Research Article

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Possible effects of clinoptilolite on small intestinal ischemia-reperfusion injury caused by experimental mesenteric artery occlusion

https://doi.org/10.1515/tjb-2021-0244
Received April 6, 2021; accepted August 21, 2022; published online September 15, 2022

Abstract

Objectives: Mesenteric ischemia is a surgical emergency caused by poor blood supply to the intestines. In ischemia, the decrease in blood flow to the tissue causes acidosis and cell death through anaerobic metabolism. Clinoptilolite is one of the most abundant natural zeolites, and it is used for its ion exchange and adsorbent properties. Clinoptilolite has been reported to have an immune-enhancing, anti-carcinogenic, and antioxidant effect *in-vitro*/*in-vivo* studies. Clinoptilolite's histological and biochemical effects on ischemic small intestines.

Methods: The experimental animals were randomly divided into sham, control, and clinoptilolite treatment group. Clinoptilolite was administered intraperitoneally after ischemia/reperfusion. Cardiac blood was stored for biochemical analysis. Total antioxidant levels and total oxidant levels were analyzed from the sera taken from groups. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) expressions in blood samples were determined by RT-qPCR. At the end of the reperfusion, terminal ileum tissues were taken for histological tests.

Results: The mean TNF-α expression level was 3.89 in the control group and 2.91 in the clinoptilolite treatment group. The mean IL-6 expression levels were 2.32 in the control group and 1.49 in the clinoptilolite treatment group.

Conclusions: clinoptilolite administration provided healing in the rat ischemia-reperfusion injury model.

Keywords: clinoptilolite; ischemia-reperfusion; ischemic small intestines; RT-qPCR; TNF-α.

Introduction

Mesenteric ischemia (MI) is a surgical emergency caused by poor blood supply to the intestines. Although it accounts for only 0.1% of hospital admissions and 1–2% of gastrointestinal diseases, its morbidity and mortality rates are very high, ranging from 60 to 80% [1]. The most important cause of mortality is that the inflammatory response caused by intestinal ischemia and reperfusion injury does not stay locally, but also affects distant organ systems. Each organ dysfunction added to the table increases mortality by 30% [2]. The mortality rate is 30–40% when dysfunction develops in a single organ. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are pro-inflammatory cytokines that play a crucial role in ischemia-reperfusion-mediated inflammatory processes. TNF-α contributes to neutrophil-mediated endothelial damage by causing neutrophil activation and releasing endothelial leukocyte adhesion molecules. Endothelial damage occurs through elastase released from activated neutrophils and free oxygen radicals. TNF-α induces apoptosis and controls the production of IL-6 secreted by

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phagocytes, T cells, and endothelial cells [3]. The increase of TNF-α has been reported to cause shock and hypoperfusion development and increases mortality [4]. IL-6 is responsible for the oxidative burst of neutrophils and the release of free radicals [3]. Therefore IL-6 blood level rises during acute trauma, stress, and hemorrhagic shock; it is often used as a systemic inflammatory response or an indicator of postoperative morbidity [5]. Intestinal ischemia occurs in the small intestinal with the occlusion of the superior mesenteric artery, resulting from many etiological factors [6]. In ischemia, the decrease in blood flow to the tissue causes acidosis and cell death through anaerobic metabolism [7]. The formation of free oxygen radicals, the release of pro-inflammatory cytokines and mediators, and complement activation occur with reperfusion [8]. These activated mediators are known to cause leukocyte and platelet accumulation and adhesion in postcapillary venules, which disrupt microcirculation [7, 8], cause local and systemic inflammation, and damage intestinal mucosa [9, 10].

The diagnosis of MI is usually a complicated and late clinical event. Supportive treatments, such as fluid resuscitation, correction of acid-base abnormalities, vasodilators, anticoagulants, and thrombolytics, can be applied. However, the emergency treatment of thromboembolic mesenteric ischemia in the necrotic intestine is revascularization and resection with surgical treatment [11].

