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Original Article

Protective and Therapeutic Effects of Betulinic Acid on Acetic Acid-Induced Experimental Colitis Model

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Abstract

Objective: The aim of this study is to investigate the protective and therapeutic effect of betulinic acid on acetic acid-induced colitis.

Methods: Thirty-two *Wistar albino* male rats were randomly divided into 4 groups as follows: sham group (n=8; physiological saline solution enema), colitis group (n=8; 2 mL/days 4% acetic acid enema for 3 days), colitis+betulinic acid group (n=8, 2 mL/days 4% acetic acid enema for first 3 days and then peroral 3 mg/kg/days betulinic acid for next 3 days), betulinic acid+colitis group (n=8, peroral 3 mg/kg/days betulinic acid for first 3 days and then 2 ml/days 4% acetic acid enema for next 3 days). On day 7, laparotomy was performed and distal colonic segment including rectum was resected. All groups were compared with respect to tissue malondialdehyde, glutathione, total antioxidant status, total oxidant status, oxidative stress index, and histopathological findings.

Results: Statistically significant differences were not found between the groups in terms of rat weight (P = .516), tissue malondialdehyde (P = .203), tissue glutathione (P = .056), tissue total antioxidant status (P = .168), tissue total oxidant status (P = .810), oxidative stress index (P = .562), and histological score (P = .244).

Conclusion: Although the protective and therapeutic effect of betulinic acid has not been reach statistically significant levels, histopathological features and levels of some biochemical parameters (glutathione, total antioxidant status, and total oxidant status) show that the betulinic acid administration before the creation of the colitis is relatively better.

Keywords: Acetic acid, betulinic acid, experimental colitis, oxidative stress, rat.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease that progresses with inflammation in the intestinal mucosa.[1] Although it is thought that many factors play a role in the etiopathogenesis alone or together, the etiology of IBD is still not clear. However, in individuals with genetic predisposition, changes occurring in the intestinal barrier functions as a result of uncontrolled and widespread inflammation developing in the gastrointestinal tract with the effect of environmental factors are the most reasonable theory to explain the pathogenesis of IBD.[2]

In inflammation developing during IBD attacks, inflammatory mediators, oxidative stress, changing colonic flora, abnormal glycoprotein accumulation in the mucosa, decreased oxidation of short-chain free fatty acids, increased intestinal permeability, increased sulfide production, and decreased methylation play a role in inflammation. It is not possible to explain the development of ulcerative colitis (UC) with a single mechanism.[3]

One of the important factors playing a role in UC pathogenesis is the butyric acid level. Butyric acid is a 4-carbon short-chain free fatty acid that is produced by fermentation of fiber and carbohydrates taken by the healthy colon. It consists of propionic acid and acetic acid. Butyric acid is the primary fuel of colonocytes. However, it is also used for ion transfer, mucus synthesis, phase II detoxification, and lipid synthesis required for cell membrane integrity.[4]

Defective butyrate oxidation plays a role in disease development. Sulfate-reducing bacteria, which are found in high levels in patients with UC, increase the amount of hydrogen sulfide. This molecule inhibits mitochondrial butyric acid synthesis in the intestinal mucosa.[5]

Betulinic acid (BA) is naturally found in herbs in 3 hydroxyl pentacyclic triterponeic acid structures.[6] It constitutes 30% of the dry weight of the birch. It is a natural tripterpene with anti-neoplastic, anti-human immunodeficiency virus, anti-malarial, and anti-inflammatory effects. Due to its biological feature, it has attracted a lot of attention in the pharmaceutical industry in recent years.[7] Betulinic acid belongs to the plants belonging to the genus *Betula* spp. (birch tree), which has been used in traditional medicine since ancient times. It is isolated from *Ziziphus* ssp, *Syzygium* ssp, *Diospyros* ssp, *Triphyophllum peltatum, Ancistrocladus heyneanus, Tovomita krukovii, Ipomea pes-caprae, Rosa canina,* and *Rosmarinus officinalis*.[8]

Although IBD is a common disease, unfortunately, there is no regressive standard treatment regimen for its treatment. As the disease progresses with relapses and the serious side effects of the drugs used in treatment, searches for new drugs or agents continue. Compounds of natural origin are drugs used clinically in the treatment of diseases by showing various biological activities. It has been shown in many studies that BA in terpenic nature, which is one of the natural origin compounds, has anti-inflammatory and anti-oxidant effects. In this study, we aimed to investigate whether BA has a protective or therapeutic effect in the experimental colitis model created by using an acetic acid enema.

