Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2020;45(4):1459-1466
DOI: 10.17826/cumj.734911

ARAŞTIRMA / RESEARCH

Protective effects of phloretin and phloridzin on indomethacin-induced gastric ulcers in mice: characterization of potential molecular mechanisms

Floretin ve floridzin'in farelerde indometazine bağlı gelişen mide ülserine karşı koruyucu etkileri: potansiyel moleküler mekanizmaların karakterizasyonu

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Cukurova Medical Journal 2020;45(4):1459-1466

Abstract

Purpose: We aimed to examine the potential protective effects of phloretin and phloridzin in indomethacin induced ulcer model in mice.
Materials and Methods: In total 54 female Balb/C mice were separated into nine groups. Famotidine was used as standard antiulcer agent. The phloretin and phloridzin was given at the doses of 50 and 100 mg/kg as a pre-treatment. After experimental procedures stomach tissue oxidative parameters (SOD, GSH and MDA), inflammatory cytokine TNF-α, and COX1 and COX2 mRNA expressions were analyzed. In addition, to clarify antiulcer effect mechanism of phloretin and phloridzin, numerical densities of ulcerative areas were analyzed.
Results: Phloretin and phloridzin inhibited indomethacin-induced ulcer formation in dose dependent manner. Tissue inflammation and oxidative stress were increased after the indomethacin administration. Phloretin and phloridzin treatment normalized all parameters compared to indomethacin treated group. After the treatments, SOD activities and GSH levels were increased while MDA levels were decreased. Phloretin and phloridzin treatments decreased TNF-α, COX1 and COX2 mRNA expressions.
Conclusion: Our results showed that phloretin and phloridzin may be an alternative treatment for peptic ulcer disease due to their potential regulatory effects against oxidative stress and inflammation.

Keywords:. Phloretin, phloridzin, ulcer, mice

Öz

Amaç: Bu çalışmada farelerde indometazine bağlı ülser modelinde, floretin ve floridzinin potansiyel koruyucu etkilerini incelemeyi amaçlanmıştır.
Gereç ve Yöntem: Toplam 54 dişi Balb/C faresi dokuz gruba ayrıldı. Famotidin standart anti-ülser madde olarak kullanıldı. Floretin ve floridzin, tedavi gruplarına 50 ve 100 mg/kg dozlarında verildi. Deneysel prosedürlerden sonra, mide dokusunda oksidatif parametreleri (SOD, GSH ve MDA), enflamatuar sitokin TNF-α ve COX1 ve COX2 mRNA ekspresyonları analiz edildi. Ayrıca, floretin ve floridzinin anti-ülser etki mekanizmasını açıklığa kavuşturmak için ülserli alanların sayısal yoğunlukları analiz edilmiştir.
Bulgular: Floretin ve floridzin, indometazine bağlı gelişen ülser oluşumunu doza bağımlı olarak azalttı. İndometazin uygulamasından sonra enflamasyon ve oksidatif stresin arttığı belirlendi. Floretin ve floridzin tedavisi indometazin ile tedavi edilen gruba kıyasla tüm parametreleri normalleştirmiştir. Tedavilerin ardından, indomethazin grubuna kıyasla, SOD aktiviteleri ve GSH düzeyleri yükselirken, MDA düzeyleri azalmıştır. Floretin ve floridzin uygulamarının TNF-α, COX1 ve COX2 mRNA ekspresyonlarını azalttığı tespit edildi.
Sonuç: Sonuçlarımız oksidatif stres ve enflamasyona karşı potansiyel düzenleyici etkileri nedeniyle floretin ve floridzinin peptik ülser hastalığı için alternatif bir tedavi olabileceğini göstermiştir.

Anahtar kelimeler: Floretin, floridzin, ülser, fare

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Geliş tarihi/Received: 10.05.2020 Kabul tarihi/Accepted: 06.09.2020 Çevrimiçi yayın/Published online: 30.12.2020

INTRODUCTION

Peptic ulcer is a chronic gastrointestinal disease that is characterized by acidic lesions of stomach and duodenum[1]. The prevalence of peptic ulcer is estimated that 3% of the world population[2]. Cigarette and alcohol consumption, emotional factors, advanced age, dietary factors, and genetic factors can cause a hyper secretion of acidic environment in stomach[3]. Also some polymorphisms in genes are related with peptic ulcer. For example, TNF-α polymorphisms cause TNF-α secretion and resulted in ulcerative colitis[4]. Near these factors, nonsteroidal anti-inflammatory drugs (NSAIDs) consumption also play important role on ulcer[5].

