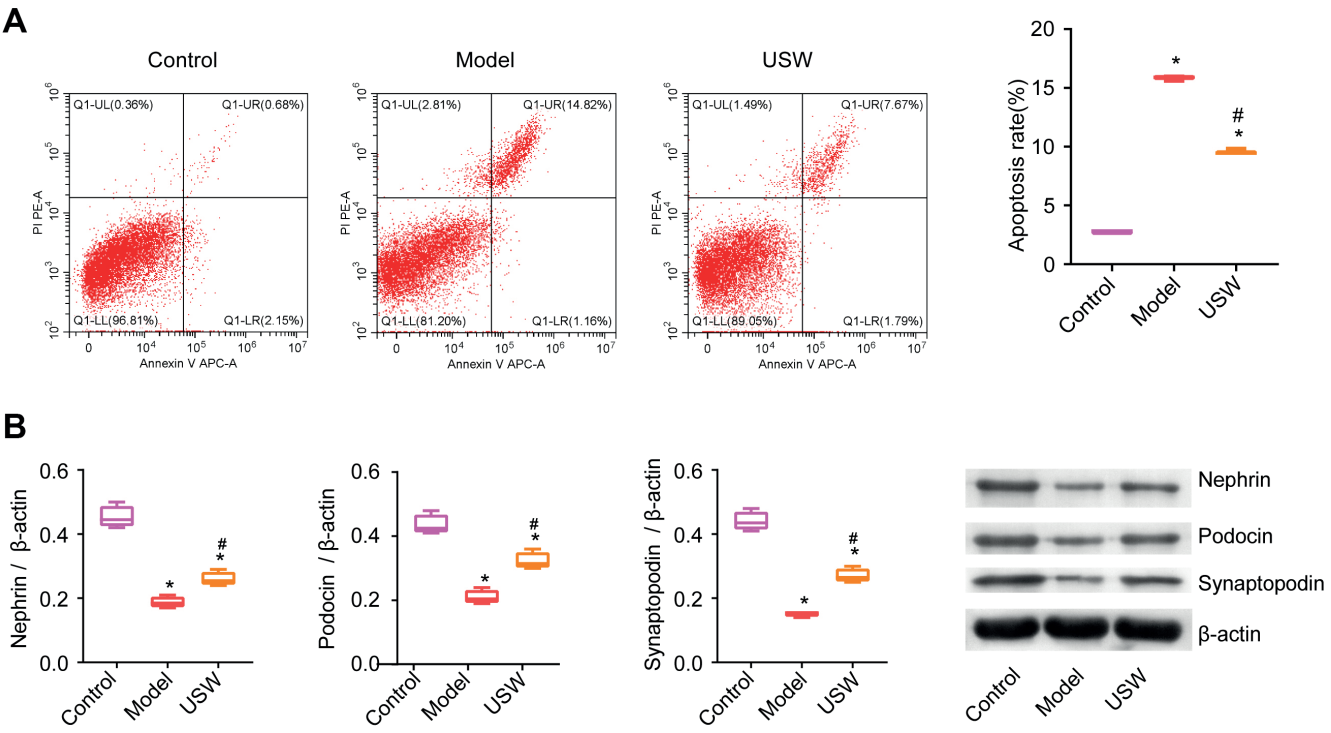

Fig. 4. Ultrashort wave (USW) relieved kidney injury in diabetic kidney disease (DKD) rats. A. The apoptosis rate of rat renal tissue was determined. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test; B. Nephrin, podocin and synaptopodin levels in the kidney tissue of DKD rats were measured using western blot. Statistical analysis was performed with one-way ANOVA and Tukey's post hoc test

*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the model group.

$p < 0.05$, ANOVA, $F = 61.15$; podocyte: $p < 0.05$, ANOVA, $F = 40.95$) levels were higher in the oe-ULK1 group compared to the oe-NC group. The p62 expression decreased in the oe-ULK1 group (RRPTEpiC: $p < 0.05$, ANOVA, $F = 21.87$; podocyte: $p < 0.05$, ANOVA, $F = 43.4$). These results indicated that ULK1 was deregulated in a HG-induced DKD cell model and that regulation of ULK1 could affect the level of autophagy.

The mTOR/ULK1 signaling axis mediated USW to regulate the autophagy level in DKD rats

To further explore the role of the mTOR/ULK1 signaling axis in USW affecting DKD, USW and L-leucine were applied to treat the DKD rats. The ULK1 ($p < 0.05$, ANOVA, $F = 90.61$) and LC3 ($p < 0.05$, ANOVA, $F = 294.9$) levels decreased in the USW+L-leucine group compared to the USW group (Fig. 7A). The activation of mTOR signaling affected autophagy levels in the DKD rats. Serum CRE ($p < 0.05$, ANOVA, $F = 109.9$) and BUN ($p < 0.05$, ANOVA, $F = 498.9$) concentrations were higher in the USW+L-leucine group than in the USW group. The urinary MAU ($p < 0.05$, ANOVA, $F = 634.3$) and blood GLU ($p < 0.05$, ANOVA, $F = 472.3$) levels of the DKD rats increased in the USW+L-leucine group compared to the USW group (Fig. 7B). The activation of mTOR signaling affected the efficacy of USW in alleviating DKD. These results hinted that the mTOR/ULK1 signaling axis might mediate USW to alleviate DKD by regulating autophagy.

Discussion

This study revealed that the levels of relevant metabolites (MAU, GLU, CRE, and BUN) decreased in the DKD rats after the USW intervention. In DKD, there is an increase in levels of metabolites such as MAU, GLU, CRE, and BUN. However, following USW intervention, there was a decrease observed in the levels of these metabolites.[43,44] When evaluating renal function, increased expression of CRE and BUN in DKD indicated more serious renal lesions.[45] Microalbuminuria was considered to be the first manifestation of DKD and a predictor of DKD progression.[46] In DKD, the imbalance between filtration load and renal tubule reabsorption of albumin may lead to an increase in MAU.[47] Since USW caused a decrease of MAU, GLU, CRE, and BUN levels in the DKD rats, it can be suggested that USW has a certain alleviating effect on kidney injury in DKD rats. Excessive epithelial injury and inflammation could promote the development of renal fibrosis.[48] The related literature has also confirmed that the increase in MAU in DKD is related to podocyte functional and pathological morphological changes.[49] Our study found that the degree of renal

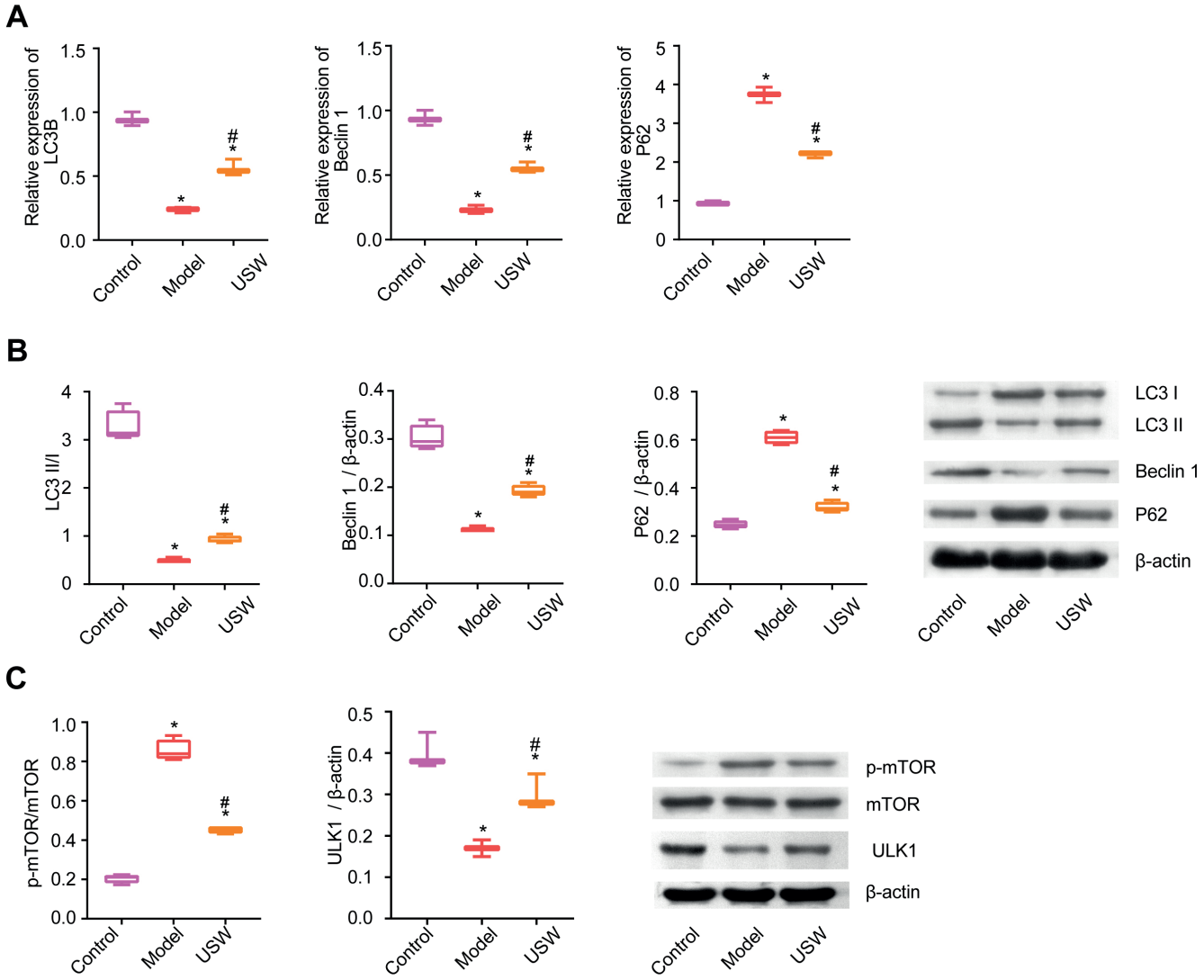

Fig. 5. Ultrashort wave (USW) alleviated the autophagy decrease induced by high-fat diet (HFD)/streptozocin (STZ) and affected the mTOR/unc-51 like autophagy activating kinase 1 (ULK1) signaling axis. A,B. Gene and protein expressions of LC3, Beclin1 and p62 were determined in the renal tissue of diabetic kidney disease (DKD) rats. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test; C. The protein levels of p-mTOR/mTOR and ULK1 were analyzed using western blot. Statistical analysis was performed with one-way ANOVA and Tukey's post hoc test

*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the model group.

interstitial fibrosis in the DKD rats was reduced after the USW intervention. These findings further proved that USW had certain therapeutic effects on the DKD rats.

Inflammation plays a key role in the progression of DKD.[50] Reduced inflammation can alleviate renal dysfunction.[43] In our study, renal inflammation (IL-1β, IL-18, TNF-α, and IL-6) was increased in the DKD rats. The USW treatment significantly downregulated the expression of IL-1β, IL-18, TNF-α, and IL-6. These results indicate that USW could alleviate the inflammatory response in DKD to a certain extent. The levels of IgM and IgG in the urine of the DKD rats were also affected by USW. Immunoglobulin G is mainly transported through large pores in the capillary wall of the glomerulus. The increased urine concentration of IgG in DKD indicated that there was an increase in the number of large pores.[51] The increase in IgM in the urine was mainly related to the size-selective barrier dysfunction of DKD glomeruli.[51] Thus, USW treatment reduced urine IgM and IgG levels. This suggests that USW might reduce the number of large pores in the kidney to some extent and alleviate the selective dysfunction of glomeruli, thereby achieving the goal of improving the DKD state.

Podocytes are important components of the glomerular filtration barrier.[52] Nephrin, the main component of the fissure diaphragm, is significantly reduced in DKD patients.[53] This was consistent with our results that nephrin expression decreased in the kidney tissues of the DKD rats. Podocin is an integrated membrane protein expressed in mature glomerular podocytes.[54] It can be combined with nephrin to form an oligomer and connect the diaphragm to the cytoskeleton.[55] In the study, the USW

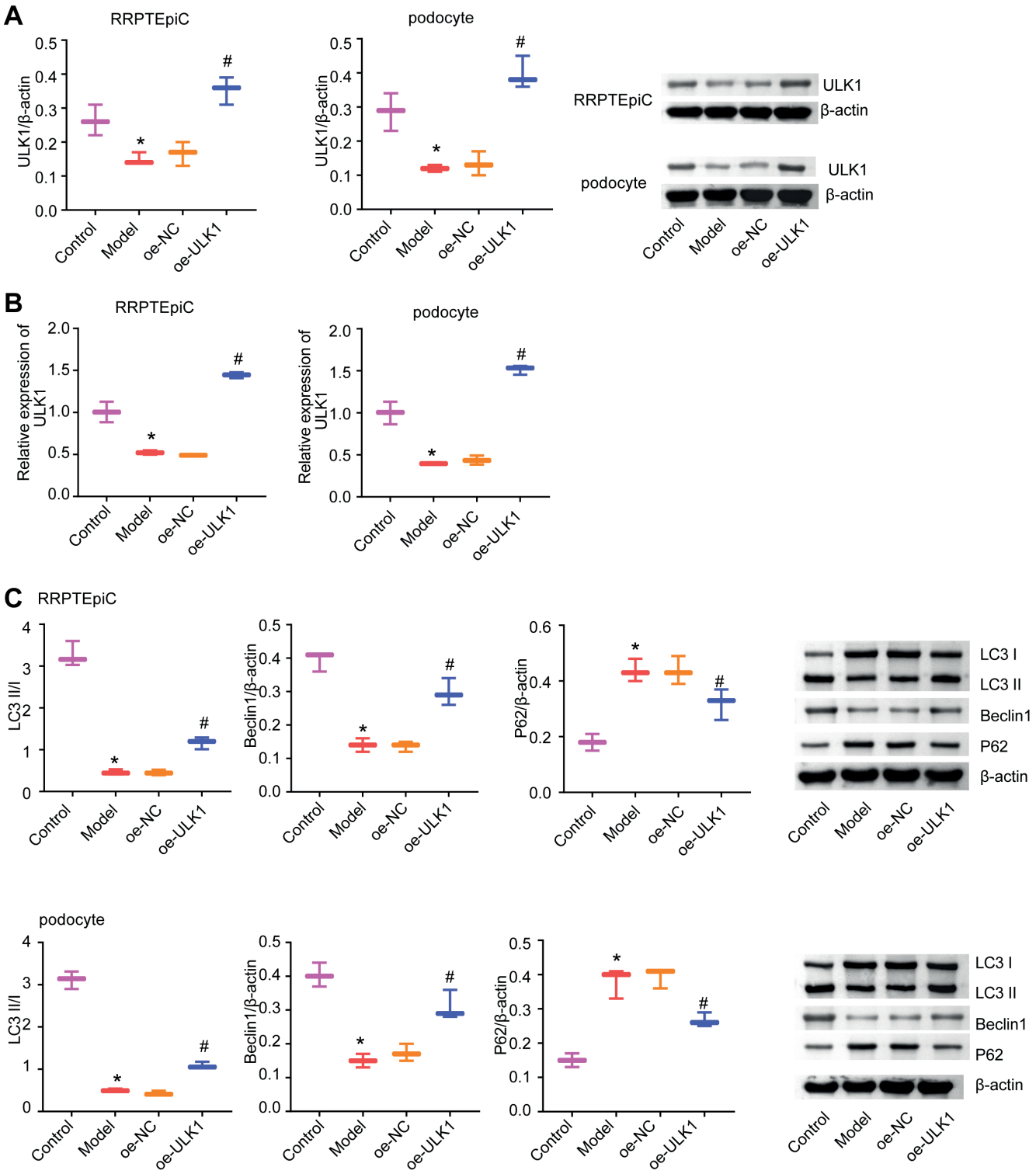

Fig. 6. Unc-51 like autophagy activating kinase 1 (ULK1) signaling affected autophagy. A,B. After high glucose (HG) and ULK1 overexpression interventions, levels of ULK1 were determined in rat renal proximal tubular epithelial cells (RRPTEpiC) and podocyte cells using western blot and quantitative real-time polymerase chain reaction (qPCR). Statistical analysis was performed with one-way analysis of variance (ANOVA) and Tukey's post hoc test; C. Levels of LC3, Beclin1 and p62 were detected in RRPTEpiC and podocyte cells after HG and ULK1 overexpression interventions. Statistical analysis was performed using one-way ANOVA and Tukey's post hoc test

*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the overexpression-negative control (oe-NC) group.

intervention increased the expression of podocin. This suggests that USW could maintain the integrity of the glomerular barrier in DKD to a certain extent. Moreover, reversing the decreased expression of nephrin and podocin can prevent renal fibrosis in diabetic nephropathy mice (also to a certain degree).[56] These findings showed that our research results have been reasonably achieved. The USW intervention restored the expression of nephrin

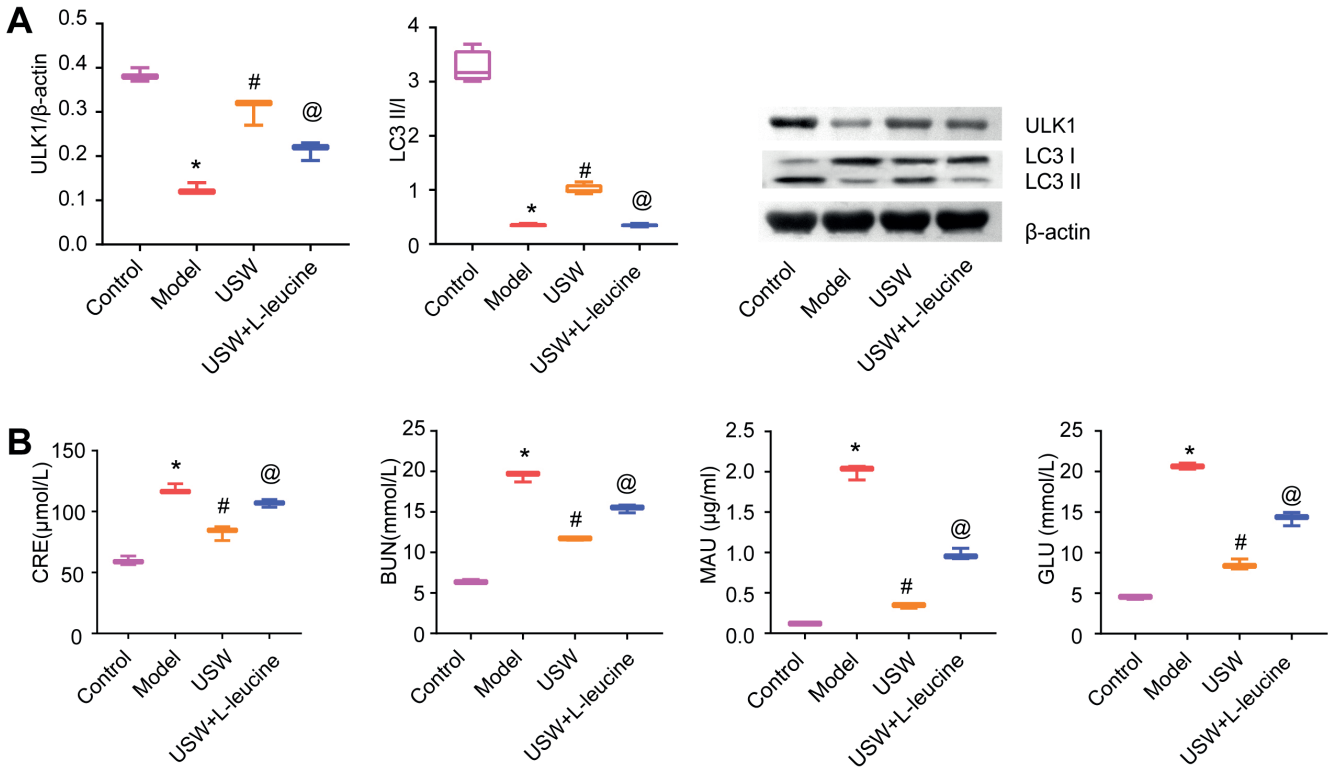

Fig. 7. The mTOR/unc-51 like autophagy activating kinase 1 (ULK1) signaling axis mediated ultrashort wave (USW) to regulate autophagy levels in DKD rats. A. After the USW and L-leucine interventions in diabetic kidney disease (DKD) rats, protein expression of ULK1 and LC3 in kidney tissue was determined using western blot. Statistical analysis was performed with one-way analysis of variance (ANOVA) and Tukey's post hoc test; B. Concentrations of creatinine (CRE), blood urea nitrogen (BUN), microalbuminuria (MAU), and glucose (GLU) were measured using enzyme-linked immunosorbent assay (ELISA). Statistical analysis was performed with one-way ANOVA and Tukey's post hoc test

*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the model group; @$p < 0.05$ compared to the USW group.

and podocin, and reduced the level of renal interstitial fibrosis in the DKD rats.

As a pathway involved in protein and organelle degradation, autophagy plays an important role in maintaining cell homeostasis.[57] Glomerular podocytes exhibited high levels of autophagy under basal conditions.[15] A HFD with STZ has been shown to induce an inhibition of autophagy activity in the renal podocytes of DKD mice.[58] This is consistent with our result that the level of autophagy in the kidneys of the DKD rats decreased. However, the level of autophagy increased in the DKD rats after the USW intervention. These results suggest that USW might affect renal autophagy in DKD rats. A previous study showed that mTORC1 activation was a key step in developing diabetic nephropathy in mice.[59] The intervention of the mTOR/ULK1 signaling axis could affect podocyte autophagy.[60,61] In addition, the mTOR/ULK1 signaling axis was evaluated. Ultrashort wave was found to inhibit the activity of the mTOR/ULK1 signaling axis. L-leucine, an activator of mTOR, was used in the DKD rats. It was found that L-leucine could reduce the therapeutic effects of USW. Thus, it could be concluded that USW affects renal autophagy in DKD rats by inhibiting the mTOR/ULK1 signaling axis, at least in part.

Limitations

This study analyzed the regulation of the mTOR/ULK1 signaling axis by USW in DKD rats in a limited way. The intrinsic pathway by which USW regulates the mTOR/ULK1 signaling axis needs to be further explored. In the future, we will analyze the effect of USW on the gene transcriptome of DKD rat kidney tissue by RNA sequencing. The relationship between differential genes and the mTOR/ULK1 signaling axis was analyzed in combination with bioinformatics. In addition, we will further combine in vivo and in vitro experiments to analyze whether USW regulates the mTOR/ULK1 signaling axis through differential genes, thereby alleviating the decrease in autophagy in the kidney tissue of DKD rats.

Conclusions

In conclusion, the study found that USW could affect metabolic and inflammatory levels in DKD rats. Ultrashort wave could alleviate kidney injury and increase autophagy activity in DKD rats. At the same time, USW might activate renal autophagy in DKD rats via the mTOR/ULK1

signaling axis. This enriches the basic experimental data on using the USW intervention to alleviate DKD and provides a scientific basis for the clinical utilization of USW in the treatment of DKD.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.7693991. The package contains the following files:

Supplementary Table 1. Normality and uniformity test results of data presented in Fig. 1.

Supplementary Table 2. Normality and uniformity test results of data presented in Fig. 2.

Supplementary Table 3. Normality and uniformity test results of data presented in Fig. 3.

Supplementary Table 4. Normality and uniformity test results of data presented in Fig. 4.

Supplementary Table 5. Normality and uniformity test results of data presented in Fig. 5.

Supplementary Table 6. Normality and uniformity test results of data presented in Fig. 6.

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Exploring the anti-glioma mechanism of the active components of Cortex Periplocae based on network pharmacology and iTRAQ proteomics in vitro

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Abstract

Background. The active components of Cortex Periplocae (CP) exert antitumor properties in many cancers. However, little is known about their effects on glioma or the related underlying mechanisms.

Objectives. The study investigated the underlying mechanism of CP in treating glioma.

Materials and methods. The U251 and TG905 cells were treated with an ethanol extract from CP. Cell proliferation was detected using Cell Counting Kit-8 (CCK-8) and a colony formation assay. The flow cytometric analysis was applied to explore the induction of cell cycle arrest and apoptosis. The expression levels of cell cycle- and apoptosis-associated proteins were measured with western blot. A network pharmacology method was performed to predict the potential mechanism underlying the effects of the active components of CP on glioma. Then, isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomics analysis was used to verify the differentially expressed proteins and pathways in order to reveal the underlying mechanisms. Furthermore, to determine the iTRAQ results, 6 candidate proteins were chosen for quantification using parallel reaction monitoring (PRM).

Results. The CP extract inhibited the proliferation of U251 and TG905 cells and induced cell cycle arrest and apoptosis. There are 16 active compounds of CP. The antitumor mechanism of CP may be related to the apoptosis pathway, p53 signaling pathway, PI3K-AKT pathway, or transcriptional misregulation in cancer pathway. Six proteins (HSP90AB1, TOP2A, ATP1A1, TGFβ1, ATP1B1, and TYMS) were determined to be key factors involved in regulating CP in glioma.

Conclusions. Our research revealed the underlying mechanism of CP in treating glioma using integrated network pharmacology and iTRAQ-based quantitative proteomics technology.

Key words: proteomics, glioma, network pharmacology, Cortex Periplocae

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Introduction

Gliomas, a group of life-threatening tumors, are the most common primary intracranial tumors.[1] They arise from precursor or glial cells of the central nervous system,[2] and the mortality rate remains high despite advancements in currently available therapeutic approaches, including surgical treatment, radiotherapy and chemotherapy.[3] It is difficult to resect the tumor completely due to its infiltrative growth and critical localization in the brain.[4] The 5-year survival rate of patients with malignant glioma was reported to be less than 25% when treated by surgery alone.[5] Gliomas are not sensitive to radiotherapy,[6] and the blood–brain barrier makes it difficult for chemotherapeutic drugs to penetrate.[7] Furthermore, radiotherapy and chemotherapy cause complications[8] that negatively impact the quality of life of patients. Thus, there is an urgent need to find more satisfactory treatment methods for glioma.