METHODS

Animals

In this study, 32 *Wistar albino* male rats, aged between 11 and 12 weeks and weighing between 230 g and 300 g, were obtained. The rats were housed in laboratories where they could freely access standard rat food and water. All procedures applied to the rats were performed in accordance with the Animal Research Guidelines of the National Institute of Health. Simple randomization technique was used to create the experimental groups.

Medicines and Chemicals

Betulinic acid was obtained from Sigma-Aldrich (Sigma Aldrich, Betulinic Acid, CAS number: 472-15-1). Serum solution containing 5% dimethyl sulfoxide (DMSO) (Dimetil sülfoksit) was used as a solvent for BA. Acetic Acid (AA) was obtained from Sigma-Aldrich (Sigma Aldrich, Acetic acid ≥99.5%, CAS number: 64-19-7). Distilled water was used as a solvent for AA.

Creating the Colitis Model

Acetic Acid was used to induce acute colitis in rats. Intrarectal and oral administration to rats was performed with a 6G nelaton catheter under mild anesthesia [mixture of ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg)]. A 6-cm catheter was inserted into the anus and 2 cc AA followed by 1 mL of air, and then the rats were held for 15 minutes in the Trendelenburg position. Betulinic acid was also administered with orogastric gavage as a 3 mg/kg dose once a day under mild anesthesia. Acetic acid and BA dosages were adjusted according to previous dose–response studies.[9]

MAIN POINTS

- Ulcerative colitis is a common gastrointestinal disease.
- This disease disrupts the patient's standard of living.
- Betulinic acid, an easily available active ingredient, can be used to control this disease.

Institutional Approvals

Ethical approval for the study has been obtained from the Committee on Animal Research at Inonu University, Malatya, Turkey (date: October 1, 2020, decision number: 2016/ A-52).

Experimental Design

Thirty-two rats were randomly divided into 4 groups, with an equal number of rats (n = 8) in each group. The rats in the control group (sham group) were given 2 mL of saline solution containing 5% DMSO rectally for 3 days. Rats in the colitis group (colitis group) were given rectally 4% AA for 3 days, with a single dose (2 mL) per day. The rats in the colitis + BA group (colitis + BA) were given rectally 4% AA in a single dose (2 mL) daily for 3 days. Twenty-four hours after the colitis model, 3 mg/kg/day BA was given orally for 3 days and once a day. The rats in the BA + colitis group (BA + colitis) were given a single dose of 3 mg/kg BA once daily for 3 days. Twenty-four hours after the end of oral BA treatment, 4% AA was given rectally with a single dose (2 mL) daily for 3 days to begin. On the seventh day of the experimental study, the rats were administered ketamine-based anesthesia and laparotomy was performed using a midline incision. During the laparotomy, a 10-cm large intestine section was resected, including the rectum. The rats were then sacrificed by administering an excess dose of ketamine. Some of the colectomy specimens were fixed with formalin for histopathological analysis, while the remaining part was stored at −70°C for biochemical analysis.

Definition of Experimental Colitis Model

Colitis created with AA may cause necrosis and transient inflammation in the epithelium or mucosa by instillation of dilute AA into the lumen, depending on the dose. In the form of an enema, 4% AA is applied for 15-30 seconds, higher concentrations often cause perforation. Excess fluid is withdrawn 30 seconds after exposure and the large intestine is washed under pressure with 1.5 mL phosphate-buffered saline or saline.[10] The first damage in this model is relatively mild epithelial necrosis and edema. As the concentration of AA increases, the damage to the external muscle layer in the lamina propria increases. Damage to the epithelium develops in proportion to the specific reaction to organic acids. Because HCl at similar pH does not cause damage in the same way.[11] Short-term local ischemia contributes to acute damage, but neutrophils do not interfere with this event in the very early phase. Inflammation in the mucosa and submucosa occurs following the initial damage and is associated with the activation of arachidonic acid pathways. Colitis created with AA is one of the easiest models to create the IBD model and is similar to IBD in terms of the profile of inflammatory mediators. The inflammatory phase shows some similarities to acute intestinal inflammation in humans.[10]