NSAIDs are known as the most preferred drug group in the world due to their analgesic and anti-inflammatory activities. But the excessive using of NSAIDs with anticoagulants and corticosteroids increase the risk of gastrointestinal bleeding[5]. NSAIDs increase the risk of ulcer disease four times compared to nonusers[6]. NSAIDs decrease prostaglandin levels by inhibiting the cyclooxygenase (COX) enzyme and cause mucosal damage as a result of increased gastric acid secretion[7]. COX can be divided into two subtypes: COX-1 and COX-2. COX-1 is a structural enzyme that exists in normal tissue cells and participates in the regulation of vascular relaxing and platelet aggregation. COX-2 is a key enzyme in the synthesis of prostaglandins which are known to participate in a variety of inflammatory reaction processes[8]. Furthermore, NSAID contains free carboxylic acid. This free acid causes NSAID to attach to the gastric mucosa, causing disruption of the structure of the stomach wall[9]. The long-term use of NSAIDs also causes oxidative stress. Increased oxidative stress causes damage to many tissues and triggers the formation of peptic ulcers[10].

Nowadays, peptic ulcer treatment is provided by inhibiting gastric acid secretion by using chemical drugs[11]. However, most of the chemical drugs have some serious side effects such as joint pain, heart rate change, hemopoietic changes, gynecomastia, impotence and systemic alkalosis[11,12]. Todays, the development of drugs with minimal side effects, are being studied for the use of natural products especially in ulcer treatment.

Phloretin and its glycosylated form Phloridzin, a dihydrochalcone, are natural compounds and members of bicyclic flavonoids[13]. These natural compounds are mainly found in unripe apples, root of apple tree and small amounts of strawberries. These dihydrochalcones can occur in natural sources in conjunction with other polyphenols such as quercetin, catechin, epicatechin, procyanidins and routine[14]. Dihydrochalcones is an unusual group of natural antioxidant and the bioactivity of dihydrochalcones has attracted the attention of scientists in recent years[15]. For example, a typical dihydrocolic phloretin has been investigated for its anti-inflammatory and hepatoprotective effects in mouse models[16,17]. It has also been suggested that glucoside phloridzin (phloretin 2'-β-d-glucoside) has neuroprotective and cytoprotective effects[18]. From the medical point of view and free radical biology, these bioactivities can be related to the antioxidant capacity of these compounds. In fact, phloretin and phloridzin flavonoids have been found to have an antioxidant effect, and even these molecules have proven to be stronger antioxidants than the well-known flavonoids due to the presence of the 2,-OH group[19].

In this study, on the basis of mentioned biological activations of phloretin and phloridzin and presence of inflammatory response and oxidative damage in peptic ulcer injury, the protective effects of these natural compounds will be investigated on indomethacin induced peptic ulcer in mice.

MATERIALS AND METHODS

Animals

For this study in total 54 female, Balb/C mice were used in the experiments. Each mouse weighed 35-45 g and was obtained from Ataturk University Experimental Animal Laboratory. The Institutional Animal Care and Use Ethics Committee of Ataturk University approved the study on 28.03.2019, which was conducted in accordance with protocol number 2019-4/72.

Chemicals

Phloretin (CAS: 60-82-2), phloridzin (CAS: 7061-54-3) and all chemicals that we used during the experimental process were purchased from Sigma Chemical (Munich, Germany); indomethacin (Endol 25 mg) was obtained from DEVA (Istanbul, Turkey); famotidine (Famodin 40 mg) was obtained from Sandoz (Istanbul, Turkey).

Experimental design and ulcer model

Totally 54 female mice were separated into nine groups (n=6). All the experimental design and procedures were shown in Table 1. The phloretin and phloridzin was given at the doses of 50 and 100 mg/kg as a pre-treatment, after the mice were fasted for 24 h, as described previously[20]. Famotidine was used as a control treatment drug at the dose of 40 mg/kg and administered to the corresponding mice groups as described previously[21]. One hour after the drug treatments, 25 mg/kg of indomethacin was suspended in isotonic saline solution and administered to all groups except the control group. Six hours after the indomethacin administration, the animals were killed with an overdose of a general anesthetic (thiopental sodium, 50 mg/kg), and the stomachs were transported to the analyze laboratory for biochemical and molecular analyses.