Cortex Periplocae (CP), called Xiangjiapi in Chinese, is the dry root of the Chinese herb *Periploca sepium* Bunge, a Traditional Chinese Medicine (TCM) medicament. It has a long history of use in the treatment of autoimmune diseases, such as rheumatoid arthritis.[9] Recently, additional research has explored the biological activity of the crude extract and the active components of CP, such as anticancer,[10] cardiotonic[11] and anti-inflammatory effects,[12,13] to determine its potential pharmaceutical value. Li et al. found that the periplocin isolated from CP significantly inhibited the proliferation of gastric cancer cells and induced apoptosis in vivo and in vitro by the ERK1/2-EGR1 pathway.[14] Moreover, periplocin inhibited the growth of pancreatic cancer by inducing apoptosis through AMPK-mTOR signaling.[10] As a potential antitumor component, periplocin has also been reported to inhibit the growth of colon cancer[15] and lung cancer[16] through β-catenin/TCF signaling and the AKT/ERK signaling pathway, respectively. Yang et al. suggested that periplogenin (PPG) isolated from CP triggers the apoptosis of colon cancer cells through the $IRE_{1\alpha}$-ASK1-JNK and BIP-$eIF_{2\alpha}$-CHOP signaling pathways.[17] The PPG also has a clear effect on the treatment of nasopharyngeal carcinoma.[18] Moreover, lupeol acetate from CP showed an inhibitory effect in esophageal tumorigenesis in rats.[19] However, the effects of CP extract and its underlying mechanisms in treating glioma remain to be determined.

Due to the rapid advancements in bioinformatics, network pharmacology and isobaric tags for relative and absolute quantitation (iTRAQ) proteomics approaches have been effectively used to reveal the active components of TCM medicaments and their potential mechanisms of action.[20] A new methodological strategy, network pharmacology, provides a significant advantage in helping to understand the therapeutic mechanism of TCM medicaments.[21] The CP has antitumor activities exerted through a multi-component, multi-target, multi-pathway, and multi-biological process, which conforms to the features of network pharmacology.[22] The iTRAQ approach has been employed to quantify and qualify proteins, as well as explore potential protein interactions.[23,24] In this study, network pharmacology and iTRAQ proteomics were applied to explore the active components of CP and its anti-glioma molecular mechanisms.

Objectives

The purpose of this study is to investigate the underlying mechanisms of CP in treating glioma.

Materials and methods

Preparation of the ethanol extract of Cortex Periplocae

The CP was obtained from Linyi People's Hospital (Linyi, China). First, 100 g of CP was crushed and mixed with 75% ethanol in a ratio of 1:10, then placed in the dark overnight. The mixture was sonicated for 30 min, removing the filtrate, and the process was repeated 3 times. The filtered solution was centrifuged at 10,000 rpm for 8 min to obtain the supernatant, using a Buchi rotary evaporator (Buqi, Gent, Belgium). The ethanol extract was freeze-dried and separated, and 92.18 g of freeze-dried powder was obtained from the extract. A total of 100 mg of ethanol extract of CP was added to 1 mL dimethyl sulfoxide (DMSO) to prepare a stock solution with a concentration of 100 mg/mL.

Cell lines and cell culture

The U251 and TG905 were obtained from the Cellular Biology Institute of the Shanghai Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640, adding 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere.

CCK-8 assay

The cells were treated with CP (0 μg/mL, 1 μg/mL, 3 μg/mL, 10 μg/mL, 30 μg/mL, and 100 μg/mL) for the corresponding time (24 h, 48 h and 72 h). Then, the cells were added to a mixture of Cell Counting Kit-8 (CCK-8) (BB-4202; Dojindo Laboratories, Kumamoto, Japan) and Dulbecco's modified Eagle medium (DMEM), and incubated for another hour. The absorbance at a wavelength of 450 nm was recorded using a microplate reader (BestBio, Shanghai, China).

Colony formation assay

A total of 200 cells were planted in each hole of a 6-well plate. After 24 h, different concentrations of CP were added to the cells for approx. 10 days. The colonies were fixed

with 4% paraformaldehyde and stained with 0.1% crystal violet. The images were captured with a digital camera (Fluorchem E; Proteinsimple, Santa Clara, USA).

Flow cytometric analysis

The cells were harvested and fixed with 75% ethanol overnight. After washing and resuspension in phosphate-buffered saline (PBS), the cells were stained with propidium iodide (PI)/RNase staining buffer for 15 min. Finally, the samples were used for the analysis in a flow cytometer (LSRFortessa™ SORP; BD Biosciences, Franklin Lakes, USA).

Apoptosis assay

An FITC Annexin V Apoptosis Detection Kit (BB-4101) (BD Biosciences) was used to measure the apoptosis rate. The cells were harvested, washed and resuspended in IX binding buffer. Five microliters of PI and Annexin X were added to the solution, respectively, and incubated in the dark for 5 min. Finally, the samples were used for analysis in a flow cytometer.

Western blot

After treatments, cells were lysed using radioimmunoprecipitation assay (RIPA) with phosphatase inhibitor. The cellular protein was extracted and quantified with the bicinchoninic acid (BCA) method. Similar amounts of protein were loaded and separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were exposed to specific primary antibodies and fluorescent secondary antibodies after being blocked in a 5% non-fat dry milk solution. The primary antibodies were monoclonal mouse antibodies against cyclin E, CDK2 (Cell Signaling Technology, Danvers, USA), poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, caspase-3 (Abcam, Cambridge, UK), and GAPDH (Santa Cruz Biotechnology, Dallas, USA). The chemiluminescent detection was used to visualize the membrane samples (MilliporeSigma, Burlington, USA). The respective densities of the protein bands were evaluated using ImageJ software (National Institutes of Health, Bethesda, USA).

Identification of candidate compounds

All of the CP compounds were retrieved from the TCM Systems Pharmacology Database and Analysis Platform (TCMSP), which includes 500 Chinese herbal medicines with 30,069 ingredients through database integration and literature mining. The pharmacokinetic properties of natural compounds involve oral bioavailability (OB), drug-likeness (DL) and Caco-2 cells, which were provided for study.

Screening of active compounds

Among the ADME (absorption, distribution, metabolism, and excretion) properties of the compounds, OB, DL and Caco-2 cells represent the most vital properties of the administered drugs. Oral bioavailability is used to assess the efficiency of the drug distribution within systemic circulation. Drug-likeness refers to a measure of similarities between the compound and known drugs. The average DL index of all of the drugs was 0.18.[25] The Caco-2 cells can be used to investigate intestinal epithelium permeability.[26] Based on the TCMSP database, molecules with Caco-2 > −0.40 corresponded to good permeability in the small intestinal epithelium.[27,28] Hence, in this study, molecules with OB ≥ 30%, DL ≥ 0.18 and Caco-2 > −0.40 were considered active compounds.

Target identification through integrated database analyses

The TCMSP database was used to identify the active components of CP, while GeneCards database (https://genecards.weizmann.ac.il/v3/) was used to discover glioma-specific therapeutic targets. The overlap between the 2 results provided drug targets for CP in treating glioma.

Protein extraction, enzymatic hydrolysis and peptide quantification

Total protein was extracted from U251 cells with SDT (4% (w/v) SDS, 100 mM Tris/HCl 1 mM dithiothreitol (DTT), pH 7.6). The extracted proteins were quantified using a BCA kit. Protein enzymatic hydrolysis was performed using the filter-aided sample preparation (FASP) procedure.[29] The enzymolysis peptide was desalted with the use of a C18 purification cartridge, freeze-dried and re-dissolved with 40 μL of the dissolution buffer. Finally, the peptides were quantified with OD280.

iTRAQ and LC–MS/MS

Equal amounts of each peptide were taken and labeled according to the AB SCIEX iTRAQ labeling kit instructions.[30] Samples were labeled as iTRAQ-113, -114, -115, and -116, -117, -118. The labeled peptides were mixed in equal amounts and graded with an AKTA Purifier 100 (GE Healthcare, Chicago, USA). Liquid chromatography–mass spectrometry (LC–MS)/MS was carried out as described by Ross et al.[30]

Mass spectrometry data processing and analysis

The original raw data obtained using LC–MS/MS were identified and quantified with mascot and proteome discoverer. Table of SI is shown in the main library parameters.

Bioinformatic analysis

The Gene Ontology (GO) analysis was performed using the Blast2 GO database (http://geneontology.org/). The KEGG Automatic Annotation Server (KASS) software (https://www.genome.jp/kegg/kaas/) was used to classify and group the identified proteins. The distribution of Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathways and whole protein sets for each GO classification were compared using Fisher's exact test. The GO annotation or KEGG pathway annotation was used to enrich the target protein set.

The complex Heatmap R package (R Foundation for Statistical Computing, Vienna, Austria) was employed to categorize the expression of proteins and samples at the same time, as well as to create a hierarchical clustering heatmap.

The information obtained from the IntAct or STRING database was used to find direct and indirect interaction relationships between the target proteins. The interaction network was generated and analyzed using Cytoscape (https://cytoscape.org/).

Parallel reaction monitoring verification

Protein extraction was carried out as described above for the iTRAQ experiment. Then, the samples were added to DTT, boiled in a water bath for 15 min, cooled down, and UA (8 M urea, 150 mM Tris-HCl, pH 8.0) buffer was added. The samples were then transferred to a 10 kd ultrafiltration tube and centrifuged at 14,000 × g for 30 min. A total of 200 µL of UA buffer was added, then the samples were centrifuged and the filtrate was discarded. Next, indole-3-acetic acid (IAA) was added, the sample was shaken and centrifuged at 14,000 × g for 20 min. Then, the NH_4HCO_3 buffer (50 mM) was added, and the sample was centrifuged at 14,000 g for 20 min twice. A new collecting pipe was used and the sample was centrifuged for 15 min. A total of 40 µL of NH_4HCO_3 buffer (50 mM) was added, the sample was centrifuged again for 30 min, and the filtrate was collected. After enzymatic hydrolysis, the collected peptide was desalted and lyophilized, then re-dissolved with 0.1% formic acid (FA). The peptide concentration was established using OD280.

According to the pre-experimental results, 10 target peptides of 6 identified target proteins were quantified using parallel reaction monitoring (PRM). In order to set up the PRM method, peptide information was entered into Xcalibur software (Thermo Fisher Scientific, Waltham, USA). The standard peptide was mixed with about 1 µg of peptide extracted from each sample for detection. High-performance liquid chromatography (HPLC) was used for chromatographic separation. The chromatographic column reached liquid equilibrium at 95% A.

The samples separated with HPLC were analyzed using PRM mass spectrometry (Q Exactive HF mass spectrometer; Thermo Fisher Scientific, Waltham, USA). The PRM was detected in 6 samples, and finally, the data from the original PRM files were analyzed using Skyline software (Skyline Software Systems, Inc., Herndon, USA).

Statistical analysis

In this study, GraphPad Prism v. 8.2.1 (GraphPad Software, San Diego, USA) was used for data analysis. Data were expressed as mean ± standard deviation (M ±SD) and were obtained from at least 3 separate experiments. The significance levels of differences were determined with independent sample t-tests for the various treatments. A value of $p < 0.05$ was considered statistically significant.

Results

CP inhibits cell viability of glioma cells

The CP with different concentrations (0 µg/mL, 1 µg/mL, 3 µg/mL, 10 µg/mL, 30 µg/mL, and 100 µg/mL) were added to the U251 and TG905 cells for 24 h, 48 h and 72 h, and cell viability was measured with a CCK-8 assay. As shown in Fig. 1A,B, CP suppressed cell viability in a time- and dose-dependent manner. For U251 cells, the IC50 values of CP at 24 h, 48 h and 72 h were 26.48 µg/mL, 11.07 µg/mL and 3.349 µg/mL, respectively. For TG905 cells, the IC50 values of CP at each of the 3 time intervals were 30.39 µg/mL, 11.1 µg/mL and 12.7 µg/mL, respectively. Furthermore, a colony formation assay showed that CP degraded the rate of colony formation in U251 and TG905 cells in a dose-dependent manner (Fig. 1C).

CP induces cell cycle arrest in glioma cells

Flow cytometry results showed that CP caused a large accumulation of glioma cells in G0/G1 phases in a dose-dependent manner, which was accompanied by a decrease in S and G2/M phases (Fig. 2A). In addition, western blotting was used to explore the expression level of cyclin E and CDK2, 2 cell cycle-associated proteins (Fig. 2C). The results showed that a high concentration (16 µg/mL and 32 µg/mL) of CP downregulated the expression level of cyclin E and CDK2, but a low concentration (8 µg/mL) of CP had no effect on the protein expression (Fig. 2B). The above results indicate that CP may have inhibited cell proliferation resulting from G0/G1 cell cycle arrest.

Treatment of glioma cells with CP induces apoptosis

To investigate whether CP induced apoptosis in glioma cells, CP (0 µg/mL, 8 µg/mL, 16 µg/mL, and 32 µg/mL)

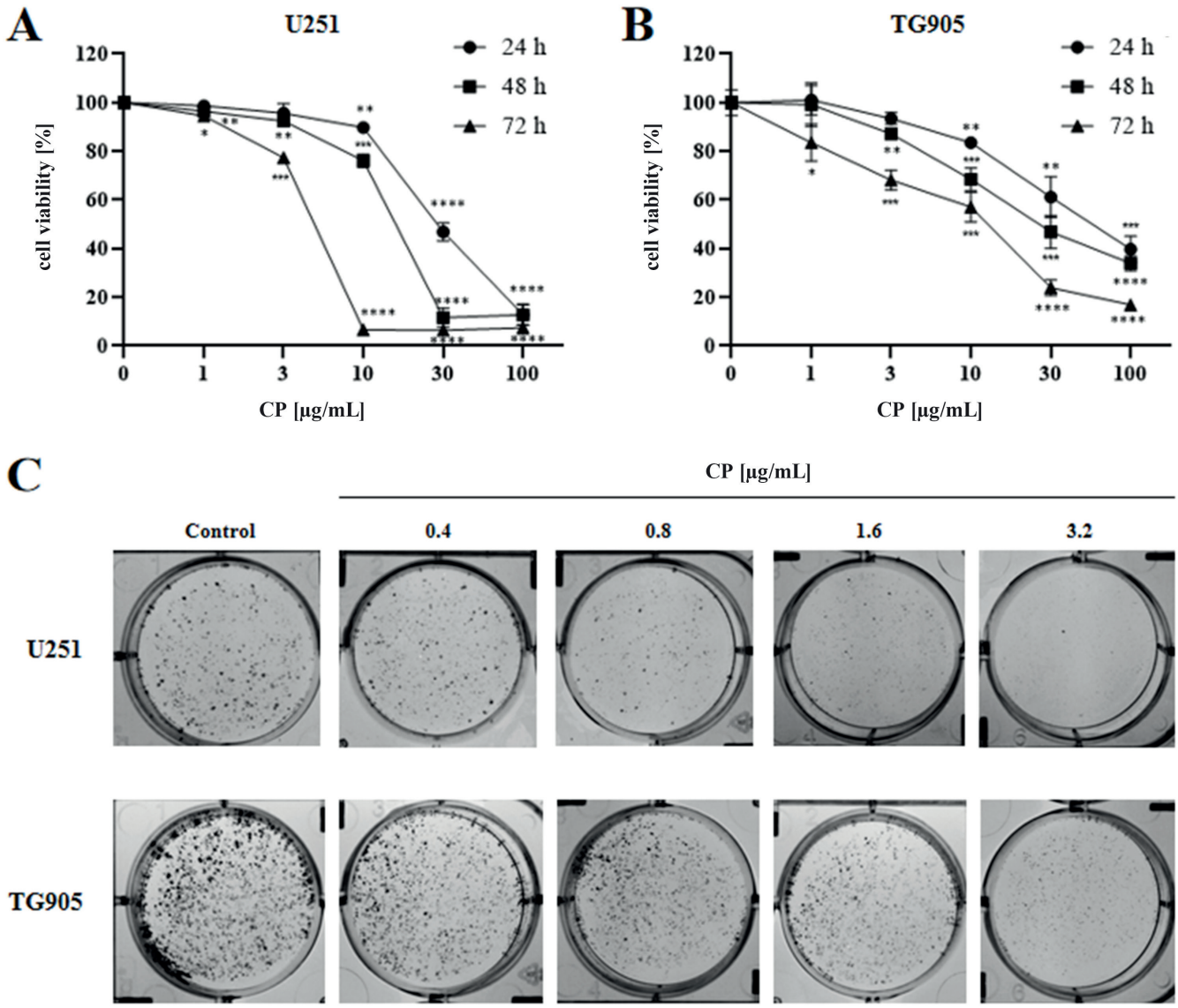

Fig. 1. Cortex Periplocae (CP) inhibits cell viability of glioma cells. A,B. U251 and TG905 cells were treated with CP (0 µg/mL, 1 µg/mL, 3 µg/mL, 10 µg/mL, 30 µg/mL, and 100 µg/mL) for 24 h, 48 h and 72 h. Cell viability was measured using Cell Counting Kit-8 (CCK-8) assay (n = 3); C. U251 and TG905 cells were treated with CP (0 µg/mL, 0.4 µg/mL, 0.8 µg/mL, 1.6 µg/mL, and 3.2 µg/mL) for approx. 10 days. The rate of colony formation was evaluated with colony formation assay (n = 3)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to the control group.

was added to U251 and TG905 cells for 48 h, and then flow cytometry with Annexin V-FITC staining was used to detect the number of apoptotic cells. The results showed that the treatment of U251 and TG905 cells with CP induced apoptosis. Apoptotic cells in the U251 and TG905 samples after CP treatment ranged from 4.1% to 64.9% ($p < 0.0001$) and from 4.04% to 86.9% ($p < 0.0001$), respectively (Fig. 3A,B). To further reveal the potential mechanisms involved in CP-induced apoptosis, western blotting was used to establish the level of apoptosis-related proteins, namely PARP, caspase-3 and cleaved caspase-3. The data showed that CP decreased the expression level of caspase-3 and PARP, while upregulating the level of cleaved caspase-3 in a dose-dependent manner (Fig. 3C).

Identification of the active compounds of CP

To identify the active compounds of CP, the TCMSP database was used, and 79 components of CP were found. Among the 79 compounds in CP, 16 satisfied the criterion of OB ≥ 30%, DL ≥ 0.18 and Caco-2 > −0.40. Detailed information for the 16 compounds is shown in Table 1. The data demonstrated that CP was chemically fit for drug development.

Drug targets of CP for treating glioma

The TCMSP database was further used to predict the putative targets of CP. Nineteen putative targets of CP remained after removing duplicates. Meanwhile, 5753 validated therapeutic targets for glioma and glioblastoma

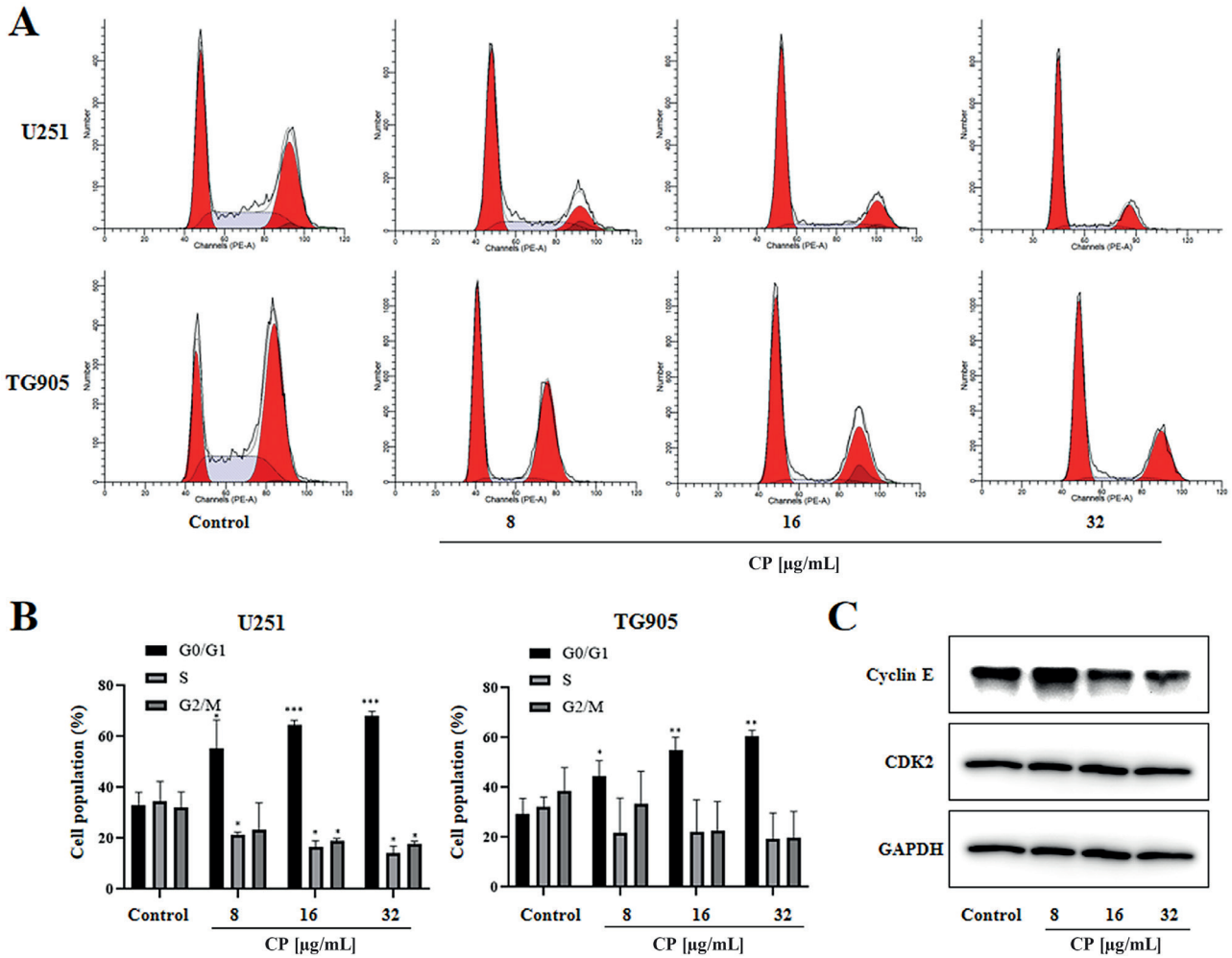

Fig. 2. Cortex Periplocae (CP) induces cell cycle arrest in glioma cells. The U251 and TG905 cells were treated with CP (0 μg/mL, 8 μg/mL, 16 μg/mL, and 32 μg/mL) for 24 h. A,B. The cell cycle distribution was analyzed with flow cytometry (n = 3); C. The expression level of cyclin E and CDK2 was detected with western blot

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to the control group.

Table 1. The active components of Cortex Periplocae (CP)

Mol ID	Molecule name	OB [%]	DL	Caco-2
MOL001771	poriferast-5-en-3beta-ol	36.91	0.75	1.45
MOL000358	beta-sitosterol	36.91	0.75	1.32
MOL000359	sitosterol	36.91	0.75	1.32
MOL005645	21-O-Methyl-5,14-pregndiene-3β,14β,17β,21-tetrol-20-one	38.52	0.57	0.08
MOL005646	21-O-Methyl-5-pregnene-3β,14β,17β,20,21-pentol	45.12	0.6	–0.04
MOL005652	glycoside K_qt	31.91	0.43	0.79
MOL005654	glycoside H2_qt	49.95	0.48	0.11
MOL005656	glycozolidal	78.07	0.2	1
MOL005658	periplogenin	36.61	0.74	0.04
MOL005664	glycoside E_qt	40.57	0.47	0.39
MOL005666	periplocoside M_qt	32	0.88	0.06
MOL005683	delta 5-pregnenetriol	35.94	0.47	0.17
MOL005686	periplocoside O_qt	32	0.88	0.1
MOL005690	periplocymarin_qt	104.15	0.74	–0.14
MOL005692	neridienone A	30.96	0.48	0.49
MOL005693	xysmalogenin	54.41	0.72	0.02

OB – oral bioavailability; DL – drug-likeness.

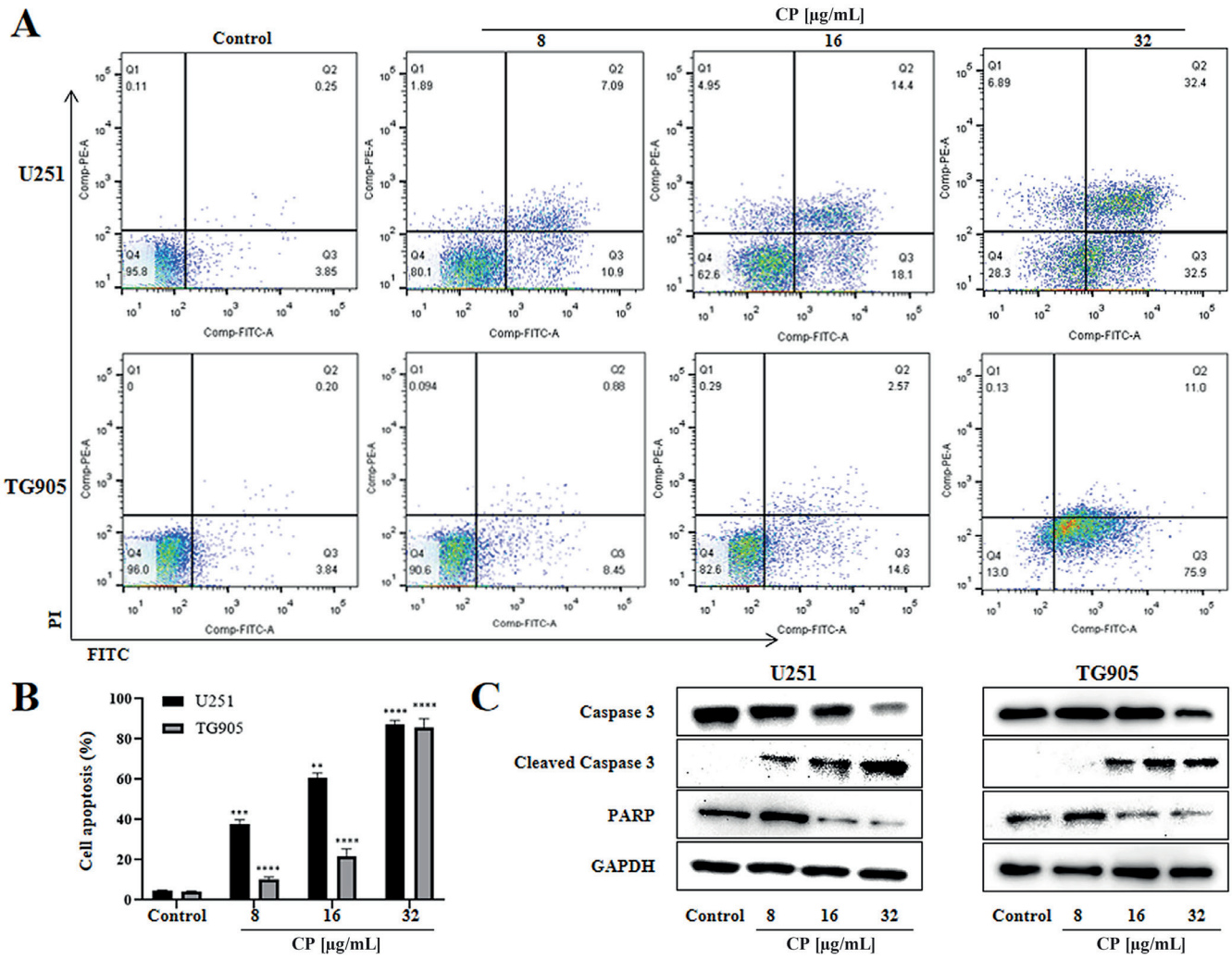

Fig. 3. Treatment of glioma cells with Cortex Periplocae (CP) induces apoptosis. The U251 and TG905 cells were treated with CP (0 μg/mL, 8 μg/mL, 16 μg/mL, and 32 μg/mL) for 48 h. A,B. Early and late apoptotic cells were analyzed using flow cytometry with Annexin V-FITC staining (n = 3); C. The apoptosis-related proteins, namely cleaved caspase-3, caspase-3 and poly (ADP-ribose) polymerase (PARP) were analyzed using western blot

** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to the control group.

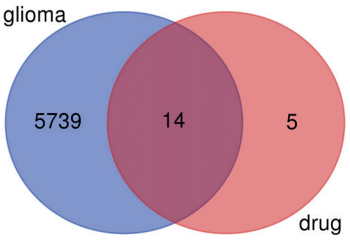

Fig. 4. Drug targets of Cortex Periplocae (CP) for treating glioma. A Venn diagram of constituent-related targets and glioma therapeutic-related targets

were acquired from GeneCards after removing duplicates. The 2 gene target groups were compared, and 14 overlapping genes were ultimately identified (Fig. 4). These 14 target genes were subjected to further analysis (Table 2).