Biochemical Analysis Techniques

Malondialdehyde Measurement: Malondialdehyde (MDA), which is the indicator of lipid peroxidation, was studied according to the method of Uchiyama and Mihara.[12] The rat tissue sample was homogenized on ice for 1 minute at 15 000 rpm to form a 10% homogenate in 1.15% KCl solution. This homogenate was used directly in MDA analysis. The contents of the sample were as follows: homogenate (250 µL), 1% phosphoric acid (1500 µL), and 0.6% TBA (1500 µL). The prepared solutions were added to the test tubes, vortexed, and the tubes were kept in boiling water (at least 95 °C) for 1 hour. Tubes were added with 2 mL of n-butanol and vortexed for 5 minutes. Then

samples were centrifuged at 3000 × g for 10 minutes. By reading the absorbances of the samples at 535 nm and 520 nm in the spectrophotometer, MDA concentrations were evaluated from the standard chart prepared with 1,1,3,3-tetrametoxypropane and the results were given as nanomolar per gram of wet tissue.

Glutathione Measurement: Glutathione (GSH) was measured according to Ellman's method.[13] Samples were homogenized for 1-2 minutes at 15 000 rpm on ice to form a 10% homogenate from the rat tissue sample. Then, the homogenate was centrifuged at 3000 rpm at +4° for 15 minutes. Trichloroacetic acid (TCA) and (5,5′-dithiobis(2-nitrobenzoic acid (DTNB) solution was added to the supernatant obtained, and mixed and the sample was prepared again for GSH analysis by centrifugation. The content of the sample was as follows: 10% homogenate (500 µL), Na_2HPO_4 (0.3 M) (4 mL), and DTNB (500 µL). The contents of the blind solution were Na_2HPO_4 (0.3 M) (4 mL), DTNB (500 µL), and distilled water (500 µL). The prepared solutions were added to the test tubes, vortexed, and the intensity of the color formed after 5 minutes was read at a spectrophotometer at 410 nm, and the results were assessed from the glutathione standard graph and given as nanomolar per gram of wet tissue.

Measurement of Total Antioxidant Status, Total Oxidant Status, and Oxidative Stress Index

Total antioxidant status (TAS) was assessed using Biotek HT Synergy Gen 5 with software, immunos plate reader, and TAS kit set (Rel Assay Diagnostics, Turkey). With this kit, the ABTS+(2,2'-azinobis (3-et hylbenzothiazoline-6-sulfonate)) molecule, which is reduced in an acidic medium (acetate buffer 30 mmol/L; pH 3.6) using only hydrogen peroxide, turns into an oxidized form. Concentrated (dark green) ABTS+molecules in the acetate buffer solution remain stable for a long time. When the higher pH is diluted with a denser acetate buffer (acetate buffer 0.4 mol/L; pH 5.8), it gradually turns lighter on its own. The antioxidants present in the analyzed sample increase the decrease in this color to some extent in proportion to their concentration. This reaction can be monitored spectrophotometrically, and the rate of opening in color is proportional to the total TAS in the sample. The test results in the absorbance change read at 660 nm in the microplate reader were calibrated with the Trolox solution, a standard antioxidant and a vitamin E analog, used for TAS measurement tests. Results were expressed in millimolar Trolox equivalent/L units.[14]

Total oxidant status (TOS) measurement was performed using Biotek HT Synergy Gen 5 immuno plate reader with software kit set and TOS (Rel Assay Diagnostics). Exemplary oxidants with this kit oxidize the complex to ferrous ion-o-dianicidine ferric ions. The oxidation reaction is carried out by glycerol molecules in the reaction medium. Ferric ions in an acidic medium form a colored complex with xylenol orange. The color density, which can be measured spectrophotometrically, is proportional to the total amount of oxidant molecules in the sample. The experiment was calibrated with hydrogen peroxide and the results were expressed in micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 equivalent/L).[15]

Oxidative stress index (OSI) is an indicator parameter of oxidative stress degree and its calculation is as in the formula. Oxidative stress index was calculated using TAS and TOS kits.