The active ingredient of Froximun Pudra® is clinoptilolite, a zeolite obtained from volcanic rocks; it is composed of a microporous mixture of silica and alumina tetrahedra. Clinoptilolite is widely available worldwide and is one of the most abundant natural zeolites used for ion exchange and adsorbent properties [12]. Thanks to its lightweight and porous structure consisting of tiny crystals, it is adsorbent, allows oxygen passage, and is used in wound care [13]. Clinoptilolite has been reported to have an immune-enhancing, anti-carcinogenic, and antioxidant effect *in vitro* and *in vivo* studies [14]. Clinoptilolite has been shown to accelerate wound healing with its antifungal, antiviral, and antibacterial activity and its effect on cytokines [15]. The antioxidative roles attributed to clinoptilolite are based on its ability to reduce free radicals and lipid peroxidation levels in the serum and increase overall antioxidant capacity [13]. Toxic effects of clinoptilolite have not been documented [14].

In this study, the effects of clinoptilolite on cytokines, antioxidant system and ischemic histological findings on ischemic small intestines ischemia-reperfusion injury were investigated.

Materials and methods

Experiment design

This study was conducted upon approval of the Animal Experiments Local Ethics Committee of Cumhuriyet University Faculty of Medicine (05.04.2019-No 272). Eighteen Wistar Albino female rats weighing 200–250 g were used in the study. Experimental animals were fed rat food and tap water under standard laboratory conditions and randomly divided into three groups of 6 rats. Group 1 (Sham); controls; Group 2 (Control); I/R injury; and Group 3; I/R injury and treated with clinoptilolite (which was performed on three groups of 6 rats each).

All animals were fasted 12 h before the experiment but allowed to drink water. The animals were anesthetized by intraperitoneal (IP) injection of sodium thiopental (90 mg/kg). The abdominal midline was cleared and opened, and the superior mesenteric artery was isolated. The artery was clamped, and ischemia was formed for 30 min [7]. Then, reperfusion was achieved for 3 h by unclamping (Supp 1a and 1b). At the end of the reperfusion, terminal ileum tissues were taken in formaldehyde for histological tests, and cardiac blood was taken for biochemical analysis.

Administration of clinoptilolite

Clinoptilolite was administered intraperitoneally at doses of 5 mg/kg after ischemia/reperfusion. No toxic effects of clinoptilolite were observed at the given doses.

Biochemical analysis

Cardiac blood was taken from rats at the end of the reperfusion, centrifuged at 4,000 rpm for 12 min, and stored at −80 °C until measurement.

Real-time PCR: TNF-α and IL-6 levels in blood samples were determined using real-time PCR according to the previously described method [16]. Total RNA was isolated with the kit (High Pure RNA Tissue kit; Roche). RNA quality was analyzed by a BioSpec-nano spectrophotometer (A260/280 ratio) (Shimadzu Biotech). cDNAs were synthesized using the first-strand cDNA synthesis kit (Transcriptor First Strand cDNA Synthesis Kit, Roche). The forward and reverse primers were as follows: 5′GGTGCCTATGTCTCAGCCTCTT-3′ and 5′ GCCATAGAACTGATGAGAGGGAG-3′ for TNF-α; 5′-GAGGATACC ACTCCCAACAGACC -3′ and 5′-AAGTGCATCATCGTTGTTCATACA - 3′ for IL-6; 5′-GCGACATGATTAATGGCACA-3′ and 5′-CAATGTCCGAG ACTTTTCAACA-3′ for β-actin.

The expression of TNFα, IL-6 was investigated by RT-PCR (Roche LightCycler® 480 system, Germany under following conditions: initial incubation at 95 °C for 5 min, 45 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Ct values were calculated from the data, and $2^{-\Delta\Delta Ct}$ was used. The sham group was used to compare the gene expression levels in the control and clinoptilolite groups.

Determination of total antioxidant status (TAS): A commercial kit manufactured by Rel Assay Diagnostics was used to determine TAS. In this method, the antioxidants in the sample are reduced from the

dark blue-green ABTS radical form to the colorless reduced ABTS form. The absorbance change at 660 nm is associated with the total antioxidant capacity of the sample. The assay was calibrated with the reference substance used as the stable standard antioxidant solution (the vitamin E analog), called Trolox Equivalent. TAS measurement was performed following the procedure on the kit. The difference between absorbances was used in the equation given below [17].

$$Calculated\ A_2 - A_1 = \Delta A \quad x = \frac{\Delta A\,(\text{sample})}{\Delta A\,(\text{standard})} x20$$

Determination of total oxidative stress (TOS): Commercial kits produced by Rel Assay Diagnostics were used to determine TOS. H_2O_2 used as the positive control for TOS [17].