Quantitative proteomic response to CP

Next, untreated and CP-treated (20 μg/mL) U251 cells were evaluated using iTRAQ/tandem mass tag (TMT) technology to identify peptides, quantify proteins and analyze

Table 2. Target gene of Cortex Periplocae (CP) for treating glioma

Molecule name	Gene symbol	Gene ID
Apoptosis regulator Bcl-2	BCL2	596
Muscarinic acetylcholine receptor M1	CHRM1	1128
Muscarinic acetylcholine receptor M4	CHRM4	1132
Progesterone receptor	PGR	5241
Muscarinic acetylcholine receptor M3	CHRM3	1131
Serum paraoxonase/arylesterase 1	PON1	5444
Caspase-3	CASP3	836
Gamma-aminobutyric acid receptor subunit alpha-1	GABRA1	2554
Caspase-9	CASP9	841
Caspase-8	CASP8	842
Glucocorticoid receptor	NR3C1	2908
Muscarinic acetylcholine receptor M2	CHRM2	1129
Prostaglandin G/H synthase 1	PTGS1	5742
Protein kinase C alpha type	PRKCA	5578

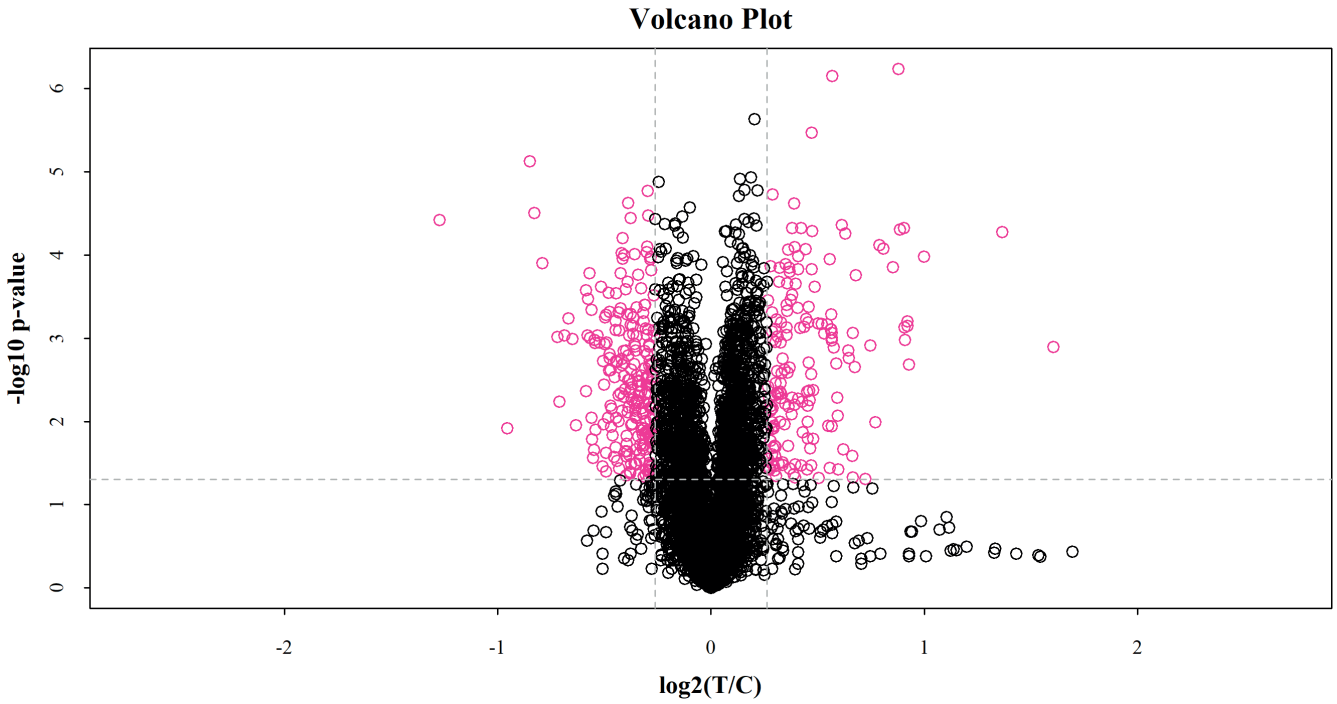

Fig. 5. Volcano plot of differentially expressed proteins in Cortex Periplocae (CP)-treated cells. All proteins were plotted with $\log_2$ fold change on the x-axis and $-\log_{10}$ (p-value) on the y-axis. The red dots indicate significantly differentially expressed proteins (up >1.2 or down <0.83). The red dots on the left indicate underexpressed proteins. The red dots on the right indicate overexpressed proteins. The black dots are proteins with no differences in the CP-treated group compared with the control group (n = 3)

Table 3. Statistics on the protein identification results

Database	Peptides	Unique peptides	Protein groups	Upregulated	Downregulated	Significantly different proteins
Swissport_Human	40,288	36,930	5482	166	230	369

differentially expressed proteins. As shown in Table 3, 40,288 peptides, including 36,930 unique peptides, were detected, and 5482 proteins were identified. Among the 5482 proteins, a change in expression greater than 1.2-fold (up >1.2 or down <0.83) and a significance level of $p < 0.05$ were the criteria set for screening the differentially expressed proteins. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) resource was used to screen differentially expressed proteins. There were 369 differentially expressed proteins, including 166 upregulated and 203 downregulated. The quantitative statistical results are presented using a volcano plot (Fig. 5).

Gene Ontology analysis

In proteomics, the collection of all proteins in a cell, tissue or organism is the major object of study. For high-throughput omics, it is a priority to understand which functions or biological pathways are significantly affected by biological treatments. Thus, proteins and their functions should be analyzed and summarized more systematically. All proteins identified in this project were subjected to GO functional annotation. Then, the GO enrichment analysis was performed for differentially expressed proteins using Fisher's exact test.

The 369 differentially expressed proteins were categorized into biological process (BP), molecular function (MF) and cellular component (CC), based on their annotation (Fig. 6). The BP analysis revealed that most of the proteins were primarily involved in cellular potassium ion homeostasis, cell proliferation, keratinization, macrophage activation, and intermediate filament bundle assembly. The significant enrichment of CC was mainly related to cytokine activity, receptor regulator activity, receptor–ligand activity, DNA-binding transcription activator activity, RNA polymerase II-specific and sodium-potassium-exchanging ATPase activity. According to the MF analysis, some proteins were clearly enriched in keratin filament, intermediate filament, sodium–potassium-exchanging ATPase complex, condensed chromosome outer kinetochore, and ATPase-dependent transmembrane transport complex. Additionally, cellular process, biological regulation, regulation of biological process, metabolic process, binding, catalytic activity, cell, cell part, and organelle changed significantly (Fig. 7).

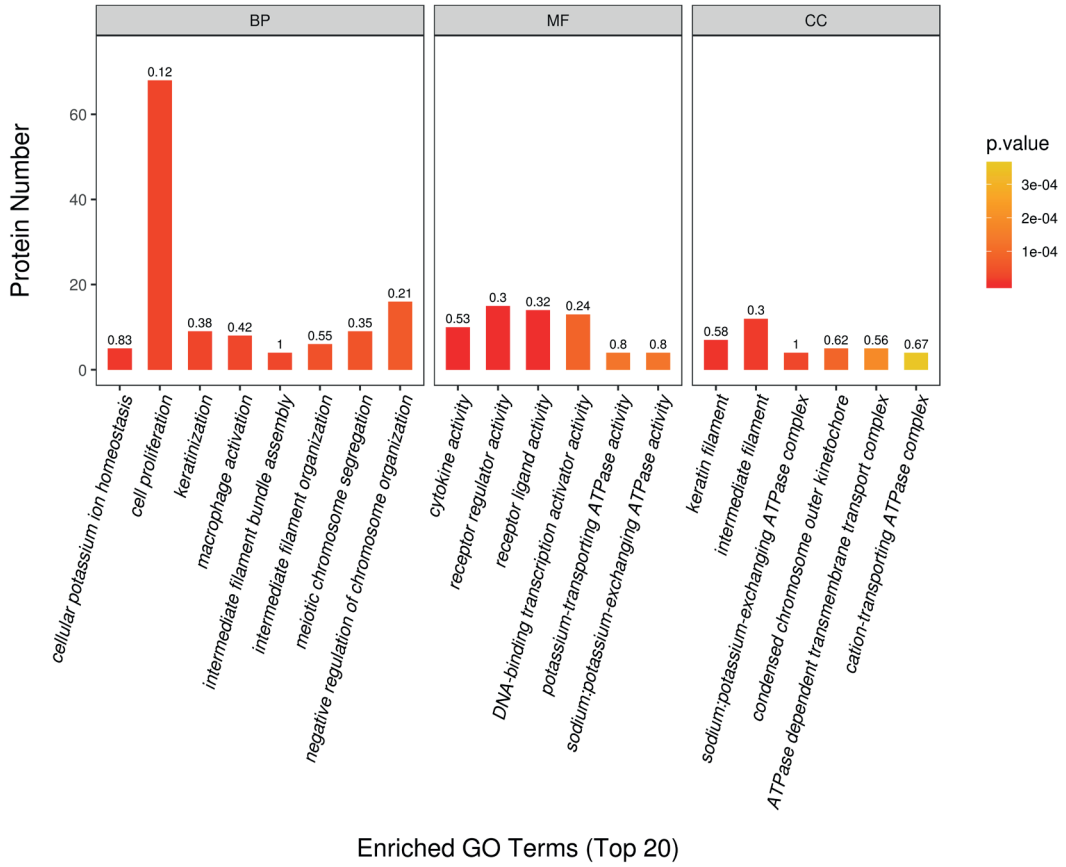

Fig. 6. The Gene Ontology (GO) functional enrichment analysis of the differentially expressed proteins. The x-axis represents the enriched GO functional classification, divided into biological process (BP), molecular function (MF) and cellular component (CC). The ordinate indicates the number of differentially expressed proteins. The color of the bar chart indicates the significance of the enriched GO functional classification. The label at the top of the bar chart shows the enrichment factor (richFactor ≤1), which refers to the ratio of the differentially expressed protein relative to all identified proteins

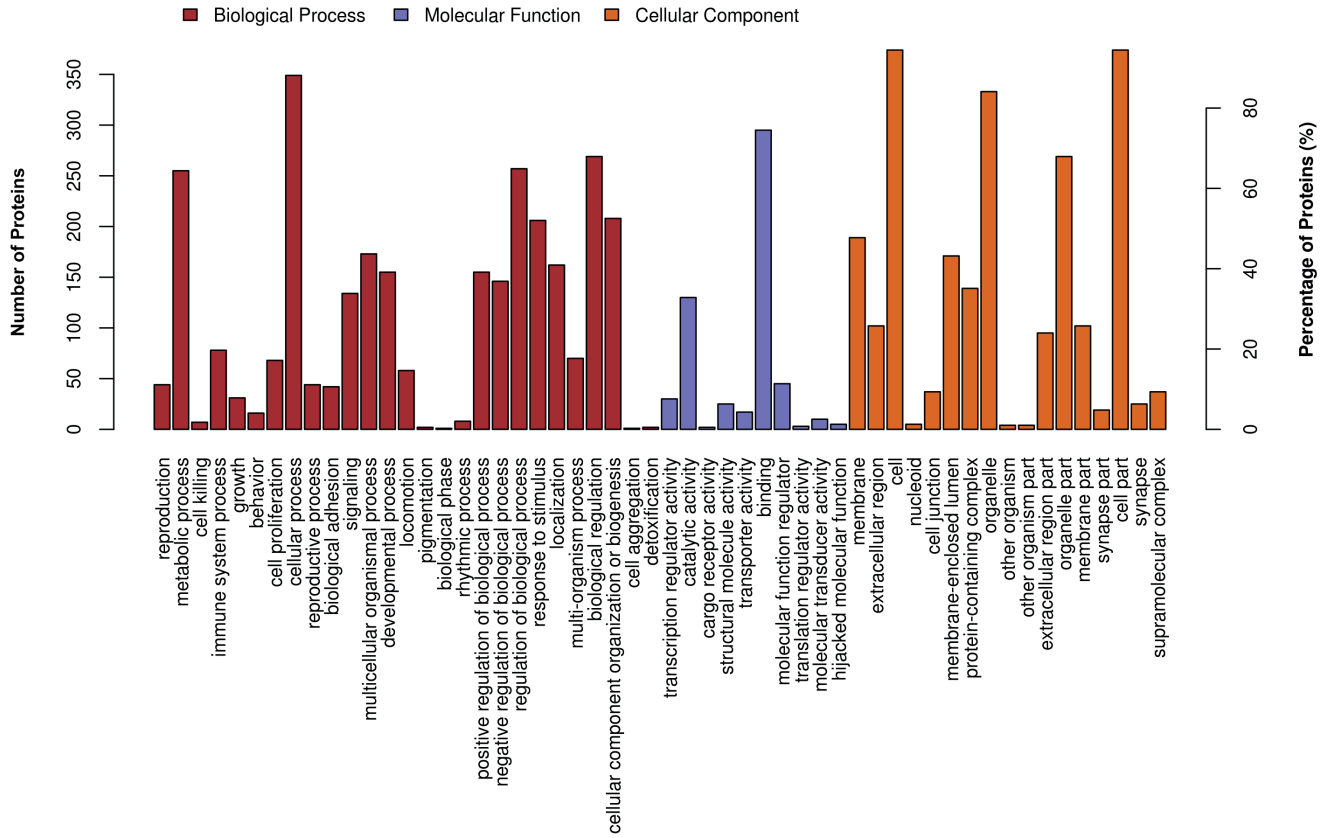

Fig. 7. The Gene Ontology (GO) analysis of 369 differentially expressed proteins for functional classification. Red, blue and orange bars represent biological processes, molecular functions and cellular components, respectively, ranking terms in the same category according to p-values. The ordinates on the left and right represent the number of differentially expressed proteins and their percentage within the differentially expressed proteins

KEGG analysis

To identify the biological pathways related to differentially expressed proteins, KEGG was conducted. The proteins were found to be involved in 261 KEGG pathways. The top 20 significant pathways are: protein digestion and absorption, transcriptional misregulation in cancer, cardiac muscle contraction, bile secretion, malaria, proximal tubule bicarbonate reclamation, aldosterone-regulated sodium reabsorption, gastric acid secretion, mineral absorption, cytokine–cytokine receptor interaction, insulin secretion, Fanconi anemia pathway, carbohydrate digestion and absorption, viral myocarditis, p53 signaling pathway, *Staphylococcus aureus* infection, estrogen signaling pathway, PPAR signaling pathway, primary bile acid biosynthesis, and bladder cancer (Fig. 8).

Differentially expressed proteins validated with PRM

In order to further confirm the iTRAQ results, we selected 6 differentially expressed proteins for quantitative PRM analysis. The PRM results (Table 4) exhibited the same trends as the data detected using iTRAQ, indicating that the originally obtained data are reliable.

Discussion

The crude extract or the active components of CP, such as periplocin and PPG, have been investigated for hindering the growth of cancers, including pancreatic

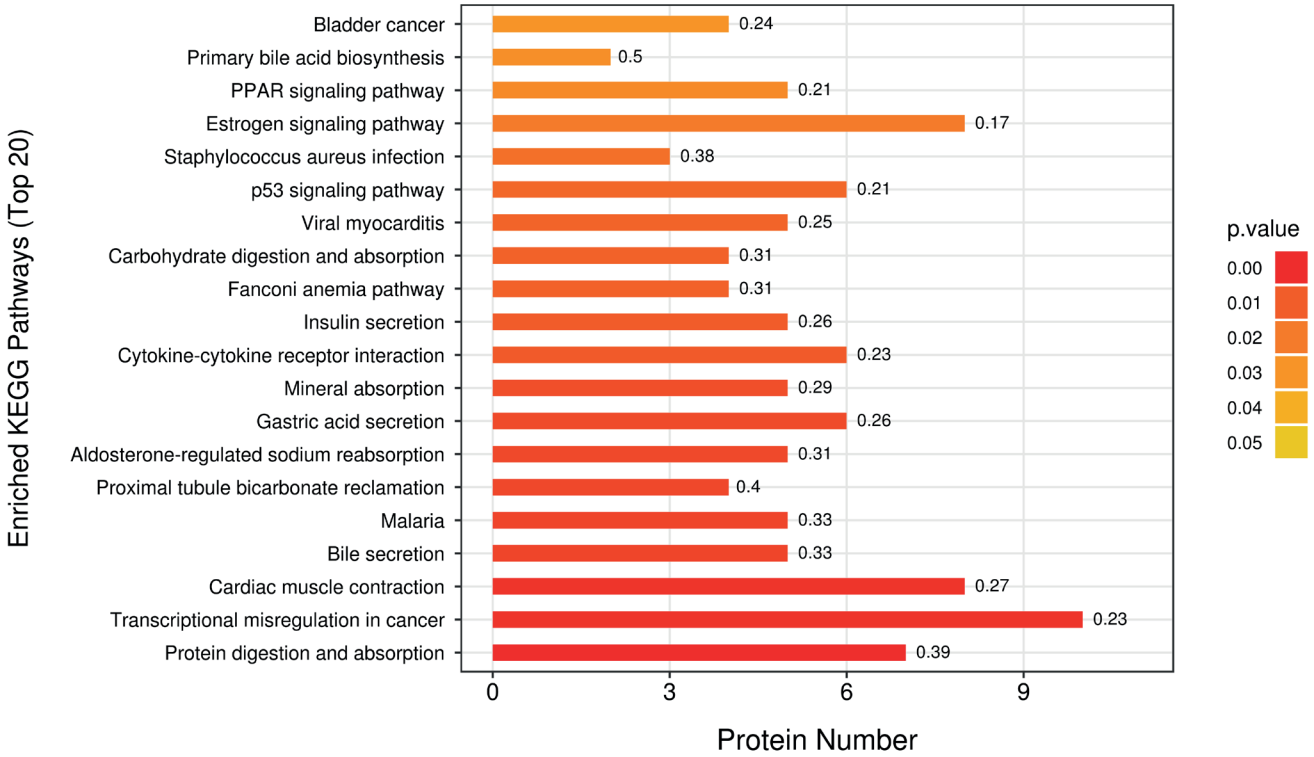

Fig. 8. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the 369 differentially expressed proteins. The KEGG pathways with significant enrichment are represented by the ordinate. The horizontal axis shows the number of differentially expressed proteins included in each KEGG pathway. The color of the bar chart indicates the significance of the enriched KEGG pathway. The label at the top of the bar chart shows the enrichment factor (richFactor ≤1), which refers to the ratio of the differentially expressed proteins relative to all identified proteins

Table 4. Quantitative results for 14 candidate proteins determined using the parallel reaction monitoring (PRM)

Protein name	Average_C	Average_T	Ratio_T/C	TTEST_T/C
P08238	43.06311	30.39073	0.705725419	0.006075415
P11388	1.6252	2.30856	1.420479392	0.087019915
P05023	6.85455	12.97942	1.893549629	0.142457108
Q15582	1.28649	3.26519	2.538055779	0.137229876
P05026	3.307	5.24357	1.585595508	0.004255904
P04818	2.09784	0.99814	0.475794078	0.000295652

Student's t-test was used to calculate the p-values of groups T and C. Average_C is the average of the 3 repetitive protein expression in group C. Average_T is the average of the 3 repetitive protein expression in group T. Ratio_T/C is the p-value of t-test.

cancer, gastric cancer, colon cancer, lung cancer, and nasopharyngeal cancer.[9] In this study, the ethanol extract of CP inhibited the proliferation of U251 and TG905 cells, and induced cell cycle arrest and apoptosis. To find the specific active components responsible for the antitumor pharmacological properties, the network pharmacology analysis was conducted. We found 16 active components, namely poriferast-5-en-3beta-ol, beta-sitosterol, sitosterol, 21-O-Methyl-5,14-pregndiene-3β,14β,17β,21-tetrol-20-one, 21-O-Methyl-5-pregnene-3β,14β,17β,20,21-pentol, glycoside K_qt, glycoside H2_qt, glycozolidal, PPG, glycoside E_qt, periplocoside M_qt, delta 5-pregnenetriol, periplocoside O_qt, periplocymarin_qt, neridienone A, and xysmalogenin.

Several reports have illustrated that beta-sitosterol inhibits the proliferation of a range of cancer cell lines related to the activation of cell cycle arrest[31,32] and stimulation of cellular apoptosis,[33] which are consistent with our findings. Beta-sitosterol isolated from various plants accelerates apoptosis by stimulating the apoptosis pathway[34] and the PI3K-AKT pathway.[35] Wang et al. reported that beta-sitosterol reverses drug resistance in colorectal cancer via the p53 signaling pathway.[36] In our study, results from both integrated network pharmacology and iTRAQ-based quantitative proteomics technology demonstrated the activation of the p53 pathway, apoptosis pathway and PI3K-AKT signaling pathway in glioma cells after treatment with CP extract. Additionally, β-sitosterol plays an important role in treating prostate, lung, ovarian, colon, breast, and stomach cancer.[37]

Periplogenin was first isolated from the chloroform extract of CP in 1987, which clearly decreased the proliferation of ascite-associated cancer S_{180} cells.[38] Li et al. reported that PPG strongly inhibited the proliferation of A2780, BGC823, PC3, Bel-7402, U937, A549, and HCT-8 cell lines in vitro with 0.66–3.16 uM IC50 values.[39] Currently, researchers have suggested that PPG may activate the ROS-ER stress pathway to stimulate apoptosis in colon cancer.[17] In nasopharyngeal cancer, PPG is related to the triggering of the PI3K-AKT signaling pathway.[18] The results reported by other researchers are consistent with our findings based on the network pharmacology and iTRAQ-based quantitative proteomics.

In addition to the abovementioned pathways, our findings showed that the Kaposi sarcoma-associated herpes virus infection pathway, the calcium signaling pathway, the transcriptional misregulation in cancer pathway, and others are involved in the CP-induced glioma treatment. The iTRAQ-based quantitative proteomics analysis also showed that there were 6 important differentially expressed proteins (HSP90AB1, TOP2A, ATP1A1, TGFβ1, ATP1B1, and TYMS) found in CP-treated U251 cells compared with the control group. The specific functions of these proteins should be investigated further in future studies.

Limitations

There are several limitations to this study. First, it is not clear which component of CP plays the anti-glioma role. Second, we only performed an in vitro cell study; further in vivo animal studies are needed to confirm this finding.

Conclusions

In this study, we revealed that the extract of CP inhibited the proliferation of U251 and TG905 cells, and induced cell cycle arrest and apoptosis. We also found 16 active compounds of CP. Additionally, 6 proteins (HSP90AB1, TOP2A, ATP1A1, TGFβ1, ATP1B1, and TYMS) were identified as the key factors involved in the regulation of CP in glioma. This study sheds light on the underlying molecular mechanisms mediated by CP effects in glioma.

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Ginsenoside Rb3 reduces ox-LDL-induced injury in human aortic endothelial cells by regulating the miR-513a-5p/ZBTB20 axis

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Abstract

Background. Atherosclerosis (AS) is a common vascular disease, and its main influencing factor is endothelial damage caused by oxidized low-density lipoprotein (ox-LDL). As one of the main active ingredients of ginseng, ginsenoside Rb3 has anti-inflammatory and anti-oxidative effects. However, the role of ginsenoside Rb3 in endothelial injury induced by ox-LDL is not clear.

Objectives. This study aimed to evaluate the effect and potential mechanism of ginsenoside Rb3 action on ox-LDL-treated human aortic endothelial cells (HAECs).

Materials and methods. The HAECs treated with ox-LDL were used to establish an in vitro AS model. The viability of the HAECs was analyzed with Cell Counting Kit-8 (CCK-8). Flow cytometry was performed to assess the apoptosis. Oxidative stress, inflammation and endothelial dysfunction were evaluated using enzyme-linked immunosorbent assay (ELISA) and western blotting. The levels of miR-513a-5p were assessed using quantitative real-time polymerase chain reaction (qPCR). A dual-luciferase assay was performed to analyze the relationship between miR-513a-5p and a zinc finger and BTB domain-containing protein (ZBTB20).