The percentage ratio of the TOS to TAC yields the OSI, an indicator of the degree of oxidative stress. OSI (arbitrary unit)=TOS (µmol H_2O_2 equivalent/L)/TAS (mmol Trolox equivalent/L). The OSI value for the lung samples was also calculated as OSI (arbitrary unit)=TOS (µmol H_2O_2 equivalent/L)/TAS (mmol Trolox equivalent/L).[16]

Histological Analysis

The tissues taken at the end of the experiment were fixed in 10% formaldehyde. After tissue follow-up, sections of 4-5 µm thickness were taken from the prepared paraffin blocks. Sections were stained with the hematoxylin–eosin staining method to determine the general morphological structure. Scoring was performed based on inflammatory cell infiltration and severity of inflammation in mucosa and submucosa. (0 score: no change, 1 score: mild, 2 score: medium, and 3 score: severe). Evaluations were made using the Leica DFC-280 research microscope and the Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Statistical Analysis

Statistical analyses were performed with IBM Statistical Package for the Social Sciences version 25.0. (IBM SPSS Corp.; Armonk, NY, USA). Quantitative variables were presented as mean ± standard deviation, median, minimum-maximum (min-max), and interquartile range. Kruskal–Wallis test was used to compare non-normally distributed quantitative variables in all groups. The chi-square test was used to compare the qualitative variables in all groups. A *P* value of less than .05 was considered statistically significant.

RESULTS

Rat Weight and Tissue Biochemical Results

The results were presented in Tables 1 and 2. Briefly, statistically significant differences were not found between the groups in terms of rat weight (*P* = .516), tissue MDA (*P* = .203), tissue GSH (*P* = .056), tissue TAS (*P* = .168), tissue TOS (*P* = .810), and OSI (*P* = .562).

Histological Results

Statistically significant differences were not found between the groups in terms of histological scores (*P* = .244). However, although it has not reached a statistically significant level, the inflammation scores in the BA+colitis group were relatively lower than in other experimental groups. When the groups were interpreted in terms of histopathological features, the colon wall was observed in the normal histological view in the control group (Figure 1A). In numerous samples belonging to the colitis group, inflammatory cell infiltration was observed in the mucous and submucosa layers (Figure 1B). On the other hand, it was noted that the number of samples with infiltration observed in BA-treated groups decreased, whereas this decrease was more pronounced in the BA+colitis group (Figure 1C-D). Histopathological score results are given in Table 3.

DISCUSSION

It is well known that UC is an inflammatory disease. This inflammation is affected by increased cytokines, free oxidant radicals, and decreased

Table 1. Comparison of the Body Weights of the Rats

Body Weight (g)	Sham	Colitis	Colitis+BA	BA+Colitis	*P*
Mean ± SD	385 ± 39	359 ± 40	366 ± 30	374 ± 45	.516
Median	398	365	368	370	
Min-Max	330-440	300-415	330-430	315-468	
IQR	67	77	38	69	

SD, standard deviation.

Table 2. Tissue Biochemical Parameters

Parameters	Sham	Colitis	Colitis+BA	BA+Colitis	*P*
MDA (nmol/g wet tissue)					.203
Mean ± SD	274 ± 132	192 ± 117	365 ± 237	239 ± 154	
Median	281	140	347	149	
Min-Max	121-456	82-403	114-786	29-436	
IQR	269	185	409	407	
GSH (nmol/g wet tissue)					.056
Mean ± SD	1037 ± 40	1095 ± 63	1090 ± 38	1061 ± 56	
Median	1050	1099	1103	1081	
Min-Max	989-1091	997-1184	1009-1140	932-1131	
IQR	76	117	61	54	
TAS (mmol Trolox Equiv./L)					.168
Mean ± SD	0.63 ± 0.19	0.87 ± 0.36	0.76 ± 0.35	0.66 ± 0.19	
Median	0.65	0.85	0.70	0.66	
Min-Max	0.36-0.84	0.57-1.84	0.08-1.30	0.37-1.00	
IQR	0.398	0.411	0.439	0.299	
TOS (μmol H_2O_2 Equiv/L)					.810
Mean ± SD	15.5 ± 6.5	18.2 ± 20	16.4 ± 8.2	13.2 ± 4.1	
Median	14.9	11.5	13.8	12.2	
Min-Max	9.2-27.8	8.4-81.2	8.8-36.6	8.9-23.3	
IQR	9.58	5.91	7.53	4.84	
OSI (arbitrary unit)					.562
Mean ± SD	27.8 ± 15.6	18.8 ± 9.2	36.6 ± 52	22.3 ± 11.1	
Median	23.2	17.6	18.5	20.9	
Min-Max	11.3-54.0	9.3-44.2	7.7-191	9.3-47.6	
IQR	29	6.5	22	17	