MDA levels: İntestinal tissues were weighted. Tissues were homogenized 1.15% KCl in ice-cold and The homogenate was centrifuged at 2000 *g* for 10 min. MDA levels were determined by Uchiyama and Mihara in tissue samples [18]. Tetramethoxypropane was used as a standard and tissue MDA levels were calculated as nmol/g wet tissue.

Histopathological examination

The terminal ileum tissue was fixed in 10% formaldehyde, embedded into paraffin, cut into 3 µm sections, and stained with hematoxylin & eosin (HE). A pathologist examined its histopathological features under a light microscope (Leica DM750, Germany) by counting 10 areas using damage measurement parameters. The damage quantification from 10 areas corresponding to the ileum tissue was graded using the following parameters and involvement percentage: cellular edema, cell apoptosis, necrocytosis, hemorrhage, and cell fracture on a four-point system (0, histopathological changes ≤10%; 1, = 11–25%; 2, = 26–50%; 3, = 51–75%; and 4, = 76–100%) [19]. The mean score for each parameter was calculated and analyzed.

Statistical analysis

Prism 8.4.3 GraphPad software was used to draw the graphs of fold change. ANOVA and the Tukey test were used to compare sham, control, and clinoptilolite groups; the significance level was set as 5% ($p<0.05$).

Results

Cytokines are relatively low molecular weight proteins secreted by stimulating immune cells. Among them, interleukins, lymphokines and interferons and tissue necrosis factor are very important. It has long been proven that proinflammatory cytokines such as interleukins 1α, 1β and 6 and TNF-α play important roles in the wound healing process [16].In our study, we used TNF-α and IL-6 to evaluate the efficacy of cytokines. We examined the TNF-α and IL-6 markers. TNF-α and IL-6 expression values were shown in Figures 1 and 2.

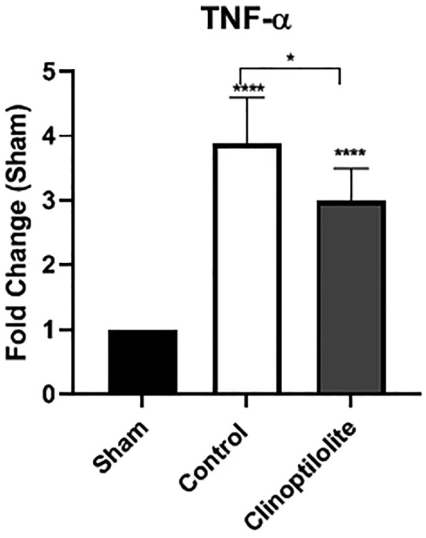

Figure 1: Comparison of TNF-α expressions of control, sham, and clinoptilolite treatment groups rat.
Data represent the mean ± standart error (SE) for 6 animals in each group. p<0.0001, a significant difference compared with the control and clinoptilolite treatment groups.

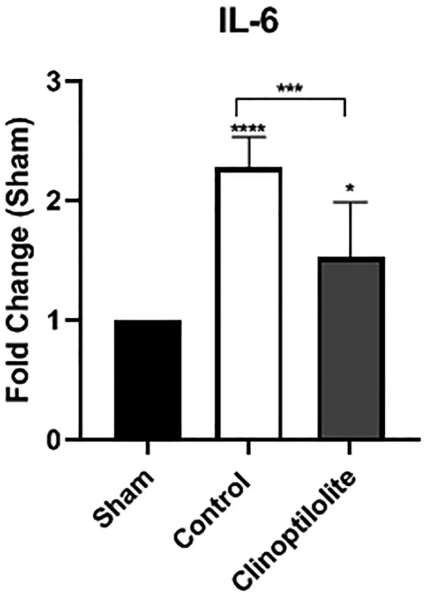

Figure 2: Comparison of IL-6 expressions of control, sham, and clinoptilolite treatment groups rat.
Data represent the mean ± SE for 6 animals in each group. p<0.0001, a significant difference compared with the control and clinoptilolite treatment groups.