Results. Exposure of HAECs to ox-LDL (50 μg/mL) reduced cell viability, superoxide dismutase (SOD) activity and endothelial nitric oxide synthase (eNOS) expression, while increasing the levels of malondialdehyde (MDA), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and soluble intercellular adhesion molecule-1 (sICAM-1). The pretreatment with Rb3 markedly enhanced cell viability and decreased ox-LDL-induced oxidative stress, inflammation and endothelial dysfunction in HAECs. The ox-LDL decreased the level of miR-513a-5p, which was reversed by Rb3 pretreatment. The ZBTB20 was a target of miR-513a-5p in HAECs, and ox-LDL upregulated ZBTB20 expression, which was reversed by Rb3 pretreatment. The protective effect of Rb3 on ox-LDL-induced HAECs was diminished by miR-513a-5p inhibition, which was reversed by ZBTB20 knockdown.

Conclusions. Ginsenoside Rb3 reduces the effects of ox-LDL on HAECs by regulating the miR-513a-5p/ZBTB20 axis, which provides a theoretical basis for the treatment of AS.

Key words: atherosclerosis, oxidized low-density lipoprotein, miR-513a-5p, ZBTB20

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Background

Atherosclerosis (AS) is a condition characterized by a lipid metabolic imbalance and persistent inflammation.[1] It is a major cause of cardiovascular disease (CVD), which is the greatest cause of death worldwide.[2] Atherosclerosis lays down the groundwork for future strokes and heart attacks.[3] Aberrant human aortic endothelial cell (HAEC) injuries are regarded as one of the pathological characteristics in the progression of AS.[4] According to research, oxidized low-density lipoprotein (ox-LDL)-induced proliferation, migration and apoptosis of HAECs are linked to the progression of AS.[5,6]

Ginsenosides Rb1, Rd, F1, Rg1–Rg3, compound K, and total ginsenosides (TG) have all been shown to be anti-AS.[7] For example, Rb2 via the GPR120/AMPK/HO-1 pathway suppressed human umbilical vein endothelial cell (HUVEC) apoptosis induced by lipopolysaccharide (LPS) and inhibited THP-1 cell adhesion to HUVEC, resulting in a reduction in inflammation and endoplasmic reticulum (ER) stress.[8] Ginsenoside Rb3 (PubChem CID: 12912363) is one of ginseng's most common active components, and has shown anti-inflammatory and anti-oxidative properties.[9] The Rb3 strongly suppresses angiotensin II (Ang II)-induced vascular smooth muscle cell (VSMC) proliferation by arresting the cell cycle,[10] implying that it may play a positive function in CVD. However, the role of Rb3 in preventing AS is still unclear.

Non-coding RNA (ncRNA) has increasingly been recognized for its role in a range of illnesses, including CVD.[11] It has been established that ncRNA plays important regulatory roles in vascular inflammation and smooth muscle cells, thus constituting a novel treatment for AS.[12] MicroRNA (miRNA) is reported to inhibit gene expression resulting in mRNA degradation or translation inhibition.[13] Existing research indicates that miRNA is directly associated with inflammation, oxidative stress and apoptosis, which suggests its involvement in the underlying mechanism of AS development.[14] Interestingly, Rb1 and Rb2 have shown anti-aging and anti-inflammatory properties through the modulation of miRNA expression.[15] Based on these findings, ox-LDL-induced HAECs were used to construct an in vitro AS model in order to investigate the regulation of miRNA by Rb3 in AS.

Objectives

This study aimed to evaluate the effect and potential mechanism of ginsenoside Rb3 action on ox-LDL-treated HAECs.

Materials and methods

Cell treatment

The HAECs (Cat. No. 6100 from 3 individuals; ScienCell Research Laboratories, Carlsbad, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Waltham, USA) at 37°C in a 5% CO_2 humidified incubator with penicillin and streptomycin (Sigma-Aldrich, St. Louis, USA), at doses of 100 U/mL and 100 mg/mL, respectively. The HAECs were then cultured in a medium containing various concentrations (0–100 μg/mL) of ox-LDL (Biosynthesis Biotechnology Company, Beijing, China) for 24 h, or 50 μg/mL of ox-LDL for 0 h, 12 h, 24 h, or 48 h to establish the in vitro AS model. For Rb3 treatment, HAECs were treated with various concentrations (0–80 μM) of Rb3 (Cat. No. A0236; ≥98%; Must Biotechnology, Chengdu, China) for 24 h. For the combined treatment, HAECs were treated with Rb3 (20 μM or 40 μM) for 24 h, and then Rb3-treated HAECs were treated with ox-LDL (50 μg/mL) for 24 h.

Cell viability

Following the appropriate treatment, Cell Counting Kit-8 (CCK-8) reagent (10 μL) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to the HAECs (5×10^4 cells/well) in a 96-well plate for 1 h, followed by the measurement of cell viability with the use of a LB960 microplate reader (450 nm; Titertek-Berthold, Pforzheim, Germany).

Cell apoptosis

Flow cytometry was used to determine the apoptosis rate after HAECs (4×10^5 cells/well in 6-well plates) were stained with fluorescein-5-isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI; Keygen, Nanjing, China) for 10 min in the dark at room temperature.

Enzyme-linked immunosorbent assay

To measure the content of malondialdehyde (MDA), superoxide dismutase (SOD), soluble intercellular adhesion molecule-1 (sICAM-1), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) in the culture media, enzyme-linked immunosorbent assay (ELISA) kits (Beyotime, Beijing, China) were used according to the manufacturer's protocol, followed by measuring the absorbance at 450 nm with the use of a LB960 microplate reader (Titertek-Berthold).

Western blot analysis

After lysing HAECs, the protein content was determined using a bicinchoninic acid (BCA) assay, and then the cells

were transferred to a polyvinylidene difluoride (PVDF) membrane and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Prior to detection using Femto enhanced chemiluminescence substrates (Thermo Fisher Scientific, Waltham, USA) and a ChemiDoc MP Imaging System (Bio-Rad, Hercules, USA), the membranes were blocked with 5% non-fat milk at room temperature for 1 h, followed by the addition of anti-endothelial nitric oxide synthase (anti-eNOS) and anti-zinc finger and BTB domain-containing protein (anti-ZBTB20) antibodies, as well as an alkaline phosphatase-conjugated secondary antibody.

Microarray data and differential expression analysis

The microarray expression profiling analysis GSE137580 (platform GPL24741; https://www.ncbi.nlm. nih.gov/geo/query/acc.cgi?acc=GSE137580) was downloaded from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/). The GEO annotation file for GPL21265 was obtained. Using the online analysis program GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r), the expression profiles of ox-LDL-treated HAECs (n = 3) and untreated HAECs (n = 3) were compared to identify differentially expressed miRNAs using the following criteria: $|\log_2 FC \text{ (fold change)}| > 1$ and $p < 0.05$.

Quantitative real-time polymerase chain reaction

Using the TRIzol reagent purchased from Invitrogen (Waltham, USA), RNA was extracted from the HAECs, and a RT First Strand Kit was subsequently used to synthesize cDNA, which was utilized as a template for quantitative real-time polymerase chain reaction (qPCR) amplification to examine miRNA expression using miRNA-specific primers (Ribobio, Guangzhou, China). Moreover, after using PrimeScriptTM RT Master Mix for mRNA expression of ZBTB20, the qPCR was carried out with a SYBR Green qPCR mix (Yeasen Biotechnology Co., Ltd., Shanghai, China) on an ABI 7500 system (Applied Biosystems, Foster City, USA). The miRNA expression was standardized to U6 while standardizing mRNAs to GAPDH. The $2^{-\Delta\Delta Ct}$ technique was used to examine the data.

Transfections

RiboBio developed and manufactured the miR-513a-5p mimics/inhibitors/negative control (NC), and Genepharma (Shanghai, China) provided small interfering RNAs (siRNAs) specific for ZBTB20 (si-ZBTB20, 5′- GAC TAG TTA AAT GGC GGA AGA TAA A-3′) and non-specific siRNA (si-NC) for the NC. Lipofectamine 2000 (Invitrogen) was used to transfect the HAECs.

Dual-luciferase assay

After using a QuikChange Kit (Qiagen, Hilden, Germany) to perform site-directed mutagenesis of the ZBTB20 3'-UTR within the probable miR-513-5p binding region, the ZBTB20-wt or ZBTB20-mut was amplified and cloned into a pMIR-REPORT luciferase vector (Ambion, Austin, USA). The HAECs were seeded in 24-well plates (2×105 cells per well) and co-transfected with ZBTB20-wt/ZBTB20-mut and miR-513-5p mimic/NC. The relative firefly luciferase activity was measured 48 h after transfection.

Statistical analyses

IBM SPSS v. 22.0 software (IBM Corp., Armonk, USA) was used to examine the data, which were expressed as mean and 95% confidence intervals (95% CIs). Each experiment was performed on 3 samples for each group, and each experiment was repeated in triplicate for every sample. To determine statistical significance, Student's t-test was used to compare the 2 groups. For the comparison of 3 or more groups, a bootstrap analysis of variance (ANOVA) with a bootstrap post hoc test was utilized. The distribution of data was analyzed using the Shapiro–Wilk test. Additionally, '0' in Fig. 1 represents the drug concentration of 0, i.e., untreated conventional HAECs. The heatmap was generated using the R package ComplexHeatmap (R Foundation for Statistical Computing, Vienna, Austria). The Venn diagram was generated using the R package Venn. A threshold p-value lower than 0.05 indicated a significant difference.

Results

Effects of ox-LDL and Rb3 treatment on HAECs

The ox-LDL is a major risk factor for AS, causing endothelial cell death and promoting the onset and progression of the disease.[16] The viability of HAECs was lowered in a dose- and time-dependent manner after exposure to ox-LDL (Fig. 1A,B), and the subsequent experiment used 50 μg/mL of ox-LDL to stimulate HAECs for 24 h in order to establish the in vitro AS model. Moreover, we found little effect of Rb3 on HAEC viability at different concentrations (0–80 μM) for 24 h (Fig. 1C,D). However, the effects of ox-LDL on HAEC activity and apoptosis were restored by Rb3 (20 μM or 40 μM, 2 h; Fig. 1E,F).

Rb3 attenuated the ox-LDL-induced injury of HAECs

Subsequently, ox-LDL-induced oxidative stress and inflammation in HAECs were found, with the increased production of MDA, the enhanced level of pro-inflammatory

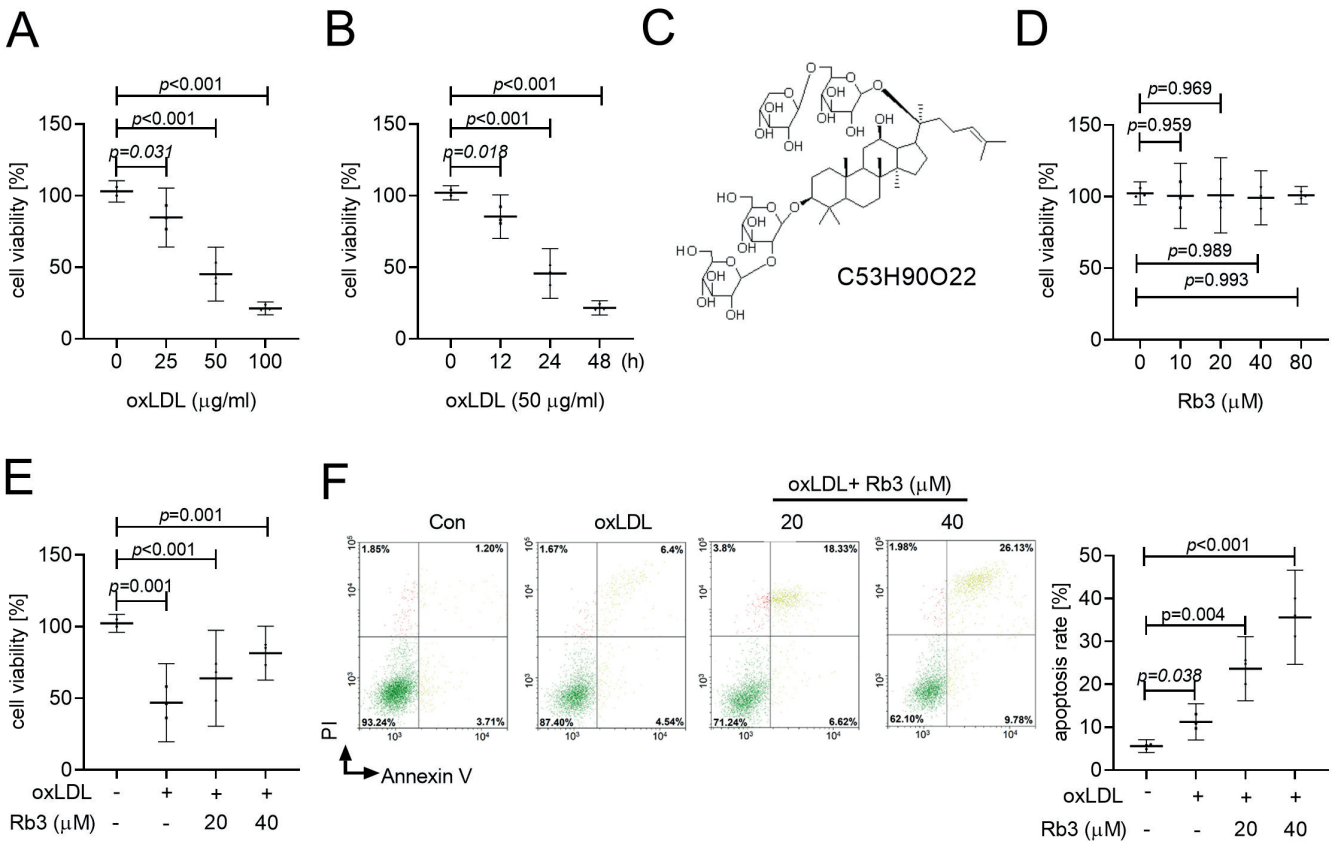

Fig. 1. The cell viability and apoptosis of human aortic endothelial cells (HAECs) after treatment with oxidized low-density lipoprotein (ox-LDL) or Rb3. A. HAECs were treated with 0–100 μg/mL of ox-LDL for 24 h, and their viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. One-way analysis of variance (ANOVA) with Bonferroni post hoc multiple analysis was utilized; B. HAECs were treated with 50 μg/mL of ox-LDL for 0 h, 12 h, 24 h, or 28 h, and their viability was assessed using the CCK-8 assay. One-way ANOVA with Bonferroni post hoc multiple analysis was utilized; C. The chemical structure of Rb3; D. HAEC viability after treatment with 0–80 μM of Rb3 for 24 h; one-way ANOVA with Bonferroni post hoc multiple analysis was utilized; E. HAECs were pretreated with 20 μM or 40 μM of Rb3 for 24 h before being exposed to 50 μg/mL of ox-LDL for 24 h, and one-way ANOVA with Bonferroni post hoc multiple analysis was utilized; F. Apoptosis of HAECs was analyzed using flow cytometry, and one-way ANOVA with Bonferroni post hoc multiple analysis was utilized. Data were expressed as means and 95% confidence intervals (95% CIs)

cytokines and the decreased activity of SOD, while the activity of SOD was attenuated by Rb3 pretreatment (Fig. 2A–D). Additionally, ox-LDL increased the secretion of sICAM-1 and downregulated the expression of eNOS (Fig. 2E,F). The Rb3 pretreatment alleviated to a certain extent the endothelial dysfunction of HAECs caused by ox-LDL (Fig. 2E,F).

Rb3 upregulates miR-513a-5p in ox-LDL-treated HAECs

We used the online analysis program GEO2R to evaluate the differentially expressed miRNAs between ox-LDL-treated HAECs and untreated HAECs from the GEO database. In total, 7 downregulated miRNAs were discovered, including miR-7110-5p, miR-1202, miR-6749-5p, miR-154-3p, miR-513a-5p, miR-6068, and miR-4697-5p (Fig. 3A). Moreover, ox-LDL-treated HAECs showed a significantly reduced expression of miR-154-3p, miR-513a-5p and miR-7110-5p in our experiments (Fig. 3B). However, miR-513a-5p was markedly upregulated in ox-LDL-treated HAECs after Rb3 pretreatment (Fig. 3C). Moreover, miR-513a-5p inhibitor (inhib-miRNA) transfection prevented Rb3-induced elevation of miR-513a-5p in ox-LDL-treated HAECs (Fig. 3D–E).

ZBTB20 as a target gene was regulated by miR-513a-5p in HAECs

Bioinformatics analysis conducted using miRDB (https://mirdb.org/), TargetScan (https://www.targetscan.org/vert_72/) and miRTarBase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php) (Fig. 4A), as well as the dual-luciferase reporter assay (Fig. 4B) confirmed that miR-513a-5p binds to the 3'UTR of ZBTB20 mRNA, and its mimics successfully upregulate the levels of miR-513a-5p in HAECs (Fig. 4C) while decreasing the expression of ZBTB20 mRNA and proteins. The transfection with an inhibitor had the opposite effects (Fig. 4D,E). Moreover, Rb3 treatment reduced ZBTB20 expression in ox-LDL-treated HAECs (Fig. 4F,G). Additionally, the upregulation of ZBTB20 expression in ox-LDL-treated

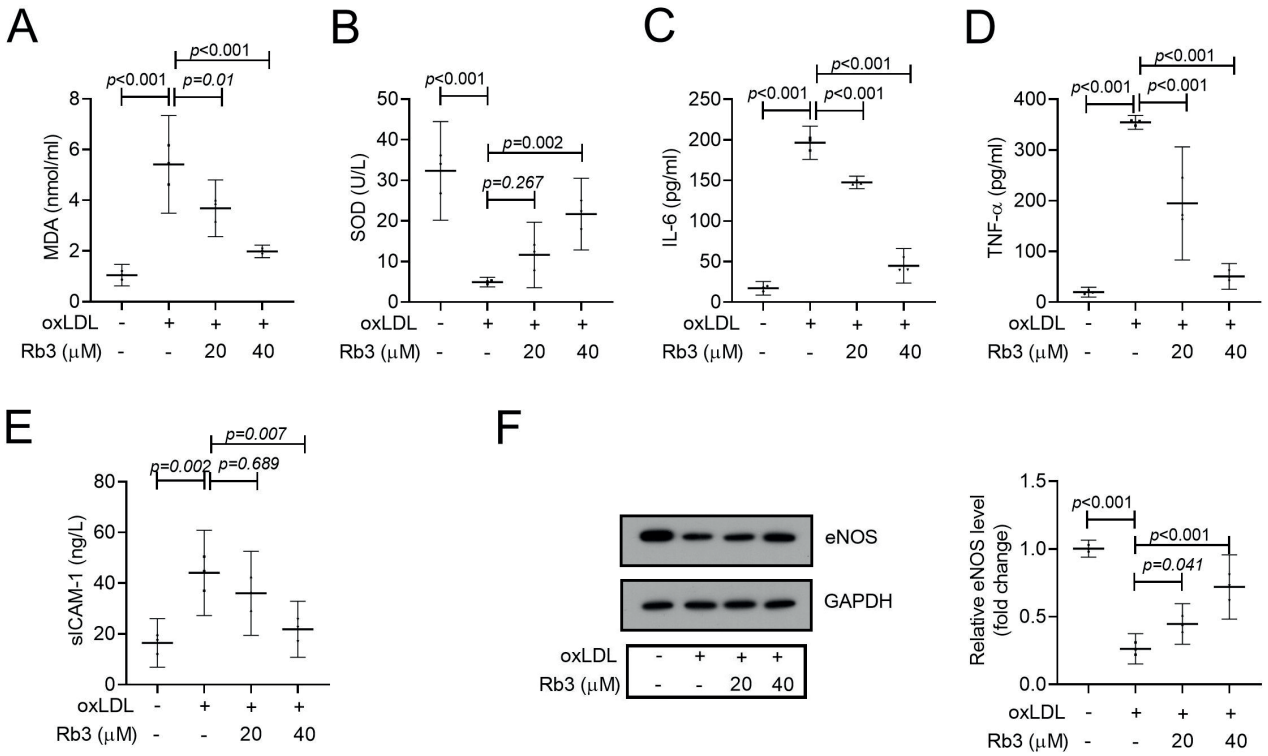

Fig. 2. Rb3 reduces human aortic endothelial cell (HAEC) injury caused by oxidized low-density lipoprotein (ox-LDL). A. The levels of malondialdehyde (MDA) were measured using enzyme-linked immunosorbent assay (ELISA); B. The levels of superoxide dismutase (SOD) were measured using ELISA; C. The levels of interleukin 6 (IL-6) were measured using ELISA; D. The levels of tumor necrosis factor alpha (TNF-α) were measured using ELISA; E. The levels of soluble intercellular adhesion molecule-1 (sICAM-1) were measured using ELISA; One-way analysis of variance (ANOVA) with Bonferroni post hoc multiple analysis was utilized; F. The expression of endothelial nitric oxide synthase (eNOS) in culture medium were determined using western blot and one-way ANOVA with Bonferroni post hoc multiple analysis was utilized. Data were expressed as means and 95% confdence intervals (95% CIs)

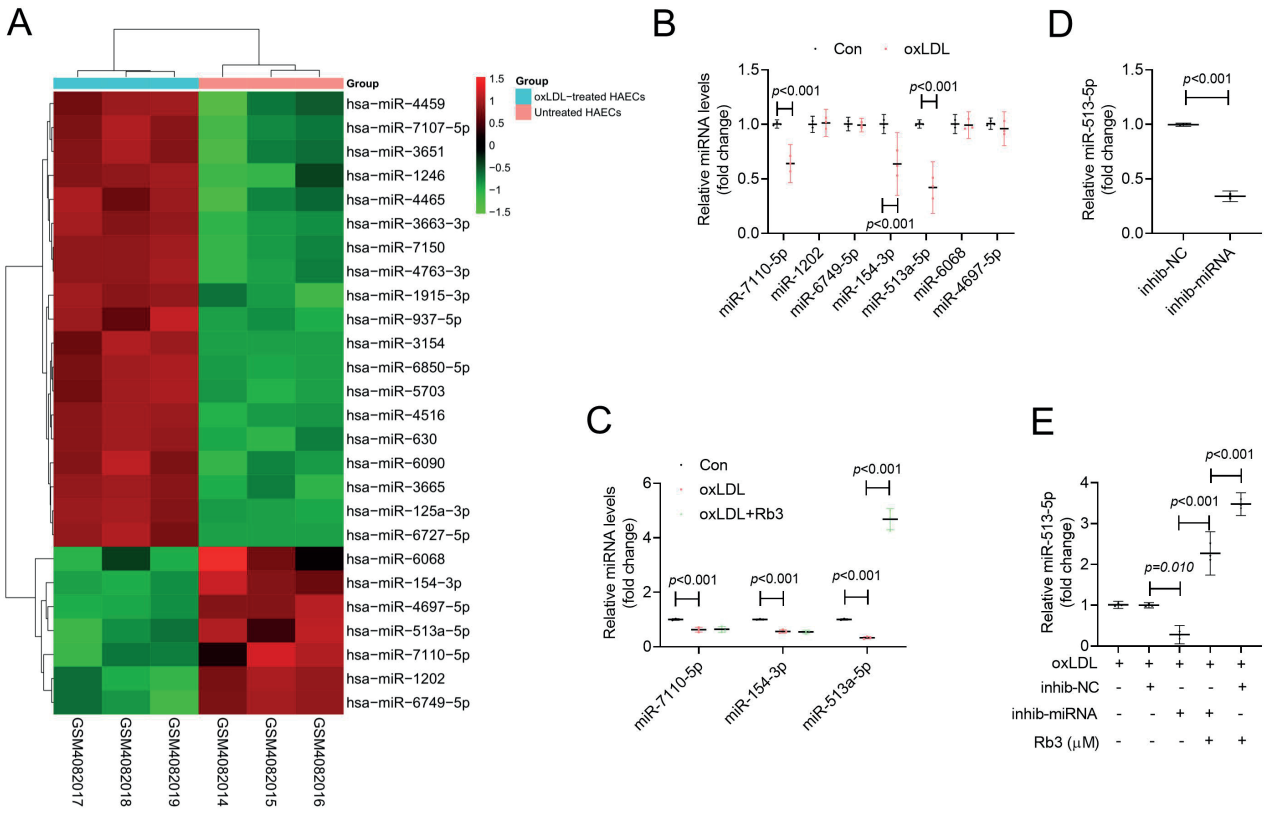

Fig. 3. Rb3 upregulated miR-513a-5p in human aortic endothelial cells (HAECs) stimulated by oxidized low-density lipoprotein (ox-LDL). A. A heatmap of 27 differentially expressed miRNAs in HAECs with or without stimulation by ox-LDL; B. The expression of downregulated miRNAs in HAECs was caused by ox-LDL, and Student's t-test was utilized; C. miRNA expression in HAECs treated with Rb3 and ox-LDL, and one-way analysis of variance (ANOVA) with Bonferroni post hoc multiple analysis was utilized; D. miR-513a-5p expression in HAECs transfected with miR-513a-5p inhibitor (inhib-miRNA) and negative control (inhib-NC), and Student's t-test was utilized; E. HAECs were transfected with an inhibitor of miR-513a-5p for 24 h; then, they were pretreated with Rb3 for 24 h before being exposed to ox-LDL for 24 h. The expression of miR-513a-5p was measured using quantitative real-time polymerase chain reaction (qPCR), and one-way ANOVA with Bonferroni post hoc multiple analysis was utilized. Data were expressed as means and 95% confidence intervals (95% CIs)