MDA, malondialdehyde; GSH, glutathione; TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index; SD, standard deviation.

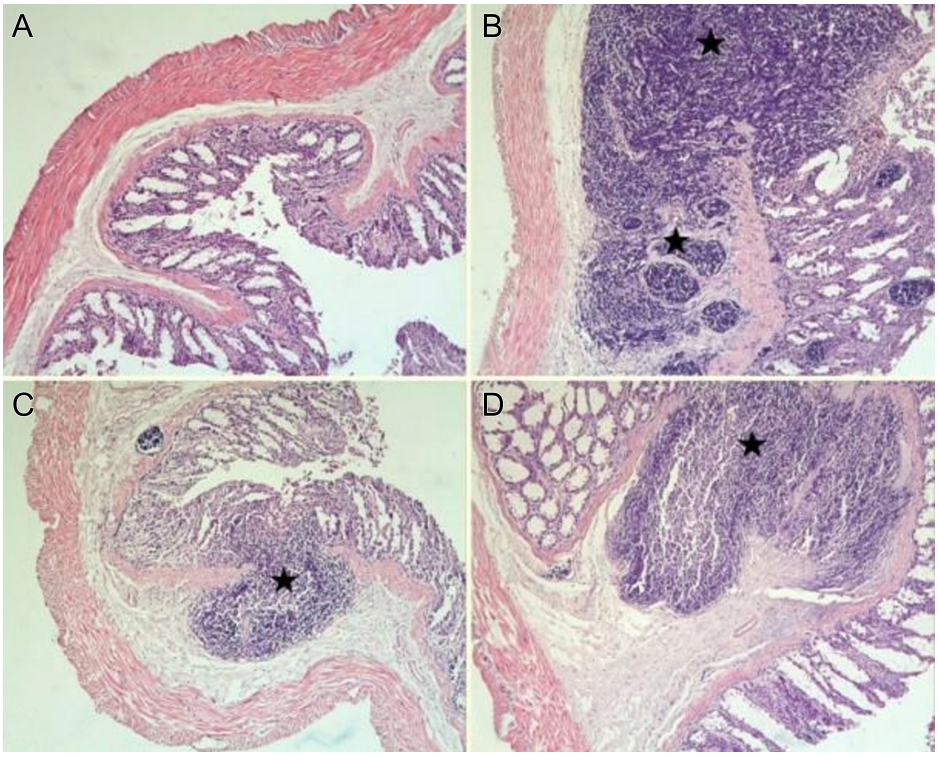

Figure 1. Normal histological view of the colon wall in the control group (A). Inflammatory cell infiltration is observed in the mucous and submucosa layers in the colitis group (B). It is noteworthy that infiltration decreases in BA+colitis (C) and colitis+BA (D) groups and this decrease is more pronounced in BA+colitis group. The stars point to infiltration areas (hmatoxylin an eosi, ×10).

Table 3. Histopathological Score Results

Histologic Score	Sham	Colitis	Colitis+BA	BA+Colitis	*P*
0 score	6 (75.0)	3 (37.5)	3 (37.5)	5 (62.5)	.244
1 score	2 (25.0)	2 (25.0)	2 (25.0)	2 (25.0)	
2 score	0 (0.0)	1 (12.5)	3 (37.5)	1 (12.5)	
3 score	0 (0.0)	2 (25.0)	0 (0.0)	0 (0.0)	

antioxidant capacity. These events cause lipid peroxidation.[17] Humans produce more or less free oxygen radicals throughout their lifetime. Free oxygen radicals trigger inflammation.[18] Patients with UC have a greater amount of free oxygen radicals than healthy individuals.[19] Ulcerative colitis is suggested to lead to lipid and protein oxidation.[9] It has been reported that BA administered orally in various doses from organs outside the intestine, especially in hepatotoxicity created with 50% alcohol in the liver, may be hepatoprotective by reducing antioxidant, lipid peroxidation and improving the tissue redox system.[20] In the study examining the effect of BA on oxidative damage, BA was reported to act by reducing oxidative damage in thymocyte apoptosis caused by dexamethasone.[21]