TNF-α expressions levels were 3.89 and 2.91 in the control and clinoptilolite treatment groups, respectively. The difference was found to be statistically significant ($p<0.0001$). IL-6 levels were 2.32 and 1.49 in the control and clinoptilolite treatment groups, respectively. The

difference was also found to be statistically significant ($p<0.001$).

TAS and TOS values were measured and tabulated to see the antioxidant properties of clinoptilolite. TAS values found as 1.09 ± 0.21 mmol Trolox Equiv./L control, 0.98 ± 0.02 mmol Trolox Equiv./L sham group, 1.02 ± 0.34 mmol Trolox Equiv./L clinoptilite treatment group. TOS values found as 1.25 ± 0.02 μmol H_2O_2/L control, 0.86 ± 0.23 μmol H_2O_2/L sham group, 1.53 ± 0.32 μmol H_2O_2/L clinoptilite treatment group. Regarding TAS and TOS results, there is no significant difference between groups.

Membrane phospholipids were affected by increased free radicals in oxidative injury. MDA is a biomarker of the lipid peroxidation process. MDA values found as 1.3 ± 0.24 nmol/mg tissue control, 2.7 ± 0.35 nmol/mg tissue sham group, 2.5 ± 0.53 nmol/mg tissue clinoptilite treatment group. MDA levels increased significantly compared to the control group. This supports the idea of damage in membrane lipids with excessive ROS production.MDA levels were not found significantly compared to the sham group.

In HE sections, histological features of cellular edema, cell apoptosis, necrocytosis, hemorrhage, and cell fracture were examined by light microscopy at 40, 100, 200, and 1,000 magnification; the percentages were calculated over 10 areas; graded according to the quadruple scoring system (Supp Table 1. Histopathological scores of the groups). Normal mucosal villi (score 0) were observed in the histopathological sections of the sham group (Figure 3a). Ischemic histological findings were prominent and severe in one sample of the control group (Figure 3b). There were score-3 changes in the ileum mucosa in two samples and score-2 changes in another two samples in the control group. The findings were focally mild in only one sample. In the clinoptilolite group, the highest score was 2 in one sample (Figure 3c). Histological findings returned to normal in most areas in two samples, and there were mild score-1 findings in three samples (Figure 3d, e). The appearance of apoptotic cells in the treatmented clinoptilolite group was demonstrated histologically (Figure 3f). According to histopathological scoring, the lowest mean score was observed in the Sham group (score = 0) and the highest in the I/R group (score = 2.5). In group comparisons, statistically significant differences were observed between the Sham and control groups ($p=0.005$) and the control and clinoptilolite treatment groups ($p=0.025$). No statistically significant difference was found between the sham and clinoptilolite treatment groups ($p=0.066$).

Discussion

Ischemic damage is seen in the intestines due to various reasons such as embolism of the arteries feeding the intestines, thrombosis or atherosclerosis-related obstruction, mechanical vascular reasons, and obstruction in the venous return of the intestine [20]. The reperfusion is essential for tissue survival after ischemia. However, reperfusion injury has been shown to cause more tissue damage than the damage caused by ischemia alone [21]. As a result of I/R, an increase occurs in oxygen-derived free radicals, and they cause injury [22].

Another reason responsible for the formation of ROS in ischemia-reperfusion injury is the interactions between endothelial cells and inflammatory cells, especially neutrophils, which come to the region with reperfusion. Inflammation occurring in the tissue during ischemia-reperfusion and pro-inflammatory mediators released by endothelial cells and other cells in the vascular bed (macrophages and mast cells) cause the migration and adhesion of polymorph nucleated leukocytes (PMNL). Apart from this, cytokines and chemokines such as IL1, IL-6, IL-12, IFNγ and TNFα and proteases such as elastase and collagenase are released from PMNL, leading to more inflammatory cell recruitment and more ROS production in the region [23].