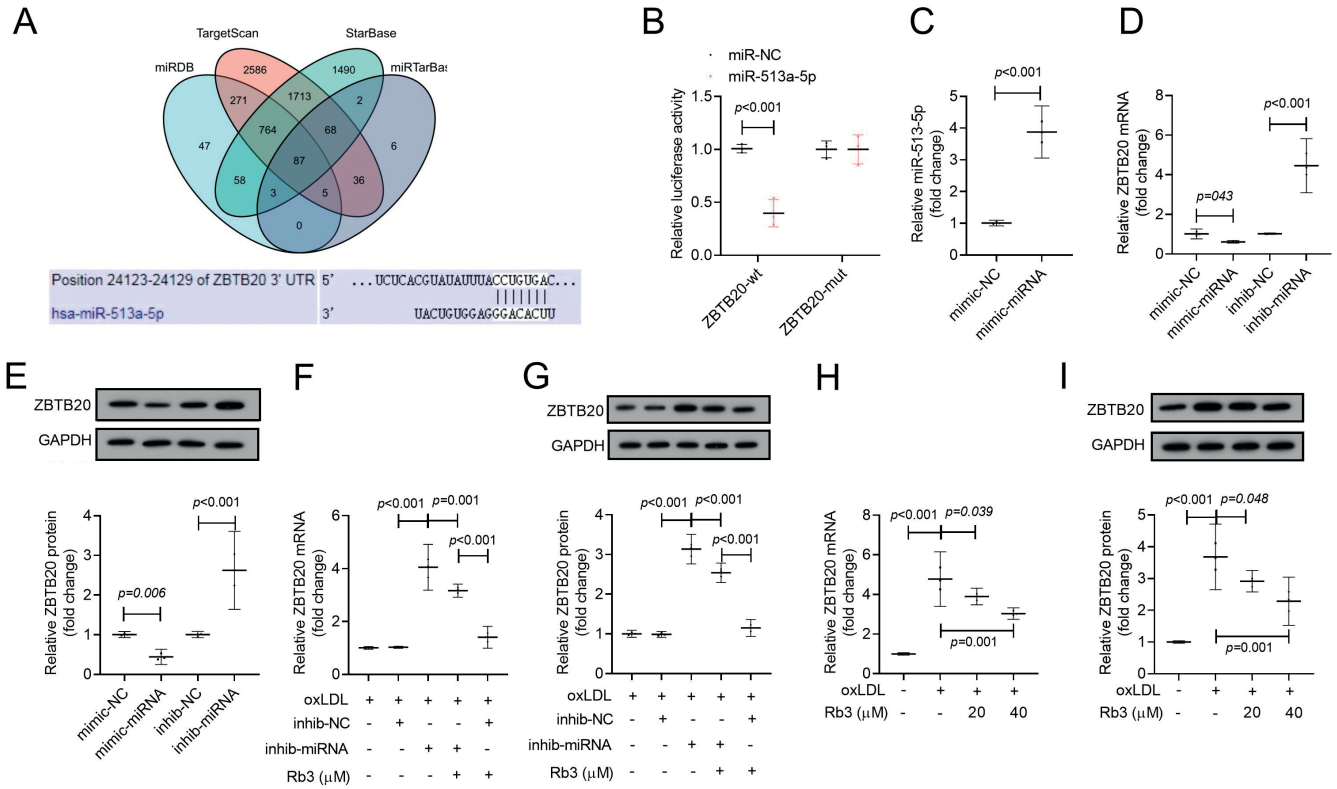

Fig. 4. *ZBTB20* as a target gene was regulated by miR-513a-5p in human aortic endothelial cells (HAECs). A. Binding sites for ZBTB20 and miR-513a-5p predicted using miRDB (https://mirdb.org/), TargetScan (https://www.targetscan.org/vert_72/) and miRTarBase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php); B. The assessment of luciferase activity in HAECs transfected with ZBTB20-wt/ZBTB20-mut and miR-513-5p mimic/negative control (NC); Student's t-test was utilized; C. miR-513a-5p expression in HAECs transfected with miR-513a-5p mimics; Student's t-test was utilized; D,E. ZBTB20 mRNA and protein expression in HAECs treated with miR-513a-5p mimics and inhibitors; Student's t-test was utilized; F,G. HAECs were transfected with miR-513a-5p inhibitor for 24 h, and then pretreated with Rb3 for 24 h, followed by exposure to ox-LDL for 24 h; the expression of ZBTB20 mRNA and protein were analyzed using quantitative real-time polymerase chain reaction (qPCR) and western blot assay. One-way analysis of variance (ANOVA) with Bonferroni post hoc multiple analysis were utilized; H,I. HAECs were pretreated with 20 μM or 40 μM of Rb3 for 24 h, and then exposed to 50 μg/mL of oxidized low-density lipoprotein (ox-LDL) for 24 h; the expression of ZBTB20 mRNA and protein were analyzed using qPCR and western blot assay, and one-way ANOVA with Bonferroni post hoc multiple analysis were utilized. Data were expressed as means and 95% confidence intervals (95% CIs)

HAECs enhanced by the miR-513a-5p inhibitor was abolished after Rb3 treatment (Fig. 4F,G). Furthermore, ox-LDL upregulated the expression of ZBTB20 in HAECs, which was reversed by Rb3 treatment (Fig. 4H,I).

The effect of Rb3 on endothelial dysfunction of HAECs induced by ox-LDL is mediated by miR-513a-5p/ZBTB20 axis

As shown in Fig. 5A, ZBTB20 siRNA transfection markedly downregulated the expression of ZBTB20 in HAECs. The inhibition of miR-513a-5p eliminated the anti-oxidative, anti-inflammatory and anti-endothelial dysfunction effects of Rb3 pretreatment in ox-LDL-treated HAECs, while the effects of miR-513a-5p overexpression were reversed by ZBTB20 siRNA (Fig. 5B–G). Collectively, Rb3 reduced oxidative stress, inflammation and endothelial damage caused by ox-LDL through the miR-513a-5p/ZBTB20 axis.

Discussion

Endothelial dysfunction and apoptosis are thought to be the initial elements in the development of AS, contributing to the pathophysiology of the disease.[3] Inhibiting ox-LDL-induced endothelial dysfunction and endothelial cell death is thought to be a possible therapeutic treatment for AS.[17] The management of the inflammatory response is critical for the treatment of AS, and inhibiting pro-inflammatory cytokines is a promising technique for preventing AS.[18] Except for the inflammatory response, which has an impact on lipid metabolism in AS and vice versa,[19] endothelial dysfunction also appears to be a frequent hallmark of AS, and the endothelial dysfunction could be induced by ox-LDL via the acceleration of oxidative stress.[20] A previous study reported that tofacitinib has shown vascular protective properties in HAECs by inhibiting ox-LDL-induced inflammation and oxidative stress.[21] In addition, saxagliptin-mediated endothelial protection is associated with the inhibition

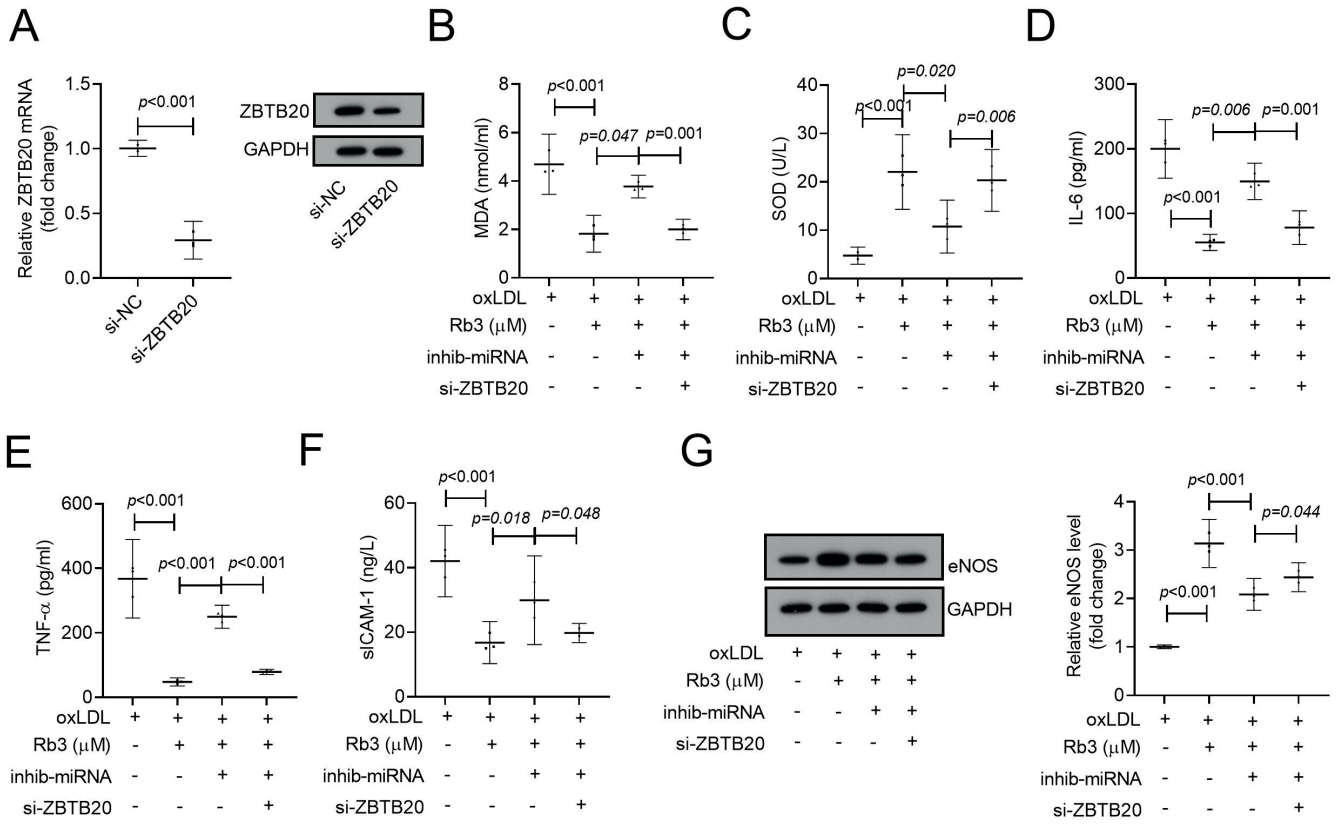

Fig. 5. The effect of Rb3 on oxidized low-density lipoprotein (ox-LDL)-caused human aortic endothelial cell (HAEC) injury is mediated by the miR-513a-5p/ZBTB20 axis. A. ZBTB20 expression in HAECs treated with ZBTB20 siRNA; Student's t-test was utilized; B. The levels of malondialdehyde (MDA) were measured using enzyme-linked immunosorbent assay (ELISA); C. The levels of superoxide dismutase (SOD) were measured using ELISA; D. The levels of interleukin 6 (IL-6) were measured using ELISA; E. The levels of tumor necrosis factor alpha (TNF-α) were measured using ELISA; F. The levels of soluble intercellular adhesion molecule-1 (sICAM-1) were measured using ELISA; one-way analysis of variance (ANOVA) with Bonferroni post hoc multiple analysis was utilized; G. The expression of endothelial nitric oxide synthase (eNOS) in culture medium were determined using western blot w, and one-way ANOVA with Bonferroni post hoc multiple analysis were utilized

Data were expressed as means and 95% confidence intervals (95% CIs).

of the inflammatory response induced by ox-LDL.[22] In the current investigation, we discovered that Rb3 pretreatment reduced MDA formation and SOD caused by ox-LDL, as well as inhibited the release of IL-6 and TNF-α, indicating that Rb3 had anti-inflammatory and anti-oxidative effects in cellular models of AS. Furthermore, we discovered that Rb3 boosted the viability and eNOS expression of ox-LDL-treated HAECs while inhibiting the generation of sICAM-1, indicating that Rb3 prevented endothelial dysfunction in cellular models of AS. Our results revealed an anti-AS effect of Rb3 in vitro.

The significance of miRNA in vascular disorders has recently gained a lot of interest.[23] The miRNA is associated with various pathophysiological processes, including growth, proliferation, apoptosis, and autophagy. Recent research has found that aberrant miRNA expression occurs at various phases of AS.[24] Increased plasma levels of miR-216a were found in elderly CVD patients, and were responsible for triggering endothelial cell inflammation via the Smad3 pathway.[25] In addition, miR-513a-5p was downregulated in human aortic VSMCs stimulated by IκBα,[26] as well as HAECs treated with ox-LDL, while the expression of miR-513a-5p could be increased with Rb3 treatment. Furthermore, it has been revealed that miR-513a-5p plays a role in TNF-α/endotoxin-induced apoptosis by suppressing the production of endothelial cell apoptosis protein X-linked inhibitors,[27] thus being a vital miRNA in regulating cell damage caused by an inflammatory response. In this study, we not only found that Rb3 treatment increased the expression of miR-513a-5p, which was inhibited by ox-LDL stimulation, but also determined that the beneficial effects of Rb3 were eliminated by miR-513a-5p inhibitors. This indicates that miR-513a-5p had a novel role in regulating Rb3-mediated anti-oxidative, anti-inflammatory and anti-endothelial damage in AS.

The ZBTB20, as a member of the large complex tramtrack bric-a-brac (BTB)/poxvirus and zinc finger (POZ) family,[28] has the ability to suppress *IκBα* gene transcription while also promoting nuclear factor-κB (NF-κB) activation.[29] In addition, ZBTB20 levels are elevated in human AS lesions and ox-LDL-stimulated macrophages. The ZBTB20 knockdown has been shown to decrease the oxidative stress level and inflammatory response of ox-LDL-stimulated macrophages.[30] Therefore, we hypothesize that miR-513a-5p could play a role by inhibiting ZBTB20. Our results showed that miR-513a-5p targeted

ZBTB20 in ox-LDL-treated HAECs. Additionally, the enhanced ZBTB20 expression caused by ox-LDL was blocked by Rb3 treatment. Moreover, the inhibition of miR-513a-5p eliminated the anti-oxidative, anti-inflammatory and anti-endothelial dysfunction effects of Rb3 in ox-LDL-treated HAECs, while ZBTB20 knockdown could have enhanced the anti-oxidative, anti-inflammatory and anti-endothelial dysfunction effects. Therefore, Rb3 might exert its anti-AS effect through the miR-513a-5p/ZBTB20 axis.

Limitations

The intracellular signal transduction pathway is very complicated. In the present study, miR-513a-5p was selected as a candidate miRNA based only on the relevant literature. In future studies, we should select more miRNAs or miRNA expression profiles to explore the protective role of Rb3 in AS. Furthermore, the role of Rb3 in animal models and clinical research needs to be further investigated.

Conclusions

In conclusion, we proved that Rb3 reduced oxidative stress, inflammation and endothelial damage caused by ox-LDL through the miR-513a-5p/ZBTB20 axis. These results supported the beneficial role of Rb3 as an anti-AS agent.

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The impact of the COVID-19 pandemic on the number of brain tumor surgeries in Poland: A national database study

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Abstract

Background. The coronavirus disease (COVID-19) pandemic has greatly affected the treatment of most medical conditions. In particular, the treatment of seriously ill patients had to be adjusted due to the limited availability of in-hospital procedures.

Objectives. The aim of this study was to evaluate the effects of COVID-19-related changes on neuro-oncological surgeries in the Polish medical system.

Materials and methods. Data from the period of 2010–2020 were collected from National Health Insurance database for 2 diagnosis-related groups: A11 (complex intracranial procedures) and A12 (large intracranial procedures). The total number of procedures and diagnoses per year, trend changes and changes in procedures grouped by medical type were analyzed, including resections/biopsies, malignant/stable (nonmalignant) lesions, elective/acute procedures, and length of stay.

Results. Mean yearly numbers of 7177 (standard deviation (SD) = 760) procedures and 5934 (SD = 1185) diagnoses were recorded. Both numbers were growing up to 9.1% per year until 2018. From 2018, a 3.1% decrease in the number of procedures was observed, with a significantly larger decrease of 10.5% observed in 2020 ($p < 0.001$). The number of diagnoses decreased in 2019 by 2.7%, and by 9.2% in 2020 ($p = 0.706$), with a statistically significant change in the annual growth rate ($p = 0.044$). The number of resections decreased by 11.5% in 2020 ($p = 0.204$), with a significant change in the annual growth rate ($p < 0.001$). The number of biopsies decreased by 2.5% in 2020 ($p = 0.018$), with the annual decrement in 2019/2020 also being significant ($p = 0.004$). Decreases were observed in 2019 and 2020 for the number of malignant (0.5% and 6.3%, respectively) and nonmalignant (5.4% and 12.9%, respectively) tumors ($p = 0.233$ and $p = 0.682$ for absolute values, and $p = 0.008$ and $p = 0.004$ for the annual growth rates, respectively). The number of acute procedures in 2020 further decreased by 9.8% from 5.5% decrease in 2019 ($p = 0.004$), and the number of elective procedures decreased by 11.8% ($p = 0.009$). The annual growth rates for both acute and elective procedures were statistically significant ($p < 0.001$ and $p < 0.001$).

Conclusions. The decrease in the number of neuro-oncological surgeries appeared to be much lower than the 20% decrease observed for general oncological surgeries in Poland during the COVID-19 pandemic. This seems to have resulted from postponing the treatment of less critical cases (i.e., nonmalignant and elective) and focusing on the treatment of the most precarious patients.

Key words: Poland, neuro-oncology, brain tumor, COVID-19, trend change

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Background

During the coronavirus disease (COVID-19) pandemic, the medical environment for the treatment of many conditions has changed. The new disease has presented itself quickly, overwhelming parts of the national healthcare system and resulting in a great number of severely ill patients. A limited number of treatment options for COVID-19 and the necessity to stop the spread of the virus have increased the burden. The treatments for medical conditions other than COVID-19 have had to be adjusted due to the limited availability of in-hospital procedures. Various treatments have been restricted due to the transformation of hospitals into infectious departments, the loss of healthcare practitioners during quarantine, the need to develop new procedures to treat COVID-19 patients, and even the availability of personal protective equipment.

This novel situation has also affected brain tumor surgery and has forced adjustments to previous treatment protocols.[1–4] One of the major changes has been a decrease in the availability of intensive care units (ICUs), which are necessary for the early postoperative period.[5] This was primarily due to a large scale increase in the number of ICU patients suffering from respiratory failure secondary to COVID-19 pneumonia.[6–8] A second important factor was an attempt to cut/stop viral spread by implementing new procedures. These protocols aimed to decrease the contact between healthcare practitioners and patients, and to limit the number of physicians involved in a single procedure.[3] These limitations have also stressed outpatient systems and basic healthcare, and decreased the number of medical examinations, resulting in an increase in the number of teleconsultations. Additionally, delays in diagnostic workups were observed.[9–11]

Objectives

The aim of this paper is to evaluate the impact of COVID-19-related changes in the Polish medical healthcare system on brain neuro-oncological surgeries. To this end, data were collected from the National Health Insurance database. The total number of procedures and diagnoses per year, changes in trends in the following years, and changes in the number of procedures grouped by their medical type were analyzed.

Materials and methods

Data on the number of brain tumor surgeries carried out in Poland were collected from the Polish National Health Fund (NHF; in Polish, Narodowy Fundusz Zdrowia (NFZ)) database. The NHF is a governmental medical insurance agency that is the sole public funding source for medical treatments in Poland. The data are publicly reported each year and are grouped according to the diagnosis-related group (DRG) system. The basic details reported for each DRG include the number of corresponding International Classification of Diseases (ICD)-9 procedures and ICD-10 diagnoses (both limited to about 50 of the most frequently reported), related length of stay (LOS), and the percentages in relation to the total numbers. Demographic data are provided for the whole DRG. Despite these limitations, it is the most representative public data source on the Polish public medical system. The NHF database is published according to relevant legal acts, and is anonymized and free to use. Therefore, ethical committee approval was not necessary for this study.

Brain tumor surgery is reported under 2 DRG procedures: A11 (complex intracranial procedures) and A12 (large intracranial procedures). The coded ICD-10 diagnosis represents the most significant medical finding reported during hospitalization, which was the target of treatment. The simultaneously coded ICD-9 procedure represents the first, most representative procedure carried out during the treatment of the patient, thus indicating the objective of hospitalization. Data on ICD-9 procedures are presented for the entire analyzed period and data on ICD-10 diagnoses are presented since 2014. For the analysis, data on ICD-9 procedures and ICD-10 diagnoses associated with neuro-oncology were collected from each DRG with the corresponding LOS. Later, the ICD-9 procedures and ICD-10 diagnoses were divided into subgroups focusing on the general way in which they are carried out. These subgroups included:

– types of ICD-9 procedures: resection (01.512, 01.595, 01.599, 04.011, 04.012, 07.62, 07.65) and biopsy (01.131, 01.132, 01.14);

– types of ICD-10 diagnoses: mostly malignant (C71, C71.0–C71.6, C71.9, C79.3), stable (nonmalignant) lesions (D32.0, D33.0, D33.1, D33.3, D35.2).

The ICD-9 procedures were additionally divided into typically highly elective treatments (e.g., cerebellopontine angle tumor removal (01.512, 04.011, 04.012, 07.62, 07.65)) and those more typically performed in a shorter time after diagnosis, such as high-grade glioma (HGG) or metastasis – acute (01.131, 01.132, 01.14, 01.595, 01.599) (Table 1).

Statistical analyses

The frequencies of each of the analyzed variables in 2020 were compared with the frequencies reported in the preceding years. Moreover, the annual growth rates were calculated (the difference between the value for a given variable observed in a given year compared to its value from the previous year), and the increase/decrease in the observed values for 2019–2020 were compared to the annual growth rates from the preceding years.

The data from the years preceding the COVID-19 pandemic were examined for normal distributions using the Shapiro–Wilk test, which has the highest statistical power for small sample sizes. After verifying the assumptions,

Table 1. Codes in International Classification of Diseases (ICD) – ICD-9 and ICD-10

ICD-9				ICD-10		
Code	description	typical aim of procedure	typical performance	code	description	lesion typically
01.512	excision of brain dura	resection	elective	C71	malignant brain tumor	malignant
01.595	excision of cerebellar tumor	resection	acute	C71.0–C71.6	malignant brain tumor	malignant
01.599	excision of brain tumor – other	resection	acute	C71.9	as above in following brain anatomic locations	malignant
04.011	acoustic neuroma excision	resection	elective	C79.3	metastatic brain and dural tumor	malignant
04.012	acoustic neuroma excision with craniotomy	resection	elective	D32.0	nonmalignant dural brain tumor	nonmalignant
07.62	partial transsphenoidal hypophysectomy	resection	elective	D33.0	nonmalignant tumor (brain, supratentorial)	nonmalignant
07.65	complete transsphenoidal hypophysectomy	resection	elective	D33.1	nonmalignant tumor (brain, subtentorial)	nonmalignant
01.131	transcutaneous brain biopsy with trepanation	biopsy	acute	D33.3	nonmalignant tumor (cranial nerves)	nonmalignant
01.132	transcutaneous stereotactic brain biopsy	biopsy	acute	D35.2	nonmalignant tumor (hypophysis)	nonmalignant
01.14	open brain biopsy	biopsy	acute			

a two-tailed Student's t-test was used to test the null hypothesis of equality of the mean value for the observations from previous years with the value observed in the given year. The alternative hypothesis was that these values were not equal. A value of $p < 0.05$ was considered statistically significant. The results are presented in Table 2.

In addition, the number of malignant tumors reported in the 1st year of observation and the number of elective procedures conducted in the 2nd year of observation were considered as outliers, and these were removed from the analyses. Later in the article, the possibility of the influence of the method of supplementing data in the collective database at the beginning of the national registry's operation is discussed, taking into account the observation values from subsequent years and the fact that the number of malignant tumors reported in 2014 was clearly underestimated (Fig. 1). The number of elective surgeries in the 2nd year of follow-up was considered an outlier based on a scatterplot (Fig. 2). On the basis of similar criteria, the number of diagnoses in the 1st recorded year (2014) and the number of resections in the 2nd year of observation (2011) could also

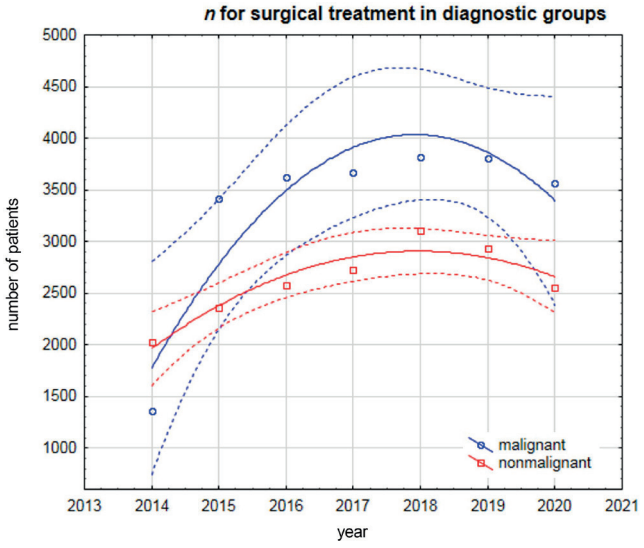

Fig. 1. Number of neuro-oncosurgical treatment in the diagnostic group. The dashed lines show the 95% confidence interval (95% CI) for the polynomial trends fitted to the data with the use of least squares method (solid line): y = –6.0042 × 108 + 5.9509 × 105x – 147.4524x2 for malignant, and y = –2.4457 × 108 + 2.42 × 105x – 60.0595x2 for nonmalignant tumors (x – year)

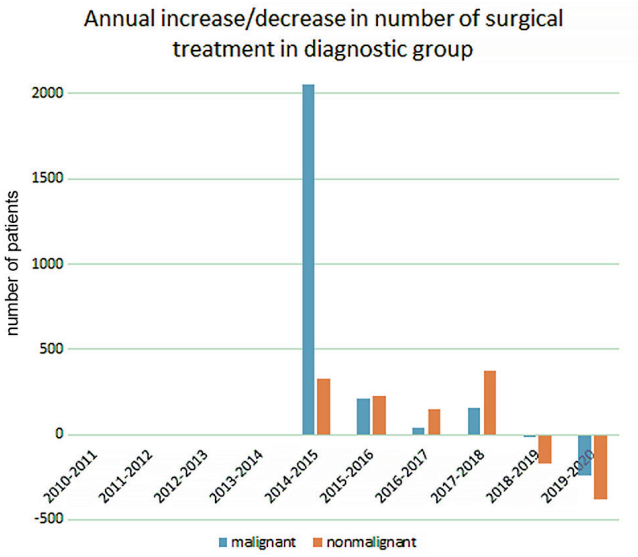

Fig. 2. The annual growth rates in the diagnostic group. The decrease in the number of operations is particularly visible for the resection of nonmalignant tumors in 2020. The analyzed database does not contain information on the diagnoses before 2014, due to Polish National Health Fund (NHF; in Polish, Narodowy Fundusz Zdrowia (NFZ)) database limits

Table 2. Results of statistical analyses for variables related to neurosurgical treatment of brain tumors.