Oxidative damage caused by free radicals occurring by physiological mechanisms in the body is protected by enzymatic or nonenzymatic pathways. Oxidative stress in the body is predicted with TAS and TOS.[14] Betulinic acid application has been proven in previous studies to show anti-inflammatory effects by reducing inflammatory symptoms such as neutrophil infiltration, edema, and septal thickening in tissue. In the same study, BA showed an antioxidant feature in vivo by increasing catalase, superoxide dismutase, GSH, and decreasing lipid peroxidation degree.[22] In our study, although BA caused improvements in the same serum parameters, it was not statistically reached a significant level. It may be necessary to further design a study with higher dose and long-term treatment of BA. It is well established that there are too many inflammatory pathways in the body. Therefore, it may be possible for BA to act with different inflammatory pathways.

As detailed before, UC is a risk factor for colon cancer. Abnormal expression of epidermal growth factor receptor (EGFR) in colon cancer is known to cause colon cancer. Mitogen activator protein kinase (MAPK) pathway is one of the main pathways that affect EGFR. Ursocholic acid can be used in the treatment of colon cancer by affecting the EGFR/MAPK pathway.[23] There are studies showing that BA has less effect on non-cancerous cells.[24] BA affects the EGFR pathway as well as ursocholic acid.[25] One of the pathways in the development of colon cancer in the background of UC is the EGFR pathway. This pathway is associated with inflammation.[26] This inflammation also reduces apoptosis, causing damage to the colon mucosa.[27] In our study, BA suppresses inflammation but does not reach a statistically significant level. It was thought that by activating apoptosis, there may be a possibility of preventing colon cancer in UC.

According to our knowledge of the use of BA in experimental colitis models, only 3 studies have been published. In the experimental colitis model created using dextran sulfate sodium, the effects of BA in 3 different doses (3 mg/kg/days, 10 mg/kg/days, and 30 mg/kg/days) were investigated. BA has been shown to have an antioxidant effect, histologically reduced colonic inflammation, and a healing effect on the disease activity index.[28] In the experimental colitis model created using trinitrobenzene sulphonic acid; The antioxidant and protective effects of BA (50 mg/kg/days) were investigated and it was shown that BA prevented oxidative damage by reducing the formation of free oxygen radicals, thereby reducing inflammation in the colonic mucosa.[29] In the experimental intestinal damage model induced by cyclophosphamide, BA was used in 3 different doses (0.05 mg/kg/days, 0.5 mg/kg/days, and 5 mg/kg/days), and BA was shown to reduce intestinal damage by reducing proinflammatory cytokines, showing anti-inflammatory effects, and reducing macrophage chemokine production.[30] In our study, it is seen that BA administered prophylactically before colitis was created suppresses inflammation at the histological level, but this effect of BA does not reach a level to show statistical significance. However, when we evaluated the results of the 3 studies summarized above together with our own results, it is observed that inflammation decreases both on the histological and biochemical basis by giving BA before the colitis model is created. However, it is clear that other studies are needed to give a stronger message on this subject.

In summary, in the study we presented here, it is in our opinion that this issue is worth researching, although it cannot be shown statistically in a way that BA can reduce inflammation in the colitis model by showing anti-oxidant and anti-inflammatory effects.

Since this is the first article related to this experimental model and investigational drug, we thought that maybe it can be a beneficial clue for readers associated with further studies. As we accepted, further studies are needed to explain the exact mechanism(s) underlying our evaluations.

Ethics Committee Approval: For this study ethical approval has been obtained from the Committee on Animal Research at Inonu University, Malatya, Turkey (Date: October 1, 2020, Desicion no: 2016/ A-52).

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

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Declaration of Interests: The authors declare that there is no conflict of interests.

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