TNFα is a pro-inflammatory cytokine whose synthesis increases rapidly after tissue damage. It is synthesized by tissue mast cells and macrophages [24]. The elevation of TNFα is the most crucial poor prognosis marker in systemic inflammatory response syndrome and multi-organ failure [25]. Studies suggest that preventing leukocyte invasion during reperfusion reduces I/R injury [26]. TNF-α, whose synthesis is increased significantly during ischemia, triggers SOR production by increasing the levels of pro-inflammatory cytokines, especially IL-6 and NO [27]. The role of cytokines in intestinal ischemia has been demonstrated in many studies, and TNF-α blockade has been suggested to reverse I/R injury [28]. IL-6 and TNF-α are predecessor cytokines that play a role in I/R injury and septic shock. Sorkine et al. showed that endotoxins that enter the blood due to disruption of the intestinal mucosal barrier increase TNF-α release [29]. In their study, Jiang et al. stated that ginsenoside, an antioxidant and anti-inflammatory component, paved the way for histological improvement by decreasing TNFα levels in the mouse intestinal ischemia-reperfusion injury model [30]. Li et al. stated in 2017 that 6-gingerol provides histopathological improvement by reducing TNFα levels in rat intestinal ischemia-reperfusion injury [31]. Our study

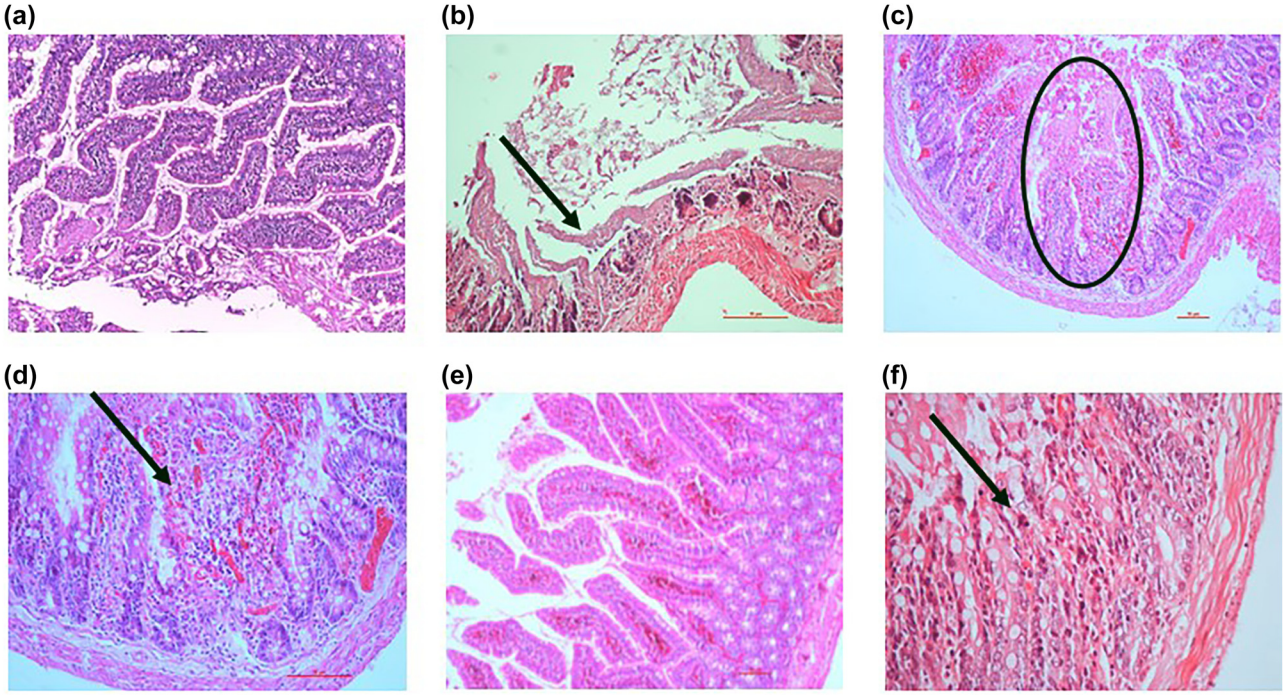

Figure 3: The histological morphology of intestinal with HE staining.
(a) Sham group, score 0, ileum mucosa of normal villi structure (HEx100); (b) Control group, score 4, prominent necrocytosis, hemorrhage, and cell fracture are seen (indicated by arrow) (HE×100); (c) Treatmented clinoptilolite group, score 2, the ischemic findings regressed to varying degrees in each area. The changes are tracked in near-normal mucosa on the right, mucosal ischemia with more necrocytosis in the middle (in the circle), mild hemorrhagic on the left (HE×100). (d) Treatmented clinoptilolite group, score1, mostly normal mucosa is observed that is occasionally very little cellular edema, cell fracture, and hemorrhage (indicated by arrow) (HE×200) (e) Treatmented clinoptilolite group, score 0, it was observed in rare areas on hemorrhage and cellular edema, mucosa with normal histology in most areas (HE×100) (f) Treatmented clinoptilolite group, apoptotic cells (indicated by arrow) (HE×100).

found that the control group's TNF-α expression levels increased significantly compared to the sham group. In contrast, the clinoptilolite treatment group's TNF-α expressions decreased significantly compared to the control group.