Examined variable		Statistical test		Interpretation
		Shapiro–Wilk test	Student's t-test	
Procedures	number per year	W = 8655; p = 0.089	t = 1.195; df = 9; p = 0.263	The number of procedures in 2020 does not differ from the mean from previous years, but their decrease between 2019 and 2020 differs from the mean growth from previous years.
	year-on-year growth	W = 0.961; p = 0.809	t = 8.481; df = 8; **p < 0.001**	
Diagnoses	number per year	W = 0.781; p = 0.059	t = –0.399; df = 5; p = 0.706	The number of diagnoses in 2020 does not differ from the mean from previous years, but their decrease between 2019 and 2020 differs from the mean growth from previous years.
	year-on-year growth	W = 0.807; p = 0.092	t = 2.906; df = 4; **p = 0.044**	
Resections	number per year	W = 0.865; p = 0.087	t = 1.368; df = 9; p = 0.204	The number of resections in 2020 does not differ from the mean from previous years, but their decrease between 2019 and 2020 differs from the mean growth from previous years.
	year-on-year growth	W = 0.971; p = 0.906	t = 5.649; df = 8; **p < 0.001**	
Biopsies	number per year	W = 0.894; p = 0.187	t = –2.890; df = 9; p = 0.018	Both the number of biopsies in 2020 and their decrease between 2019 and 2020 differ from their mean values from previous years.
	year-on-year growth	W = 0.920; p = 0.395	t = 3.982; df = 8; **p = 0.004**	
Malignant tumors	number per year	W = 0.909; p = 0.463	t = 1.403; df = 4; p = 0.233	The number of diagnoses of malignant tumors in 2020 does not differ from the mean from previous years, but their decrease between 2019 and 2020 differs from the mean growth from previous years.
	year-on-year growth	W = 0.955; p = 0.747	t = 6.464; df = 3; **p = 0.008**	
Nonmalignant tumors	number per year	W = 0.981; p = 0.9575	t = 0.434; df = 5; p = 0.682	The number of diagnoses of nonmalignant tumors in 2020 does not differ from the mean from previous years, but their decrease between 2019 and 2020 differs from the mean growth from previous years.
	year-on-year growth	W = 0.887; p = 0.344	t = 5.865; df = 4; **p = 0.004**	
Elective	number per year	W = 0.797; p = 0.056	t = 7.994; df = 5; **p < 0.001**	Both the number of elective surgeries in 2020 and their decrease between 2019 and 2020 differ from their mean values from previous years.
	year-on-year growth	W = 0.920; p = 0.391	t = 3.414; df = 8; **p = 0.009**	
Acute	number per year	W = 0.860; p = 0.075	t = 3.862; df = 9; **p = 0.004**	Both the number of acute surgeries in 2020 and their decrease between 2019 and 2020 differ from their mean values from previous years.
	year-on-year growth	W = 0.970; p = 0.892	t = 7.151; df = 8; **p < 0.001**	

For each variable, their absolute numbers in subsequent years and annual increments were analyzed, and the observation from the period of change caused by the coronavirus disease (COVID-19) pandemic (2020 and the decrease in 2019–2020) was compared to the mean values from previous years. The table presents the results of the Shapiro–Wilk test of normality for observations from previous years (values of the test statistic W and the p-value), which is a prerequisite for the correct application of the Student's t-test, and the results of the Student's t-test (values of the test statistic (t), the number of degrees of freedom (df) and the p-value). Statistically significant p-values at the significance level of 0.05 are in bold.

be considered outliers. However, their elimination did not change the statistical significance for the examined changes in the year of the pandemic; hence, the results are presented without their elimination.

Changes in the values of the analyzed variables over time are presente graphically in plots in the Results section. Scatterplots were used for the absolute values of the observed quantities, presenting their changes over time with the fitting of an illustrative nonlinear (polynomial) trend. For the plotting, it was assumed that a second-degree polynomial would be fitted to the data, and the method of least squares was applied for approximation. Bar graphs were used to present the annual differences.

Results

In the whole Results section, the following symbols are used for statistical measures: M – mean, SD – standard deviation, p – p-value, W – the Shapiro–Wilk test statistic, t – the Student's t-test statistic, df – degrees of freedom. All p-values are presented with a test name.

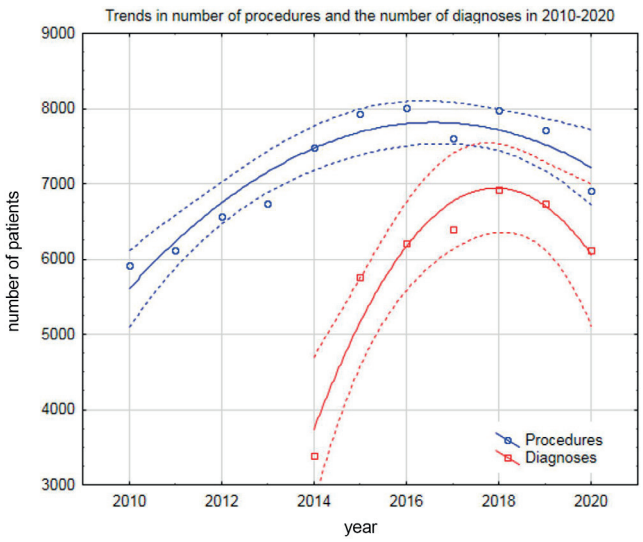

Fig. 3. Changes in the number of neuro-oncological surgeries performed and the number of diagnoses made in the years 2010–2020. The dashed lines show the 95% confidence interval (95% CI) for the polynomial trends fitted to the data with the use of least squares method (solid line): y = –2074 × 108 + 2.057 × 105x – 51.0023x2 for procedures, and y = –8.4499 × 108 + 8.3749 × 105x – 207.5119x2 for diagnoses (x – year)

Table 3. Number of patients per year in relation to ICD-9

Code		Procedure	2010		2011		2012		2013		2014		2015		2016		2017		2018		2019		2020	
			n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS
ICD-9	01.131	percutaneous brain biopsy with trepanation	91	7.0	128	7.0	146	8.0	155	7.0	211	7.0	215	9.0	231	7.0	205	7.0	190	6.0	231	6.0	209	6.0
	01.132	stereotactic brain biopsy	323	5.0	330	4.0	380	4.0	380	4.0	416	4.0	462	4.0	470	4.0	450	4.0	491	4.0	494	3.0	464	3.0
	01.14	open brain biopsy	79	9.0	75	9.0	93	9.0	91	10.0	93	10.0	108	11.0	125	8.0	116	10.0	120	9.0	111	8.0	142	7.0
	01.512	dura matter removal	628	10.0	773	10.0	1200	10.0	1293	10.0	1755	10.0	1755	10.0	1891	9.0	1756	9.0	2041	8.0	2102	8.0	1844	8.0
	01.595	cerebellar tumor excision	324	14.0	312	14.0	298	14.0	275	13.0	272	13.0	293	13.0	260	12.0	263	11.0	247	11.0	163	11.0	159	10.0
	01.599	other brain tumor excision	3594	12.0	3531	11.5	3527	12.0	3565	12.0	3675	10.5	4044	10.5	4064	11.0	3917	10.5	4078	10.0	3841	10.0	3390	9.0
	04.011	acoustic schwannoma excision	126	16.0	128	16.0	133	15.0	121	16.0	116	13.0	116	13.0	106	13.0	–	–	–	–	–	–	–	–
	04.012	acoustic schwannoma excision with craniotomy	170	13.0	155	11.0	151	12.0	144	11.0	105	11.0	105	11.0	106	13.0	139	11.0	138	10.0	124	12.0	106	11.0
	07.62	transsphenoidal partial hypophysectomy	587	8.0	684	8.0	644	8.0	711	8.0	741	7.0	741	7.0	749	7.0	755	7.0	667	7.0	655	7.0	590	6.0
	07.65	transsphenoidal total hypophysectomy	–	–	–	–	–	–	57	7.0	92	8.0	92	8.0	–	–	–	–	–	–	–	–	–	–
Total			5922	10.4	6116	10.1	6572	10.2	6735	9.8	7476	9.4	7931	9.7	8002	9.3	7601	8.7	7972	8.1	7721	8.1	6904	7.5

ICD – International Classification of Diseases; LOS – length of stay.

For groups A11 and A12 of the DRG related to brain tumor surgery, mean numbers of 7177 (SD = 760) procedures and 5934 (SD = 1185) diagnoses per year were observed (Table 3,4). During the whole analyzed period, a marked diversity in the number of cases per year can be seen, with a wide spread in the numbers. These changes were visualized using a scatterplot with a nonlinear trend (Fig. 3).

The annual growth rates in diagnoses and procedures are shown in Fig. 4. The disproportionally large increase in the number of diagnoses between the years 2014 and 2015 was caused most probably by a partial failure in reporting to the NFZ, due to the implementation of a new reporting tool. Until 2018, the total number of oncological procedures and diagnoses grew at a rate of 3.2–9.1% per year. After 2018, a decrease in percentages was observed compared to the previous year. The decrease of 3.1% in the number of procedures conducted in 2019 intensified to 10.5% in 2020, with a further down-bending of the curve representing the number of cases. The whole previous period was statistically insignificant for both procedures ($t = 1.195$, $df = 9$, $p = 0.263$) and diagnoses (Student's t-test, $t = -0.399$, $df = 5$, $p = 0.706$). These changes in time are shown in Fig. 3. The lack of differences is in large part caused by a high variability in the observations in individual years. However, when comparing the annual growth rates from 2020 to previous periods (Fig. 4), the decreases in the number of both procedures and diagnoses were significant (Student's t-test, $t = 8.481$, $df = 8$, $p < 0.001$, and $t = 2.906$, $df = 4$, $p = 0.044$, respectively).

The length of hospitalization related to all procedures steadily decreased during the whole analyzed period (Fig. 5). While there were some increases in LOS in the years 2015 and 2019, the overall trend decreased monotonically. When analyzing the year-to-year changes, the LOS decrease started with 1% per year, with a general rate of 6% per year. This process accelerated to 7% in 2020.

For procedures subdivided into resection or biopsy, the mean numbers per year were 6404 (SD = 767) and 711 (SD = 123), respectively. The number of resections initially grew between 2010 and 2018 at a rate of 2–9.5% per year. It started to decrease in 2018 by 3.9%, with a marked decrease in 2020 by 11.5% (Fig. 6). Although no statistical significance (Student's t-test, $t = 1.368$ $df = 9$, $p = 0.204$) was found for the absolute values, the relative

Table 4. Number of patients per year in relation to ICD-10 codes

Code		Diagnosis	2010		2011		2012		2013		2014		2015		2016		2017		2018		2019		2020	
			n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS	n per year	median LOS
ICD-10	C71	malignant brain tumor	–	–	–	–	–	–	–	–	–	–	72	6.0	74	3.0	86	6.0	79	3.0	–	–	–	–
	C71.0	malignant tumor (except for lobes and ventricles)	–	–	–	–	–	–	–	–	86	13.0	86	13.0	83	12.0	135	12.0	207	10.0	202	9.0	164	8.5
	C71.1	malignant tumor (frontal lobe)	–	–	–	–	–	–	–	–	340	11.0	891	10.5	854	8.5	932	9.5	886	9.5	892	8.5	932	8.0
	C71.2	malignant tumor (temporal lobe)	–	–	–	–	–	–	–	–	284	10.0	701	10.0	702	9.0	680	9.5	735	9.0	683	8.5	638	8.5
	C71.3	malignant tumor (parietal lobe)	–	–	–	–	–	–	–	–	238	10.0	575	10.0	581	10.0	544	10.0	564	9.0	575	9.0	468	8.5
	C71.4	malignant tumor (occipital lobe)	–	–	–	–	–	–	–	–	–	–	107	11.0	192	9.0	198	9.0	213	10.0	240	8.5	202	7.5
	C71.6	malignant tumor (cerebellum)	–	–	–	–	–	–	–	–	101	13.0	277	12.0	297	12.0	277	11.5	313	11.0	265	10.0	236	10.0
	C71.9	malignant tumor (brain, unspecified)	–	–	–	–	–	–	–	–	137	8.0	296	8.0	313	8.0	329	8.0	285	8.0	409	8.5	343	7.0
	C79.3	secondary malignant brain and dura tumor	–	–	–	–	–	–	–	–	177	9.0	408	9.0	530	9.5	487	10.0	540	9.5	538	8.5	580	8.5
	D32.0	nonmalignant dura matter tumor	–	–	–	–	–	–	–	–	550	10.0	653	11.0	779	10.5	827	10.5	831	10.0	915	9.0	773	8.5
	D33.0	nonmalignant tumor (brain, supratentorial)	–	–	–	–	–	–	–	–	528	12.0	750	11.5	720	11.0	795	10.5	1128	10.0	922	8.5	847	8.0
	D33.1	nonmalignant tumor (brain, infratentorial)	–	–	–	–	–	–	–	–	184	13.0	184	13.0	244	12.0	284	12.0	303	12.5	258	12.0	246	10.5
	D33.3	nonmalignant tumor (cranial nerves)	–	–	–	–	–	–	–	–	768	9.5	119	12.0	139	12.0	140	11.0	175	10.0	144	11.0	111	11.0
	D35.2	nonmalignant tumor (hypophysis)	–	–	–	–	–	–	–	–	–	–	649	7.0	697	7.0	682	7.0	665	7.0	694	7.0	575	6.0
Total											3393	10.8	5768	10.6	6205	10.0	6396	10.0	6924	9.7	6737	9.1	6115	8.5

ICD – International Classification of Diseases; LOS – length of stay.

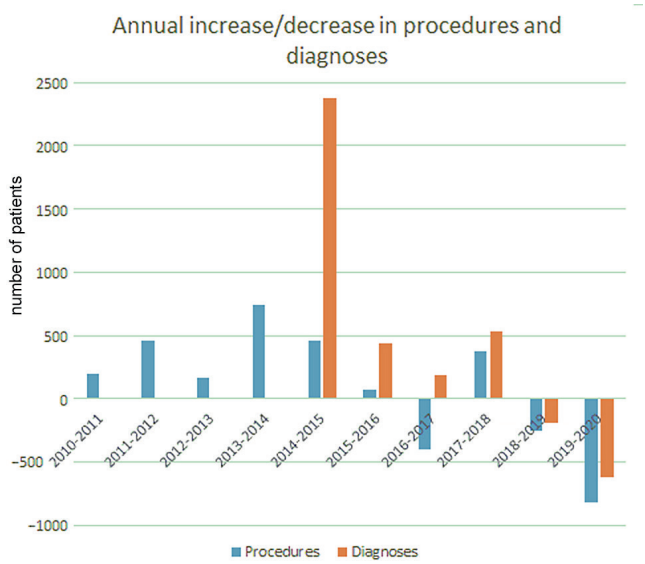

Fig. 4. The annual growth rates in diagnoses and procedures. In the years 2018–2020, there is a clear decrease in both values. The analyzed database does not contain information on the diagnoses before 2015, due to Polish National Health Fund (NHF; in Polish, Narodowy Fundusz Zdrowia (NFZ)) database limits

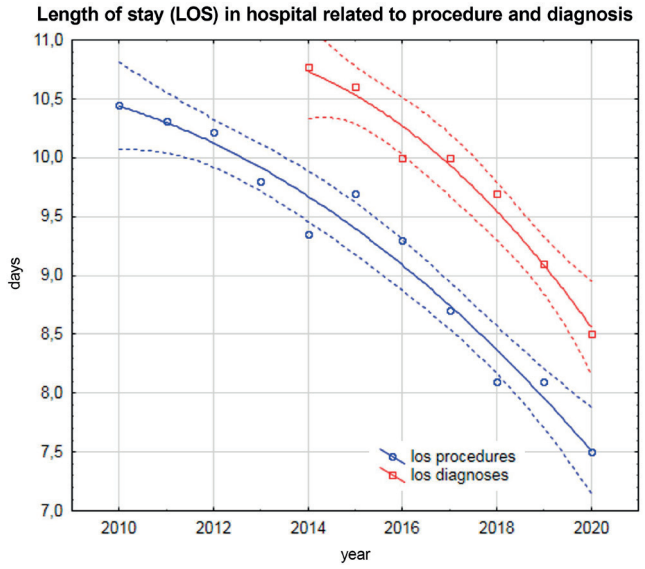

Fig. 5. Changes in the length of stay (LOS) in hospital for procedures and diagnoses in the years 2010–2020. The dashed lines show the 95% confidence interval (95% CI) for the polynomial trends fitted to the data with the use of least squares method (solid line): y = –68162.4098 + 67.957x – 0.0169x2 for procedures, and y = –1.3179 × 105 + 131.0492x – 0.0326x2 for diagnoses (x – year)

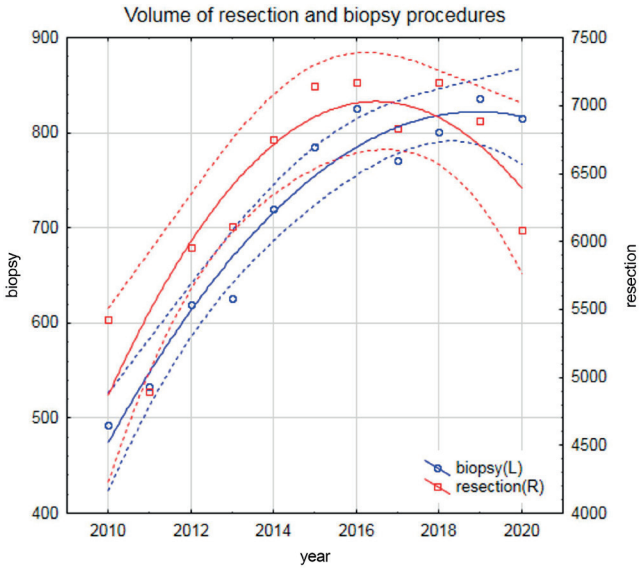

Fig. 6. Changes in the number of resection and biopsy procedures. The dashed lines show the 95% confidence interval (95% CI) for the polynomial trends fitted to the data with the use of least squares method (solid line): y = –1.7373 × 107 + 17201.003x – 4.2576x2 for biopsies, and y = –1.5773 × 108 + 1.563 × 105x – 38.7197x2 for resections (x – year)

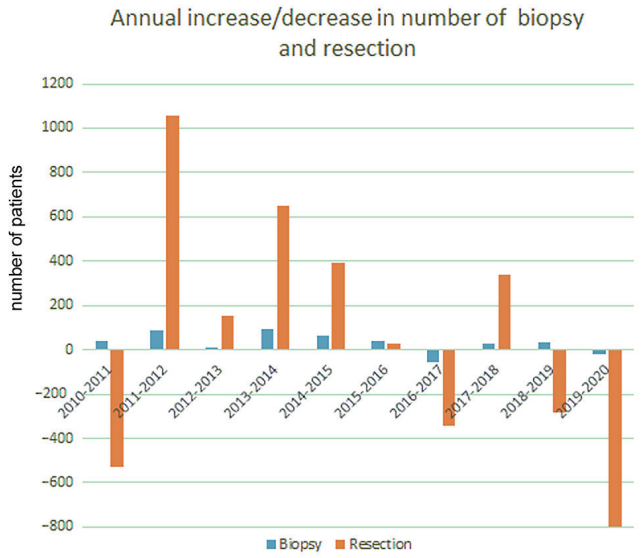

Fig. 7. The annual growth rates for resection and biopsy procedures. There is a relatively greater decrease in the number of resections than in the number of biopsies as a result of the coronavirus disease (COVID-19) pandemic

decrease in the number of resections in 2020 compared to the previous years was significant (Student's t-test, t = 5.649, df = 8, p < 0.001). The total number of biopsies in 2020 also significantly decreased compared to previous years (Student's t-test, t = –2.890, df = 9, p = 0.018). The increase in biopsies seemed to be more stable over the years than the increase in resections, with an average of 8% and a minimal decrease of 2.5% in 2020, which was statistically significant (Student's t-test, t = 3.992, df = 9, p = 0.004). These trends are shown in Fig. 7.

When looking at the diagnosis and comparing procedures related mostly to malignant (M = 3323, SD = 875) or stable (nonmalignant) lesions (M = 2611, SD = 357), both were increasing at the beginning of the analyzed period. A decrease started in 2019, followed in 2020 by increases of 0.5% and 6.3% for malignant, and 5.4% and 12.9% for nonmalignant lesions, respectively. The annual growth rates are shown in Fig. 2, and their changes over time are outlined in Fig. 1. The number of diagnoses for both subgroups in relation to the average numbers in previous years

did not differ significantly (Student's t-test, t = 1.403, df = 4, p = 0.233 for malignant, and t = 0.434, df = 5, p = 0.682 for nonmalignant tumors). However, the decrease in diagnoses for both malignant and nonmalignant tumors between 2019 and 2020 was statistically significantly different from their mean growth in the previous years (Student's t-test, t = 6.464, df = 3, p = 0.008, and t = 5.865, df = 4, p = 0.004, respectively). As can be seen in Fig. 1, there is an outlier value for the reported cases in the first year of data collection (2015), which may have impacted the results. Therefore, this observation was excluded from the analysis.

The procedures divided by typical performance showed fewer highly elective cases (M = 2400, SD = 611) than those performed in a shorter time since diagnosis (acute) (M = 4702, SD = 335; Fig. 8). The absolute number of acute procedures in 2020 was significantly higher (Student's t-test, t = 3.862, df = 9, p = 0.004) compared to previous years and decreased by 5.5% in 2019, and by 9.8% in 2020. Also, the decrease in the number of acute surgeries between 2019 and 2020 differs from the mean value reported in previous years (Student's t-test, t = 7,151, df = 8, p < 0.001).

Elective procedures showed a decrease in 2020 at a rate of 11.8%, which was statistically significant (Student's t-test, t = 3.414, df = 8, p = 0.009). The decrease between 2019 and 2020 in comparison to the mean value from previous years was also significant (Student's t-test, t = 7.994, df = 5, p < 0.001). This time series is presented in Fig. 9.

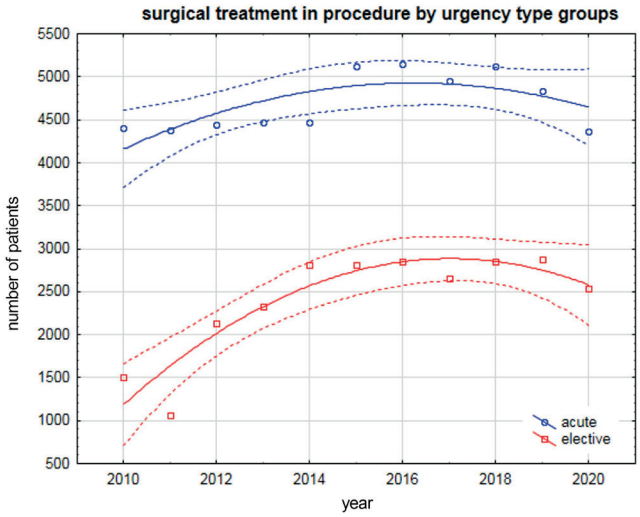

Fig. 8. Number of acute and elective procedures in the years 2010–2020. The dashed lines show the 95% confidence interval (95% CI) for the polynomial trends fitted to the data with the use of least squares method (solid line): y = –8.018 × 107 + 79539.8788x – 19.7249x2 for acute, and y = –1.3979 × 108 + 1.3862 × 105x – 34.3613x2 for elective procedures (x – year)

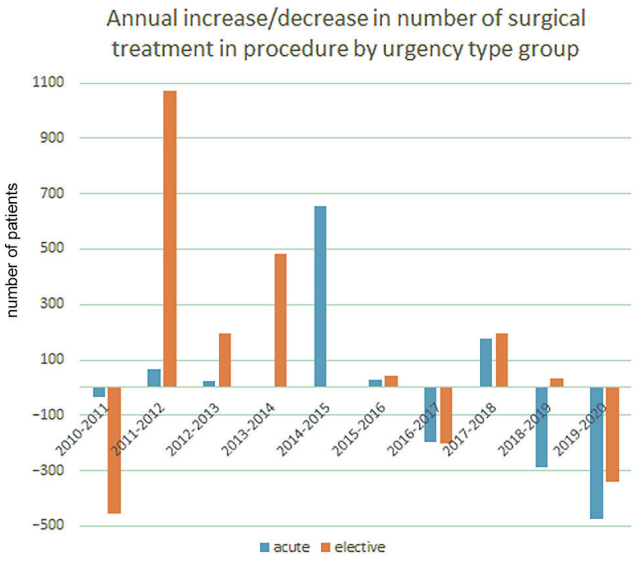

Fig. 9. The annual growth rates for acute and elective procedures. The decrease in the absolute number of procedures as a result of the coronavirus disease (COVID-19) pandemic is visible

Discussion

The COVID-19 pandemic has had a significant impact on the human community. In Poland, the first patient was diagnosed in March 2020. With growing numbers of COVID-19 patients, preventive actions were initiated by the government, which were predominantly focused on social distancing. The growing knowledge of the biological character of COVID-19, its routes of transmission, medical treatment, and, most importantly, progress in vaccination has lowered the need for social distancing. Nevertheless, social distancing and polymerase chain reaction (PCR) testing are still the main methods for disease prevention, and these strategies influence many stages of medical treatment.

One group of patients that requires urgent treatment is those with oncological diagnoses. In the case of malignant tumors, a delay in treatment is the main cause of a worsening prognosis.[12] Indeed, a 4-week delay impacts morbidity and mortality for all treatment methods, including surgery, radiotherapy and systemic treatment (6–8%, 9% and 13%, respectively).[13] Before the COVID-19 pandemic, there were 2 main causes of delayed treatment. The most common patient-related reason was financial (28%), followed by problems with travel (living far away from treatment facilities (12.7%), dependency on help of others (9%)), and ignoring the disease (16%).[10] On the medical side, the delay was found to be significant if the patient was initially diagnosed outside of a large specialist center.[10] The burden induced by these factors has increased during the pandemic.[14] Changes in the number of surgeries and oncological therapies, rescheduling, and delays in outpatient treatment appear to be a global problem. These issues have affected most medical centers and their supply chains, including personnel availability (up to 79%).[11] The impact of COVID-19-related healthcare system changes on oncological patients was not uniform. Multiple factors (e.g., age, comorbidities, type of treatment, etc.) played a role in the final influence of COVID-19 restrictions on oncological treatments. Depending on the type of diagnosis, some patient

groups showed no changes in survival, and new solutions, such as telemedicine in the case of breast cancer outpatient treatment, were applied with very good results.[9,15–18]

A special report by the Polish Oncological National Board focused on the influence of COVID-19 did not detect a long-term significant change in the general availability of treatment for oncological patients in Poland during the pandemic.[9] The problems that emerged during the first few months of the pandemic have diminished. The most profound impact on oncology was observed during spring of 2020, when the first restrictions were put into place. During this period, the availability of ambulatory diagnostics was reduced and some procedures were completely suspended. Telemedicine was advocated as the primary method for contacting a physician.[18] During the 2nd part of the year, the situation improved. However, many oncological patients were afraid of leaving their homes as these patients tend to be at higher risk for infection. The COVID-19 pandemic and the associated restrictions resulted in a decrease in new tumor detection by 10–20% in 2020, depending on the tumor type. Nevertheless, in the current study, no significant differences were observed in the number of chemo- and radiotherapy procedures in comparison to the previous period, except for the early spring. These results are in contrast to surgical treatment, which decreased by up to 20%. One of the reasons for this latter outcome is the fact that a large number of oncological surgical treatments in Poland are conducted at large multidisciplinary hospitals, many of which were transformed into infectious disease centers. Thus, chemo- and radiotherapy, mostly carried out at dedicated oncological centers, were not affected in the same way. It is expected by the board that the number of new oncological diagnoses may show a compensatory increase after normalization of the pandemic situation. However, initial data from 2021 do not support this hypothesis, with the numbers of diagnosed and treated cases comparable to those observed in 2019.[9]

In 2020, the general decrease in the number of NFZ-reported onco-neurosurgical procedures (10.5% decrease) was lower than the decrease in the number of oncological surgeries in general (20% decrease).[9] The National Oncology Boards reported that the 10.5% decrease in neuro-oncology surgeries is comparable to other countries.[19] This change in practice was present worldwide, with a reduction in neuro-oncological surgery reported to be up to 50% in some situations, due to a focus on COVID-19-negative cases or even only emergency cases for a period of time.[20] According to our observations, the total mean number of procedures and diagnoses in Poland in 2020 did not change compared to previous years. However, when looking at the trends, there was a marked decrease in general procedures, acute and elective treatments, and nonmalignant diagnoses. However, the trend remained stable for malignant diagnoses, which suggests that, in the Polish medical system, stable treatment and diagnostic plans were provided to oncological patients. Patients already going through diagnostic procedures were allocated to treatment, which is why the mean number may have remained stable. For comparison, a UK study showed a change in treatment programs up to 10.7% for neuro-oncological patients, mostly due to stoppages in surgery or patient referrals for the best supportive care. The major parameter affecting the decision process was a poor prognosis. Treatment of low-grade lesions could be planned after the acute stage of the pandemic. The scale of changes in treatment plans decreased after the initial months of the pandemic.[19,21]

In addition, our observations showed a marked decrease in the general trends in the number of patients and subgroups. These numbers most probably represent new diagnoses in patients who experienced an extended time to diagnosis and start of treatment. This extension, in many cases, was caused by the limited availability of emergency procedures due to the lockdown, decreased effectiveness of operating rooms (ORs) and decreased availability of imaging diagnostics. Neurosurgical centers have reported a decrease in the number of oncological patients due in part to treatment plan delays, but no change in outcome has been observed.[18,22] The decrease in the number of neuro-oncological procedures was partially related to limited access to ICUs, which shifted to treating COVID-19 patients.[5,6] Interestingly, Azab and Azzam reported that the rate of hospital admissions for patients with glioma who tested positive or negative for COVID-19 was similar, but the rate of complications among negative patients was higher.[23] Observations of the Polish database over time may answer the question of whether the decrease in trends is just a temporary situation or a long-term effect.

The trend observed for the total number of procedures and diagnoses correlates with the subgroup analysis. Although the trend for both resection and biopsy procedures showed a decrease, the mean volume of resections per year remained stable. The number of biopsy procedures seems to represent a general change in neurosurgical practices across most departments. The shift in the availability of ORs and ICUs forced medical providers to focus on the most critical patients (i.e., those experiencing trauma or oncological issues).[3,5,6,19] This, in part, may be explained by a decrease in biopsy procedures that were more likely to be omitted in patients treated from the beginning with resection or allocated to palliative care.

Pituitary adenoma surgery is a particularly interesting neuro-oncological procedure from the perspective of COVID-19. It has been reported that, in the years 2019–2020, the decrease in the use of this procedure was 10.5%, similar to the general decrease in neuro-oncological surgery. The treatment of pituitary lesions is mainly transsphenoidal and, in the early part of the pandemic, was expected to present a higher risk for surgical personnel.[24] However, the implementation of safety protocols appeared to provide a safe way for treatment in many countries.[25–28] This effective shift of the surgical organization most likely prevented a more visible change in the number of operated patients.

Length of stay represents procedural organization and is reflective of the general push to shorten in-hospital treatment. Previously, an ongoing decrease in LOS was observed in 2020. This decrease in hospitalization time is a natural consequence of the pandemic restrictions and the implementation of social distancing. It is interesting to note that the change in the number of procedures and diagnoses is statistically insignificant; however, when taking into consideration the general trend, it turned out to be significant for both of them but the LOS for diagnosis did not change. It seems that ongoing improvements in the quality of care did not enable medical staff to perform more procedures at the same time, which is represented by the decreased number of procedures. It is also interesting to note that the LOS for oncological diagnosis was longer than that for procedures in each of the analyzed years. This may be because the cases reported with oncological procedures had unfinished diagnostic workups.

The overall reaction of the neurosurgical Polish medical system during the pandemic seems to have focused on malignant cases and a tendency to perform resective procedures. Unfortunately, the treatment effort has been reallocated from nonmalignant and nonemergency groups, which may represent a sort of reserve capacity in the healthcare system. Therefore, in the future, it will be necessary to better prepare the logistics of treating infectious patients without destabilizing the treatment of "common" diseases in the event of another pandemic or other comparable overload of the healthcare system. In addition, there is a rationale to try to increase the efficiency of oncological diagnostics and qualification for procedures in oncological surgery by increasing the role of expert committees that can assist with setting the time priority for procedures. It is difficult to interpret the slight trend towards a decrease in the number of diagnoses and neuro-oncological procedures already present in the years preceding the pandemic. This observation will need to be assessed taking into account the data from subsequent years, which may allow for the identification of the cause.

Limitations

Several limitations of this study stem from the use of different types of medical reporting systems throughout Poland. The different ways of coding may produce a number of patient cases not included in this report. Also, the NFZ database only includes the most frequently coded procedures and diagnoses. However, it can be assumed that local coding protocols have remained unchanged throughout the years; therefore, the published data represent general trends in the country that are representative of all medical centers. Thus, the trends are more valuable to assess than the total numbers provided. Different codes represent procedures and diagnoses, and are secondary to reporting protocols that differ throughout the country. The NFZ database reports only the most common ones; hence, those less often used or those unspecific or indirectly related to oncological diagnosis are not listed. Finally, some of the patients underwent more than 1 procedure. Due to these factors, we decided to group diagnoses and procedures to achieve more comprehensive results for analysis.

An important limitation of the current study is also the relatively small sample size. The NFZ database contains only annual observations from 2010, but they are not complete in the years 2010–2014.

Conclusions

The decrease in the number of neuro-oncological surgeries was much lower than the general decrease in the number of oncological surgeries in Poland, mostly resulting from postponing operations on less critical cases and focusing on the most severely ill patients. This trend was visible when focusing on malignant diagnoses and more elective surgeries, with a decrease in acute and biopsy procedures. Further observations are needed to determine the long-term impact of these trends on oncological and nononcological treatments.

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Pain management in children with burns before admission to the ward: Analysis of selected parts of pre-hospital medical records

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Abstract

Background. The extent of pre-hospital medical care (PHMC) given to burned patients affects both the patient's condition and the effectiveness of treatment.

Objectives. To improve the quality of PHMC of burns in children, based upon an analysis of the selected parts of pre-hospital medical records, with particular emphasis on analgesia.

Materials and methods. Medical records were used to analyze how PHMC was given to 117 burned children aged 0–18 years, treated at the Pediatric Surgery Ward between January 1, 2014 and December 31, 2017.

Results. In 41/85 cases, PHMC was delivered by Emergency Medical Teams (EMTs), in 42 in Emergency/Admission Rooms (ARs) and in 2 by Primary Health Care (PHC). Monotherapy was predominant. Medical records from ARs included the following information: the administration of analgesics with the name in 95% (21) of the cases, the route of drug administration in 45% (10), insertion of intravenous access and dressing in 33% (14), and fluid transfusion in 43% (6) cases. The way in which the EMTs provided assistance was described in 34% (14/41) of medical records, the administration of analgesics in 86% (12) cases, cooling and dressing in 43% (6), and the establishment of intravenous access with fluid transfusion in 36% (5) cases.

Conclusions. In burned children, access to analgesic and combined pain therapy is still random, limited and deviates from current recommendations. Prior to the admission to the ward, pain relief with 1 agent administered rectally prevails. There is a need to standardize the procedures for pre-hospital medical assistance provided to burned children, including the method of pain management in line with the Polish recommendations. It is necessary to make the medical staff aware of the obligation to keep medical records in a reliable and legal manner.

Key words: pain, burn, child, pre-hospital care

Cite as
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Background

Emergency Medical Teams, Admission Rooms (ARs) and Primary Health Care (PHC) staff provides medical assistance in pre-hospital conditions to people with burns. The scope of this assistance includes analgesia.[1,2] In burned children, analgesia is both a priority and an integral part of pre-hospital medical care (PHMC). The analgesia within this support is overlooked, limiting to non-pharmacological methods or a single analgesic agent, which negatively affects the child's somatic and mental spheres.[3–13] The consequence of small burns is a local defensive body's reaction. Extensive thermal injuries cause systemic consequences leading to the development of burn disease and the systemic inflammatory response syndrome (SIRS). Thus, in the case of extensive burn lesions, it proceeds as a systemic inflammatory reaction resulting in, e.g., burn shock, sepsis and multiple organ dysfunction syndrome (MODS). Under the conditions of controlled inflammation, pro-inflammatory cytokines induce the formation of anti-inflammatory cytokines, cytokine inhibitors, and the symptoms of inflammation gradually subside – and compensatory anti-inflammatory response syndrome (CARS) is then diagnosed. However, if adaptive anti-inflammatory mechanisms are insufficient, the process becomes generalized inflammation. Thus, it is extremely important to implement analgesia in order to prevent the clinical complications.[13]

The medical services provided and the type of analgesia implemented should be carefully recorded in the medical records. Inaccurate completion of medical records and non-standards procedures are not uncommon, which hinders further management.[14,15]

Objectives

The aim of study was to improve the quality of PHMC in burned children based on the analysis of selected parts of medical records, with particular emphasis on analgesia. Additionally, specific objectives were formulated:

– Is the pain in burned children relieved at the PHMC stage in accordance with the applicable recommendations?

– Is a single analgesic agent or a multiple therapy used for analgesia prior to admission to the burn unit?

– Using what route are painkillers given to burned children before admission to the ward?

– Did the PHMC staff properly complete the medical records before patients admission to the ward?

Materials and methods

The retrospective analysis comprised 117 medical histories of children, aged 0–18 years, admitted to the Department of Pediatric Surgery on the first day after sustaining a burn between January 1, 2014 and December 31, 2017. The analysis excluded 15 cases with burns occurring more than 24 h before admission. The medical records written by ARs doctors were subject to an analysis in terms of PHMC provided by the EMTs, in the ARs and in the PHC, with particular emphasis on analgesia. Medical procedures of ARs and EMTs affecting analgesia were compared. Percentage values of the studied variables were calculated and the descriptive method of statistical analysis of the studied parameters was used. The study was approved by the Bioethics Committee of the Medical University of Lodz, Poland (approval No. RNN/94/18/KE).

Results

In the study group of 117 children, thermal burns were predominant (97.43%), as a result of dousing (87.18%), mild, I/II degree, including up to 10% of the total body surface area (84.61%) (Table 1). The most common burns involved the head and neck, upper limbs and chest. The provision of PHMC was noted in the medical records of 85 children – 42 in the ARs, 41 by the EMTs and 2 in the PHCs. In the ARs medical records, the following was noted: administration of analgesics in 22 children, including the name of the agent administered in 21. In 1 case, there was only an entry "analgesics administered" but without specifying the name, dose and route of administration. Pain was most commonly alleviated with a monotherapy – paracetamol in 17 out of 21 children. In isolated cases, morphine was administered in 2 children, Dolargan in 1 child and Fentanyl in another 1. The dose of analgesic administered was recorded only in 3 children out of 22. A combination intravenous (iv.) treatment was used in only 1 severely burned child. Paracetamol was administered to 16 children. The dose of the drug was recorded only in 2 cases and the route of administration in 8. Morphine was given in 1 case subcutaneously at an undetermined dose, and in the other case the dose was specified but the route of administration was not marked. Dolargan was given to 1 child intramuscularly, also at an unspecified dose. Administration of other drugs was recorded in 4 out of 42 children: Luminal without specifying the dose in 1 case and Relsed in 3 cases, specifying the dose in only 1 child; establishment of iv. access, entered into the medical histories of 14 out of 42 children; transfusion of intravenously fluids in 6 children; indicating the name and volume of fluids transfused in only 1 case; protection of the burn wound with a dressing in 14 children; a note: "dressing" in 10 children, sterile dressing in 3, Argosulfan in 1 child; electrocardiography (ECG) examination in 1 child with electrical burns. A referral letter with a request for further treatment in the ward was issued for all AR patients.

The EMTs transported 20 children to hospital. The remaining 22 children were transported by parents/guardians.

Table 1. Clinical characteristic of study group

Category	Study period (January 1 – December 31)				
	2014 n (%)	2015 n (%)	2016 n (%)	2017 n (%)	Total n (%)
Type of burn, n = 117					
Thermal	34 (30)	24 (21)	25 (22)	31 (27)	114 (97)
Electrical	2	–	–	–	2
Chemical	–	–	–	1	1
Total, n (%)	36 (31)	24 (21)	25 (21)	32 (27)	117 (100)
Mechanism of injury, n = 117					
Dousing	25 (25)	24 (23)	23 (23)	30 (29)	102 (87)
Contact with hot object	7 (70)	–	2	1	10 (8)
Contact with flame	2	–	–	1	3
Electrical	2	–	–	–	2
Total, n (%)	36 (31)	24 (21)	25 (21)	32 (27)	117 (100)
Depth of injury, n = 113/117 (97)					
Partial thickness (I/II°)	34 (31)	24 (21)	24 (21)	30 (27)	112 (99)
Full-thickness (III°)	1	–	–	–	1
Total n (%)	35 (31)	24 (21)	24 (21)	30 (27)	113 (100)
Size of burns area (%), n = 114/117 (97)					
1–6	22 (31)	16 (23)	13 (19)	19 (27)	70 (61)
7–10	9 (31)	3	7 (24)	10 (34)	29 (25)
11–20	2	5 (36)	4	3	14 (12)
Over 20	–	–	1	–	1
Total, n (%)	33 (29)	24 (21)	25 (22)	32 (28)	114 (100)
Burns severity, n = 117					
Minor	32 (33)	19 (20)	20 (21)	25 (26)	96 (82)
Moderate	2	5 (28)	4	7 (39)	18 (15)
Severe	2	–	1	–	3
Total, n (%)	36 (31)	24 (20)	25 (21)	32 (27)	117 (100)

The EMTs assisted 41 children at the scene and 20 during transport from the ARs to the ward. The EMTs provided assistance at the scene described in only 14 of the 41 children. No information about the way assistance had been provided was available in the medical records of the remaining 27 children and the 20 transported from the ARs to the ward. In the medical records of 14 patients, the EMTs recorded administration of analgesics in 12 out of 14 children. The name of the agent administered was indicated in 11 out of 12 cases: paracetamol in 3 children, ibuprofen in 3, morphine in 3, and dolargan in 2 children. In the records of 1 child, apart from the entry 'analgesic', the name, dose and route of administration were not found. The doses of analgesics administered were recorded in only 2 out of 12 children. Pain was alleviated with monotherapy in ¾ of cases. Combination treatment was reported in 1 child. The route of analgesics administration was determined in 2 cases; administration of other drugs without indicating their names, doses and routes of administration in 2 cases; establishment of iv. access and fluid transfusion in 5 of 14 children; the fluid name and volume in only 1 case, and the information on infusion in 4 cases; and cooling and care of the burn wound in 6 of 14 children (Table 2).

Medical assistance was provided to 2 children at the PHC facilities. In both cases, there was no note of the analgesics administration. The children were transported to the ward by their parents/guardians.

Discussion

Each burn causes tissue damage and pain and affects the psycho-physical sphere; hence, analgesia should be a priority at every stage of medical assistance.[2,4,11–13] Pain causes systemic vegetative reactions in the somatic and mental spheres. No analgesia or ineffective analgesia provision (oligoanalgesia) causes early and late consequences. Patients who experience pain are more likely to also experience anxiety, depression, sleep disturbances, delirium, or persistently lowered pain threshold. In children suffering from pain, there is an increased risk

Table 2. Medical assistance provided in Admission Rooms and by Emergency Medical Teams at the scene of accident

The scope of medical assistance	ARs (n = 42)		EMT (n = 14)	
	n (%)	ND, n (%)	n (%)	ND, n (%)
Administration of analgesics	22/42 (52)	20/42 (48)	12/14 (86)	2
Name:	21/22 (95)	1/22	11/12 (92)	1
Paracetamolum	17/21 (81)	–	3	–
Morphini sulfas	2	–	3	–
Pethidine hydrochloride	1	–	2	–
Fentanylum	1	–	–	–
Ibuprofenum	–	–	3	–
Dose:	3/22	19/22 (86)	2/12	10/12 (83)
Pethidine hydrochloride 20 mg	1	–	–	–
Pethidine hydrochloride 10 mg	–	–	1	–
Paracetamolum 250 mg	1	–	–	–
Paracetamolum 10 mL	1	–	–	–
Ibuprofenum 2.5 mL	–	–	1	–
Single agent:	19/21 (91)	–	8/12 (67)	–
Paracetamolum	16/19 (84)	–	–	–
Morphini sulfas	2	–	–	–
Pethidine hydrochloride	1	–	–	–
Multimodal analgesia:	1	–	1	–
Paracetamolum and fentanylum iv.	1	–	–	–
Ibuprofenum, morphini sulfas, and pethidine hydrochloride	–	–	1	–
Route of drug administration:	10/22 (45)	12/22 (55)	2/14	12 (86)
Rectally (paracetamolum)	7/10 (70)	–	1	–
Subcutaneously (morphini sulfas)	1	–	–	–
Intravenously (paracetamolum)	1	–	–	–
Intramuscularly (pethidine hydrochloride)	1	–	–	–
Orally (ibuprofenum)	–	–	1	–
Other drugs:	4/42	38/42 (91)	2	2
Phenabarbitalum	1	–	–	–
Diazepamum	3	–	–	–
Intravenous access	14/42 (33)	28/42 (67)	5/14 (36)	9 (64)
Fluid transfusion	6/14 (43)	8/14 (57)	5/14 (36)	9 (64)
Name and fluid volume (0.9% NaCl 100 mL):	1	–	1	4
Method of wound dressing:	14/42 (33)	28/42 (67)	6/14 (43)	8 (57)
Dressing	10/14 (71)	–	2	–
Sterile dressing	3	–	1	–
Dressing with sulfathiazolum argentum	1	–	–	–
Hydrogel	–	–	2	–
Wet dressing	–	–	1	–
ECG	1	–	–	–
Admissions to ward	42 (100)	–	–	–
Ambulance transport order for further examination	20/42 (48)	–	–	–

ND – no data; ARs – admission rooms; EMT – emergency medical teams; iv. – intravenously.

of modifying their endogenous regulatory mechanisms. This may lead to enlargement of the somatosensory area of the cerebral cortex responsible for the perception of pain, which causes neurosensory changes, hypoalgesia to thermal stimulus, and hyperalgesia in areas affected by inflammation.[11,12,16]

Despite the progress in medical science, the importance of medical assistance including analgesia is not always understood by medical staff authorized to administer painkillers.[17] An analysis of how medical assistance is provided to burned children in the PHMC period in terms of analgesia may better highlight this problem and further improve the quality of this assistance.

In our study, similar to other researchers, thermal burns resulting from a dousing were predominant, and almost all of the cases were superficial (99%), involving up to 10% of total body surface area (TBSA).[4,8] Pre-hospital medical care was received by 85 children. The remaining 32 children received aid from their caregivers. From the obtained medical records, it was evident that the PHMC in the ARs boiled down to the administration of analgesics – in only half of children, the placement of iv. access, burn wound care and dressing in ⅓ of cases, and issuing of a referral letter for further treatment in the ward in all 42 children. Intravenous access was provided in 14 children, iv. fluids were transfused in every 7 patients, and the name and dose of the fluid transfused was given in 1 case. The recorded PHMC consisted of administering analgesics in 12 cases, applying a dressing to the wound and cooling the burn in 6 cases, and setting up iv. access and fluid transfusion in 5 cases. The name of the analgesic administered was indicated in most cases – 11, the dose in 2 and the name of the fluid transfused in 1 case. In 1 case, the assistance provided in the PHC facilities was limited to referring patients to hospital and protecting the burn wound with a dressing.

Over half of the children in the ARs received analgesics. Preferred was mainly monotherapy with paracetamol, most often including patients with minor burns. Morphine was given only to 2 children with moderate-to-severe and minor burns. Dolargan was administered to 1 child with a minor burn. Multimodal analgesia was recorded in only 1 child with a moderate-to-severe burn. The administration of analgesics was not recorded in the medical histories of 18 children with minor burns, and of 2 moderately burned children.

We found some irregularities in the manner in which the EMTs staff handled the pain. Multimodal therapy was used in only 1 case (Table 2), which indicates that the choice of analgesics was discretionary and not always in line with current recommendations.[18–22]

It could be considered erroneous to administer dolargan or morphine to a child with a minor burn and to desist from giving any drug from the first step of the World Health Organization Analgesic Ladder (WHOAL). According to the WHOAL recommendations, it is optimal for the patient to be administered analgesics of increasing efficacy and in line with the intensity of the pain. For alleviating minor pains, non-opioid agents are recommended, e.g., paracetamol or ibuprofen. In moderate-to-severe burns, a combination therapy is suggested, e.g., paracetamol and a weak opioid drug, e.g., Tramal or Fentanyl administered iv., bucally or intraosseously. To treat severe pain, the combination of an opioid preparation, e.g., Morphine, Dolargan or Fentanyl, with a non-opioid such as ibuprofen, paracetamol or ketamine is recommended.[2,21,23,24] Currently, ketamine cannot be administered by basic EMT staff in Poland.[19] An accepted practice in the treatment of acute pain is to choose the agent and route of administration, including the iv. route, depending on the origin and intensity of the pain experienced, its mechanism, the point of uptake, and the duration time of its action.[2,19,21] It is unreasonable to administer analgesics subcutaneously and rectally, or opioids intramuscularly, especially in moderate-to-severe burns, due to the delayed absorption and peak concentration of rectally administered substances (in the youngest children in particular).[19] The choice of the intramuscular route for Dolargan and the subcutaneous for morphine may have been due to the ease of administration, lack of experience and fear of adverse effects.[3,25] Paracetamol with Metamizol, non-steroidal anti-inflammatory drugs (NSAID) and weak opioids have been shown to reduce opioid doses and to increase analgesic effect and efficacy.[23]

The choice of the appropriate analgesic and the route of its administration does not always correspond to the current recommendations and needs of a child. Medical staff often prefer oral route or non-pharmacological methods. Kiszka et al. noted the relief of burn pain with a hydrogel dressing alone in 15 out of 16 children described.[9] Noskiewicz et al. reported the use of a hydrogel dressing in 96% of 310 children.[8] Different observations were made by Baartmans et al., showing an increase in the administration of analgesics in a group of 622 patients from 68% to 79%, mainly of paracetamol and morphine, especially in burns above 10% of TBSA.[26] The contradiction of the abovementioned observations may be due to doubts among medical staff about when and which analgesics should be used, depending on the type of burn, and when to relieve pain only locally and when in general. According to the literature, monotherapy of pain prevails in burned children during the pre-hospital period. Holak et al. showed monotherapy of pain by the EMT in 53% of 89 burned children, and combination therapy in only 20%.[7] The current recommendation for pain scores of 1–3 on the numerical rating scale (NRS) is to administer non-opioid analgesics orally or per rectum. Instead, to relieve pain of 4–6 points, the NRS recommended the administration of a weak opioid and NSAIDs.[21–24,27] To relieve severe pain of 7–10 points, a combination therapy is recommended, e.g., morphine/fentanyl with paracetamol or iv. metamizole. This strategy has an advantage of reducing the risk of side effects and increasing the extent of the analgesic effect.[21–24,27] In 2019, the Polish Ministry of Health released recommendations for the treatment of pain in children for basic EMTs, which indicate that post-traumatic pain should be relieved regardless of intensity, with iv. or intraosseous

opioids and non-pharmacological methods.[19] In terms of the choice of analgesics, these recommendations are inconsistent with the WHOAL recommendations.[21]

Among healthcare professionals, the problem of oligoanalgesia may result from insufficient understanding of the need for pain management and commitment to pain relief, insufficient education, lack of regular assessment of pain intensity, and misconceptions.[3,7,28,29] Other researchers point to emotional, social, organizational, and environmental causes of pre-hospital oligoanalgesia in children.[11] Giving up or avoiding iv. opioids may be due to the child's age, difficulties in assessing pain intensity or providing iv. access, lack of experience, or the so-called opioidophobia.[5,6,8,14,25] Those concerns were confirmed by Hartshorn et al., who reported more frequent analgesia in children provided with with non-opioid drugs (98%, 3802/3888), oral drugs (48%, 1880), paracetamol (29%, 1118), and ibuprofen (19%, 734).[30]

If iv. access is difficult to provide, fentanyl can be used safely by the sublingual or buccal routes.[21,24] Oligoanalgesia in children can be counteracted by periodic training of the medical staff, by reminding them of their obligation to treat pain, and through stronger enforcement of existing recommendations in clinical pracitce.[12,16,17] Pain management among children and access to analgesics can be improved by a greater emphasis on teaching pain management in training standards.[10]

A series of deficiencies in the way medical records were kept also made it difficult to assess the extent of assistance provided. The ascertained deficiencies included the way in which emergency medical services were provided by the EMTs in all children transported from the ARs to the ward, the administration of any analgesic in almost half of the children, the way in which assistance was provided, the name of the drug administered, the dose, route of administration, as well as the establishment of iv. access, fluid transfusion and the burn wound care. These are problems frequently observed in medical facilities in other regions.[4,31] Holak et al. proved that information on pain management was missing in the medical records in 99% of 2,452 EMT patients.[7]

Pain management in burned children still leaves much to be desired. It requires a coordinated action and systemic interventions at the legislative, organizational and educational levels. The obtained results are the basis for further analyzes and improvement of clinical practice for comprehensive care for children with burns.