IL-6 is generally used to indicate systemic inflammatory response or postoperative morbidity, as blood levels increase during acute trauma, stress, and hemorrhagic shock. IL-6 levels are correlated with mortality in shock. In their study, Grotz et al. showed that IL-6 value in tissue and serum increased in line with TNF-α after IRR [32]. Our study found that IL-6 expressions of the control group increased significantly compared to the sham group, and IL-6 expression levels of the clinoptilolite treatment group decreased significantly compared to the control group.

Increased free radical production in oxidative damage affects membrane phospholipids [33]. MDA is the most important and most studied biomarker of polyunsaturated fatty acids in ischemia and oxidative stress [34]. The MDA level increased significantly in the treatment group compared to the control group.This supports the idea of damage to membrane lipids by excessive production of ROS. In the clinoptilolite-treated group, tissue MDA levels were decreased compared to the sham group, but not significantly. In our study, it was shown histopathologically that clinoptilolite prevents lipid peroxidation in the membrane.

The essential feature of clinoptilolite is allowing oxygen passage to the wound [35]. In this way, clinoptilolite increases collagen, fibronectin, and granulation on the tissue and protein synthesis [36]. In addition, studies have shown that clinoptilolite accelerates wound healing by macrophage activation and T-cell stimulation that increases with complement activation [37]. The administration of dietary zeolite has been suggested to enhance the hypertrophic function of intestinal villi and epithelial cells in the duodenum and ileum. It leads to a greater surface area in nutrient absorption [38]. The leukocyte-endothelium interaction is one of the strategies used to reduce the damage of intestinal ischemic syndromes that may result in mortality. It is based on

blocking the effects of cytokines that cause a systemic inflammatory response [39]. Thus, although it has not yet entered the medical literature, clinoptilolite was thought to play a protective role in the pathophysiological mechanism of intestinal ischemic syndromes with its antioxidant and anti-inflammatory properties and lack of known toxicity and interaction.

In the study, the effects of clinoptilolite (Froksimun®) on cytokines were shown. The cytokine activity network realizes post-injury complement formation, platelet aggregation, blood coagulation and hemostasis, the release of cell activation and growth factors, neovascularization, granulation tissue formation, and wound healing. Class II MHC is activated by clinoptilolite particles, activating macrophages and other mononuclear phagocytic cells [14]. Pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α are released [40]. Cytokines are one of the essential mediators that play a role in inflammation. Cytokines are a family of proteins that regulate the functions of cells involved in natural and acquired immunity in growth, differentiation, and immune response. In this study, pro-inflammatory cytokines including TNF-α and IL-6 expressions levels were studied.

Many physiological and pharmacological agents have been studied to prevent I/R injury. Marquie et al. tested pentoxifylline after performing ischemia for 120 min and reperfusion for 60 min in the small intestine of rats and reported that this agent reduced I/R injury [41]. Schmeling et al. used diclofenac sodium which has an anti-inflammatory effect after performing ischemia for 120 min and reperfusion for 60 min in the small intestine of rats. They also reported that this agent reduced I/R injury [42].

Conclusions

In the study, it was shown that administering clinoptilolite provided healing in the rat ischemia-reperfusion injury model. Clinoptilolite can be used as a good agent in ischemia-reperfusion medically, with further study in this field.

Research funding: None declared.

Author contributions: All authors have accepted respon sibility for the entire content of this manuscript and approved its submission.

Conflict of interest: No conflict of interest was declared by the authors.

Informed consent: Not applicable.

Ethical approval: The study was conducted upon approval of the Animal Experiments Local Ethics Committee of Cumhuriyet University Faculty of Medicine (05.04.2019-No 272).

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/tjb-2021-0244).