Conclusions

1. In burned children, access to analgesic and combined pain therapy is still random, limited, and deviates from current recommendations.
2. Prior to the admission to the ward, pain relief with 1 agent administered rectally prevails.
3. There is a need to standardize the procedures for pre-hospital medical assistance in relation to burned children, including the method of pain management in line with the Polish recommendations.
4. It is necessary to make the medical staff aware of the obligation to keep medical record in a reliable and legal manner.

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Evaluation of patients' awareness and knowledge regarding dental implants among patients of the Department of Prosthetic Dentistry at Wroclaw Medical University in Poland

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Abstract

Background. As dental implants become a more popular treatment option for restoring missing teeth, it is significant for a dentist to understand patients' level of knowledge toward implants, to avoid potential miscommunication and unrealistic expectations.

Objectives. To determine the knowledge level regarding dental implants among patients applying for prosthetic treatment.

Materials and methods. The study was conducted among patients from the Department of Prosthetic Dentistry at Wroclaw Medical University, Poland. A questionnaire composed of 11 questions was distributed to 232 patients, of which 225 were qualified for the study. The chi-square test of independence (χ^2) was used to analyze the association between parameters of interest and groups of patients. The strength of studied relationships was measured with Cramer's V.

Results. Patients showed limited knowledge of implants. Of the respondents, 75.6% heard about dental implants; however, 40% considered the dental implant as a set of a screw with a fixed crown. The major concern was the high cost (69.4%), followed by the need for surgery (31.2%). The Internet was the most popular source of information. The source of treatment financing has a strong correlation to the willingness to undergo treatment.

Conclusions. The study group demonstrated not a satisfactory extent of knowledge regarding dental implants. Dental professionals should make an effort to provide sufficient information to patients to avoid misunderstandings about the treatment. Measures should be taken to reduce the cost of the procedure and thus increase its availability.

Key words: dental implants, prosthetic treatment, patient knowledge

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Background

According to the World Health Organization (WHO) Global Oral Health Status Report (2022), the frequency of edentulism for people aged 60 years and older is 23%, while the rate of people above the age of 20 with complete tooth loss was almost 12% in Poland. World Health Organization report has indicated a proportional and direct relationship between population groups with lower socioeconomic status (social class, lower education level, and income) and oral disease severity (including edentulism), regardless of their country's wealth. The most common causes of tooth loss are untreated dental caries, periodontal disease, dental trauma, and poor health services.[1] The burden of severe tooth loss not only affects masticatory function and appearance but also has an impact on the level of comfort and social life, with many edentulous individuals struggling with involvement in society due to reduced self-esteem and the embarrassment of smiling and speaking.[2]

The most traditional prosthodontic treatment options for replacing missing teeth include removable partial prostheses, complete prostheses, and fixed appliances such as bridges and implants.[3] Dental implants are screws surgically placed in jawbones that provide alternative options for restorations of single tooth gaps, implant-supported overdentures for fully edentulous individuals, and maxillofacial prostheses.[4,5] The broad popularity and acceptance of dental implants as a method of rehabilitation of missing teeth are due to their high rate of treatment success demonstrated by long-term research.[6–9] Thus, it is important to understand patients' level of knowledge and comprehension of dental implants to enhance patient-provider communication and accept the treatment plan.

In Poland, the differences in the oral health status of the population may result from a limited degree of insurance-covered dental procedures. The Polish public health system only finances prosthetic treatment for removable dentures (replacing 5 or more missing teeth). Other prosthetic procedures, such as crowns, bridges, metal frame partial prostheses, and implants, are fully paid for by patients. Consequently, advanced prosthetic care with dental implants is not accessible for the majority of the Polish population, and beneficiaries of the treatment are mainly the wealthy patients.[10]

The most common sources of knowledge about dental implants, indicated in different papers, are relatives/friends, dentists, and the Internet.[11–14] However, expectations and willingness to undergo an implant procedure reflect the adequacy of the patient's awareness.[15] Therefore, it is essential for dental professionals to understand patients' level of knowledge regarding dental implants to avoid potential miscommunication and unrealistic expectations.

Objectives

The present research was conducted to evaluate the level of knowledge and awareness regarding dental implants among patients admitted for prosthetic treatment. There are no reports on this subject in the literature, so this is the first study of this kind on the Polish population. Nevertheless, there are many related studies in other countries. The study hypothesis was that patients admitted for prosthetic treatment had an inadequate extent of knowledge regarding dental implants.

Materials and methods

Study design, area, and population

The study was conducted in the form of a self-administered survey among patients of the Department of Prosthetic Dentistry at Wroclaw Medical University, Poland. Data were collected from November 2022 to February 2023. The Department of Prosthetic Dentistry is responsible for preparing prosthetic treatment plans and directing patients to the Department of Dental Surgery if implants are planned. The inclusion criteria were patients interested in participating in the study and those aged 20 and above. The exclusion criteria were age below 20, mental disability, and returning incomplete questionnaires.

Participants and sample size

The total number of participants involved in the study who met the requirements was 225 (146 women and 79 men). Every patient in the Department of Prosthetic Dentistry waiting hall was invited to participate in the study to avoid sampling bias. All respondents were informed about the aim of the study, which was conducted in accordance with the principles of the Declaration of Helsinki, and all patients consented to participate.

Survey tool

A total of 232 questionnaires were distributed to the patients, of which 225 were qualified for the study. The survey consisted of 11 questions and was designed to obtain data about socio-demographic characteristics (5 questions on gender, age, and educational background), awareness of dental implants, and attitude to implant treatment (6 questions regarding dental implants) (Table 1).

Statistical analyses

The χ^2 test was used to analyze the association between parameters of interest and groups of patients. The strength of studied relationships was measured with phi coefficient

and Cramer's V. Phi was used to examine the association between 2 dichotomous variables and Cramer's V when there was more than a 2×2 contingency. A p-value of <0.05 was considered statistically significant. The graphics were produced using Microsoft® Excel® 2019 (Microsoft Corporation, Redmond, USA).

Results

Demographic structure of the study group

The total study group was comprised of 146 women (64.9%) and 79 (35.1%) men. The largest response group represented people between the ages of 61–70 (36%), the 2nd largest group was over 70 (25.3%), 15.1% were between 51–60, 13.8% between 20–40, and the smallest group was between 41–50 (9.8%). Of all participants, 29.3% had a higher level of education, 63.6% gained secondary education, and 7.1% completed only primary education. Table 1 summarizes the demographic structure of the group.

Table 1. Demographic structure of the sample

Parameter		Number of patients	(%)
Age [years]	20–40	31	13.8
	41–50	22	9.8
	51–60	34	15.1
	61–70	81	36.0
	>70	57	25.3
Gender	Female	146	64.9
	Male	79	35.1
Education	Primary	16	7.1
	Secondary	143	63.6
	High	66	29.3

Knowledge and attitude

In the present study, 75.6 % of the respondents heard about dental implants, of which 65.9% were female and 34.1% were male. There was no significant difference between men and women (p = 0.583) (Table 2), although 24.4% of patients were not aware of implants. The general knowledge of dental implants was measured with 2 questions on what an implant is and where it is anchored in the mouth. Most participants (40%) considered dental implants to be a screw with a fixed crown. The answers "a screw replacing tooth root" and "a denture" were given by 21.8% of the respondents, followed by 6.2% who answered "a crown," while 10.2% of respondents answered "I had no idea". The difference between genders was insignificant (p = 0.543).

Table 2. The effect of gender on the respondents' awareness of dental implants

Is the patient aware of the implants?	Female, n (%)	Male, n (%)	Total, n (%)	Significance
Yes	112 (76.7)	58 (73.4)	170 (75.6)	$\chi^2 = 0.301$ df = 1 p = 0.583 Φ = 0.036
No	34 (23.3)	21 (26.6)	55 (24.4)	

Regarding anchoring of dental implants in the mouth, 60.4% of participants responded "in the jawbone," 14.7% thought it is placed "in the gum," and almost 1/4 (24.9%) did not know the answer. There was a significant difference between men and women (p ≤ 0.001) in favor of women. Only 17.8% of respondents correctly answered both questions, indicating a basic knowledge of implants. The chi-squared test indicated a significant relationship between participants' knowledge about dental implants and education level (p = 0.002; V = 0.240) (Fig. 1), with results being in favor of higher education. Participant age affected knowledge (p = 0.002, V = 0.276) (Fig. 2), as most correct answers were presented in the 41–50 group and showed a downward trend to the lowest score in those over 70. However, the effect size was considered small for both. There were no significant differences between men and women (p = 0.727) (Table 3).

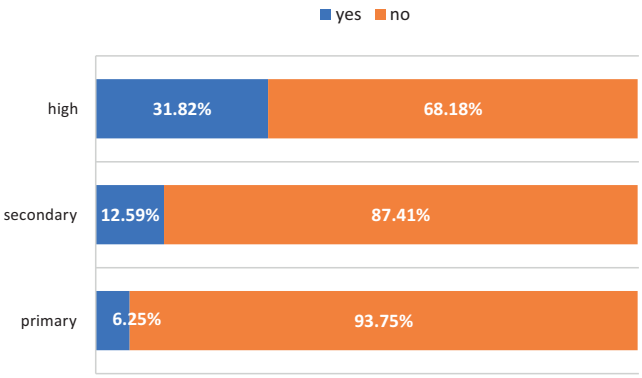

Fig. 1. Percentage of correct answers on general knowledge about implants depending on the education level of the patients

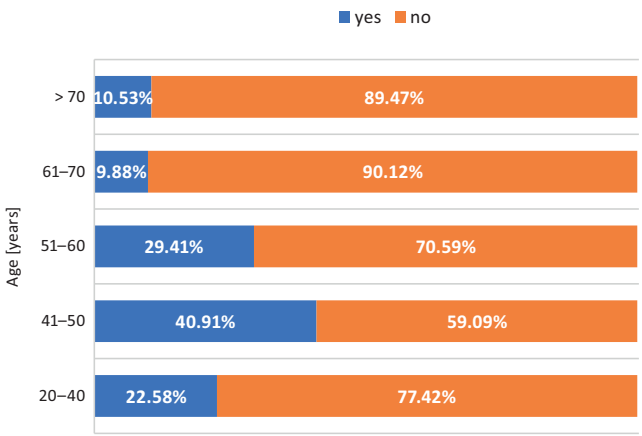

Fig. 2. Percentage of correct answers on general knowledge about implants depending on the age

Table 3. The effect of gender, age and education on the respondents' knowledge about dental implants

Parameter		Yes	No	Significance
Gender	Female, n (%)	25 (17.1)	121 (82.9)	$\chi^2 = 0.122$ df = 1 p = 0.727 Φ = 0.023
	Male, n (%)	15 (19.0)	64 (81.0)	
	Total, n (%)	40 (17.8)	185 (82.2)	
Age [years]	20–40, n (%)	7 (22.6)	24 (77.4)	$\chi^2 = 17.200$ df = 4 p = 0.002 V = 0.276
	41–50, n (%)	9 (40.9)	13 (59.1)	
	51–60, n (%)	10 (29.4)	24 (70.6)	
	61–70, n (%)	8 (9.9)	73 (90.1)	
	>70, n (%)	6 (10.5)	51 (89.5)	
Education	Primary, n (%)	1 (6.2)	15 (93.8)	$\chi^2 = 12.991$ df = 2 p = 0.002 V = 0.240
	Secondary, n (%)	18 (12.6)	125 (87.4)	
	High, n (%)	21 (31.8)	45 (68.2)	

n – number; df – degrees of freedom; V – Cramer's V.

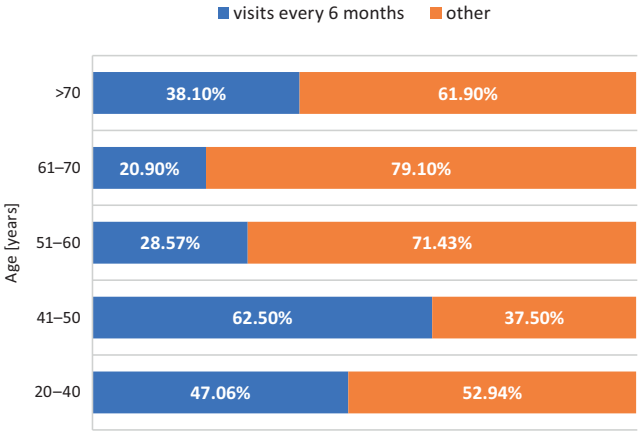

Fig. 3. Follow-up appointments

Out of the 170 participants who were aware of dental implants, almost half (48.2%) indicated that implants require a similar hygiene routine as natural teeth, 15.3% thought that implants need less care, and 11.8% thought more care would be needed. However, 24.7% of respondents had no idea about how to take care of implants.

Regarding the follow-up visit frequency, only 32.4% of participants thought a dental check-up is needed every 6 months, including 31.3% in the female group and 35.1% in the male group (Fig. 3). The difference between genders was not significant (p = 0.669). Few patients (17%) would visit the dentist once a year, while 5.9% saw no need to have a check-up, and most respondents (44.7%) had "no idea" how often they should attend. Nevertheless, the χ^2 test indicated a significant relationship between participant age and knowledge of the frequency of follow-up appointments (p = 0.011; V = 0.277), with results shown in Table 4. There was no correlation between the level of education (secondary and higher) and knowledge (p = 0.180).

To assess patients' major concerns regarding implant treatment, we asked a multiple-choice question. The 2 issues stated by the participants were high cost (69.4%) and

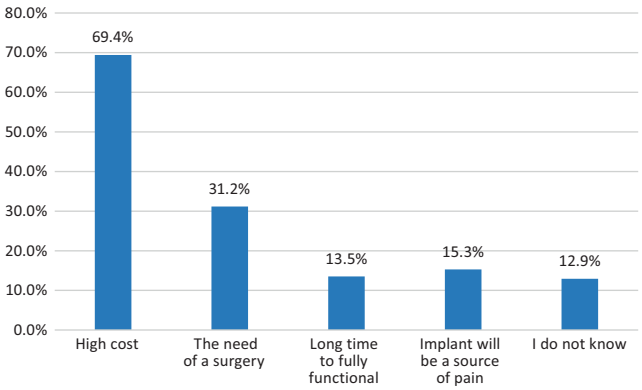

Fig. 4. Respondents' major concerns regarding implant treatment

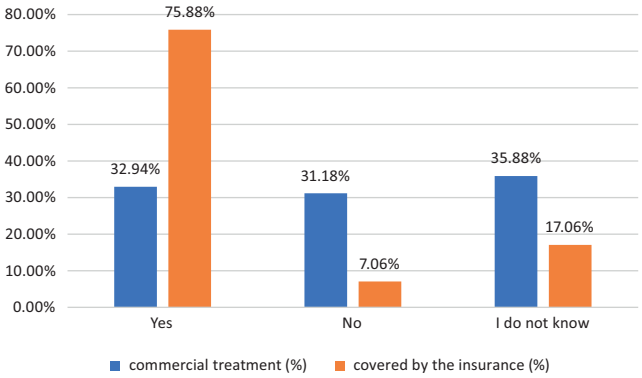

Fig. 5. Patient willingness to undergo an implant treatment depending on the source of financing

the need for surgery (31.2%), with 15.3% of respondents replying: "I'm afraid the implant will be a source of pain," 13.5% stating: "It takes time to complete the treatment and have a fully functional implant," and 12.9% (22 out of 170) replying: "I do not know" (Fig. 4).

Patient willingness to undertake an implant treatment depending on the source of financing is shown in Fig. 5. Regarding commercial treatment, 32.9% would like to have the implant if offered, 31.2% rejected it, and 35.9% did not know. However, when we asked if they would undergo treatment if the procedure was covered by the insurance, 75.9% answered affirmatively, only 7.1% rejected it, and 17% did not know. The χ^2 test indicated a strong relationship between the respondent's willingness and the source of treatment financing (p < 0.005). There were insignificant differences between men and women in commercial (p = 0.703) and insurance-covered treatment (p = 0.447). We did not find a relationship between the education level (secondary and high) and willingness for commercial (p = 0.143) or insurance-covered treatment (p = 0.111).

Sources of information

Regarding the question on the source of information about dental implants, $^1/_3$ of the respondents (33.5%) indicated the Internet. The 2nd common source was a dentist

Table 4. Patient questionnaire to evaluate knowledge regarding implants

Question	Answers	Female, n (%)	Male, n (%)	Total, n (%)	Significance, n (%)
What is, in your opinion, a dental implant?	a screw replacing tooth root	30 (20.6)	19 (24.1)	49 (21.8)	$\chi^2 = 0.369$ df = 1 p = 0.543 Φ = 0.041
	a screw with fixed crown	64 (43.8)	26 (32.9)	90 (40.0)	
	a denture	31 (21.2)	18 (22.8)	49 (21.8)	
	a crown	9 (6.2)	5 (6.3)	14 (6.2)	
	I do not know	12 (8.2)	11 (13.9)	23 (10.2)	
Where is dental implant placed in the mouth?	in the jawbone	93 (63.7)	14 (17.8)	107 (47.5)	$\chi^2 = 43.449$ df = 1 p ≤ 0.001 Φ = 0.044
	in the gum	19 (13.0)	43 (54.4)	62 (27.6)	
	I do not know	34 (23.3)	22 (27.8)	56 (24.9)	
How would you describe the care of the implant compared to natural tooth?	similar as natural teeth	57 (50.9)	25 (43.1)	82 (48.2)	$\chi^2 = 0.879$ df = 1 p = 0.348 Φ = 0.073
	more care	16 (14.3)	4 (6.9)	20 (11.8)	
	less care	17 (15.2)	9 (15.5)	26 (15.3)	
	I do not know	22 (19.6)	20 (34.5)	42 (24.7)	
How often should you visit your dentist?	every 6 months	35 (31.3)	20 (34.5)	55 (32.4)	$\chi^2 = 0.182$ df = 1 p = 0.669 Φ = 0.033
	once a year	20 (17.8)	9 (15.5)	29 (17.0)	
	no need to have a checkup	4 (3.6)	6 (10.3)	10 (5.9)	
	I do not know	53 (47.3)	23 (39.7)	76 (44.7)	
Would you agree to have an implant if it was a commercial treatment?	yes	38 (33.9)	18 (31.0)	56 (32.9)	$\chi^2 = 0.145$ df = 1 p = 0.703 Φ = 0.029
	no	34 (30.4)	20 (34.5)	53 (31.2)	
	I do not know	40 (35.7)	20 (34.5)	61 (35.9)	
Would you agree to have an implant if it was covered by the insurance?	yes	87 (77.7)	42 (72.4)	129 (75.9)	$\chi^2 = 0.579$ df = 1 p = 0.447 Φ = 0.058
	no	8 (7.1)	4 (6.9)	12 (7.1)	
	I do not know	17 (15.2)	12 (20.7)	29 (17.0)	

n – number; df – degrees of freedom; Φ – phi coefficient.

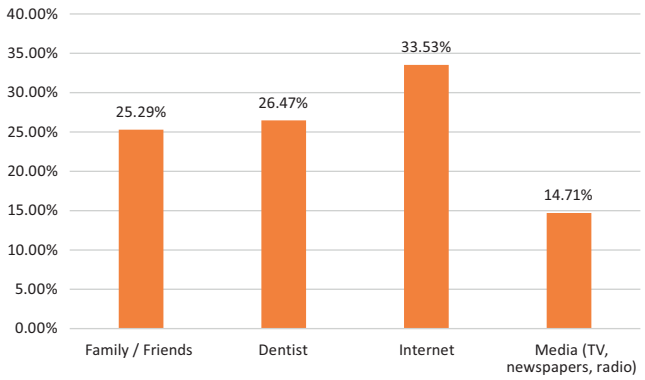

Fig. 6. Respondents' sources of information about dental implants

(26.5%), followed by family/friends (25.3%), and the media (television, newspapers, and radio) (14.7%) (Fig. 6).

Discussion

Today, dental implants are receiving widespread attention from professionals and patients due to their many advantages over traditional prosthetic restorations.[16] Using implants to treat partial and complete edentulous patients brings many benefits through improving biting force, preventing alveolar bone loss, enhancing phonetics, better esthetics, and high treatment rate success.[3,17] Indeed, implant-supported prostheses provide better masticatory efficiency, retention, and higher comfort of use compared to conventional dentures.[3,15,16] Moreover, a recent study among elderly people in Japan indicated an association between reduced chewing ability and impaired cognitive processes.[18] Therefore, it could be stated that implant-based restorations not only significantly improve oral functions but also broadly defined quality of life.

Appropriate patient knowledge of implants is crucial for avoiding negative perceptions of the procedure and treatment process that could arise due to a lack of reliable information.[3,19] This study showed that 75.1% of respondents had heard about dental implants, which is similar to results from other countries, where the implant awareness rate was 77% in the USA,[20] 72–79% in Austria,[21,22] 70.1% in Switzerland,[13] and 70.7% in Norway.[23] The study group demonstrated an unsatisfactory level of dental implant knowledge, meaning the research hypothesis was accepted. The outcome could be related to the low socioeconomic background of the group, as most respondents (61%) were above the age of 60 and without a higher education status (71%). As shown in the results, only 40 participants (18%) knew that the implant is a screw that replaces the tooth root and is anchored in a jawbone. We also found that 40% of patients considered dental implants as a whole, the screw and

a crown. Therefore, clear communication between dental professionals and patients is crucial to avoid misunderstanding and confusion during treatment planning and financing.

Figure 1 demonstrates that the number of correct answers increased with increasing education levels. Regarding patient age, most correct answers were given by the 41–50 group and decreased with increasing age. This finding may be due to the greater awareness in this age group of their dental needs, as they are at the peak of their professional activity, are more interested in various treatment options, and are willing to seek information on this subject. These findings agree with other studies indicating that people with a higher degree, aged over 50, and living in urban areas had better implant knowledge.[15,24] However, it is worth considering that in this study the patients were randomly selected in the Department of Prosthetic Dentistry. Thus, the results could be different among patients who received implants in the past. Indeed, studies focused on comparing patients' perception of implants to their experience with such treatment indicated that people with implant treatment history were better informed and more willing to undergo another procedure in the future.[16,19,25,26] On the other hand, dental implants were described as "scary," "expensive," and "painful" by patients who have never had one.[19]

Of the respondents, almost 45% stated that they do not know how often they should have a dental check-up, and around 6% thought that there is no need for further dental appointments after implant treatment. This could be explained by findings in other studies, which reported patients' problem-oriented check-up behavior and pain as a common reason for visiting the dentist.[27,28] In contradiction to our findings of insignificant differences between genders, some studies[29–31] indicated that women visit the dentist more frequently than men and show greater awareness of oral health.

Our survey showed that the primary source of information about implants was the Internet (33.5%), followed by the dentist (26.5%), relatives or friends (25.3%). It is reported that wide Internet access encourages patients to seek online health information,[11,32] even among those aged over 75,[33] which is consistent with our findings. In contradiction to our study, different results were shown in other countries, with family and friends as a primary source of information and dental professionals a secondary source in Switzerland (52.3% friends and 40% dentist),[13] Saudi Arabia (45.5% friends and 36% dentist),[34] Nepal (30.2% friends and 17.7% dentist),[12] and Sudan (38.2% friends and 35.7% dentist).[35] Conversely, studies in Austria,[21] India,[36] and China,[37] indicate dentists as a main source of knowledge (68%, 54.6%, and 42%, respectively). Thus, dental professionals should demonstrate adequate knowledge of implantology and be aware of the importance of their role as patient educators.

In this study, most patients stated the high cost (69.4%) of implant therapy as their main concern, followed by anxiety about the need for a surgical procedure (31.2%). Comparable results were reported in similar studies from other countries.[12,19,38–40] Due to a significant number of elderly people participating in this study, the results of Müller et al. are noteworthy.[13] It was stated that objections to implants of geriatric respondents were the expense of the procedure, the perception that undergoing such dental treatment was meaningless, and that they were too old.

Limitations

The findings of this study have to be seen in light of some limitations. The 1st is the limited access to patients with various socioeconomic status because the study was conducted in the Faculty of Dentistry at Wroclaw Medical University. To reach Polish patients with different socioeconomic backgrounds, the survey should be conducted in many dental offices in other cities and small towns. More diversified sample testing would better reflect the general population, which leads to the second limitation, i.e., the insufficient sample size. The group of patients with primary education levels with awareness of dental implants was too small to run a statistical test and be considered representative of the population. Thus, we could not identify a relationship regarding patients' education level. To overcome this limitation, the study should be carried out over a longer period of time and on a larger sample.

Conclusions

This study showed that patients were not adequately informed about implants, and variables such as age, education level, and gender influenced their awareness. As the Internet becomes a prevalent source of health information, dental professionals must provide sufficient information to patients to fill gaps and correct their knowledge on implant treatment. Misunderstandings about the treatment and its costs could be due to the terminological gap between the dentist and the patient. Since most patients fear the expense of implant treatment, measures should be taken to reduce the cost of the procedure to increase its availability.

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