Table 1. Drug administrations and Experimental procedures of ulcer model

Groups	24h before INDO treatment	1h before INDO treatment	INDO treatment	6h after INDO treatment
Healthy	fasted	water	-	sacrified
Healthy+PH2	fasted	100 mg/kg Phloretin	-	sacrified
Healthy+PZ2	fasted	100 mg/kg Phloridzin	-	sacrified
INDO	fasted	water	25 mg/kg Indomethacine	sacrified
INDO+FAM	fasted	40 mg/kg Famotidine	25 mg/kg Indomethacine	sacrified
INDO+PH1	fasted	50 mg/kg Phloretin	25 mg/kg Indomethacine	sacrified
INDO+PH2	fasted	100 mg/kg Phloretin	25 mg/kg Indomethacine	sacrified
INDO+PZ1	fasted	50 mg/kg Phloridzin	25 mg/kg Indomethacine	sacrified
INDO+PZ2	fasted	100 mg/kg Phloridzin	25 mg/kg Indomethacine	sacrified

Healthy: Nontreated group, INDO: Indomethacine, FAM: Famotidine, PH: Phloretin, PZ: Phloridzin

Biochemical investigation

Stomach tissues were cleaned with PBS and homogenized with liquid nitrogen and stored at -80°C. To analyze biochemical parameters, approximately 100 mg of all dust tissue samples were first perfused with 1 ml PBS and all samples were centrifuged. Supernatants were used as sample. Superoxide dismutase (SOD) activity[22], glutathione (GSH) levels[23], and malondialdehyde (MDA) levels[24] from each sample supernatant and standards were measured at room temperature in duplicate according to the modified methods with multi well plate reader[25,26]. SOD, GSH, and MDA levels were expressed as U/mg protein, nmol/mg protein, and nmol/mg protein, respectively and all results were given per mg of protein. Total protein levels were determined by the Lowry Method, according to commercial kit's procedures (Sigma Aldrich, Total protein kit-TP0300-1KT-(USA)).

Total RNA extraction and cDNA synthesis

Stomach tissues (25 mg) were stabilized with RNA stabilization reagent (RNAlater, Qiagen). First, liquid nitrogen was used to freeze tissues and then Tissue Lyser II (Qiagen, 2 x 2 minutes for all samples) was used to disrupt the samples. Total RNA was purified using RNeasy Mini Kit Qiagen according to the instructions of the manufacturer in QIAqube (Qiagen, Hilden, Germany). The RNA samples were reverse-transcribed into complementary DNA using a high-capacity cDNA reverse transcription kit (Applied Biosystem). The cDNA concentrations were assessed and quantified using the Epoch Spectrophotometer System and Take3 Plate (Biotek)[27,28].

Relative quantification of gene expression

Relative, COX1, COX2 and TNF-α expression analyses were performed with StepOne Plus Real Time PCR System technology (Applied Biosystem) by using synthesized cDNAs. A qPCR was run using TaqMan Probe mix and Probe-based technology (Applied Biosystem). Real-time PCR was performed using primers generated for mouse TNF-a Mm00443258_m1, mouse COX-1 Mm04225243_g1, mouse COX-2 Mm03294838_g1 and mouse β-actin Mm02619580_g1. Beta-actin was used as endogenous controls. For each group, triplicate determinations were performed in a 96-well optical PCR plate and all quantification of gene expression steps were performed as described previously[29]. Data were expressed as fold changes in expression, which are calculated by using the $2^{-\Delta\Delta Ct}$ method[30].

Statistical analysis

For statistical analysis, we used SPSS 20.0 software. The results were presented as means ± standard deviation (SD). Comparisons between groups were performed using one-way ANOVA and the Tukey multiple comparison test. Significant differences were detected between all groups, compared to Healthy group (*p<0.05, **p<0.01, ***p<0.001), compared to INDO group (#p<0.05, ##p<0.01, ###p<0.001) and compared to INDO+PZ2 group (δδ p<0.01, δδδ p<0.001).

RESULTS

Ulcerative hematoma areas of mice stomach tissues were shown in Fig. 1. Numerical densities of ulcerative areas in INDO group was found be higher than other groups (Fig. 1B). PH and PZ treatment (Fig. 1F and Fig. 1J) in healthy mice did not effect on the ulcerative area compared the Healthy group (Fig. 1A). Positive control Famotidine treatment (Fig. 1C) caused anti-ulcer effect when compared with INDO treated group. It was shown that PH2 (Fig. 1E) decreased ulcerative areas compared to PH1 treatment (Fig. 1D). It was also found that PZ1 (Fig. 1G) and PZ2 (Fig. 1H) treatments decreased ulcerative hematoma areas of mice when compared with INDO group. As a result of the present study, it was found that high dose of PZ showed a strong anti-ulcerative effect and cleaned all ulcerative areas in stomach compared to INDO+FAM group.

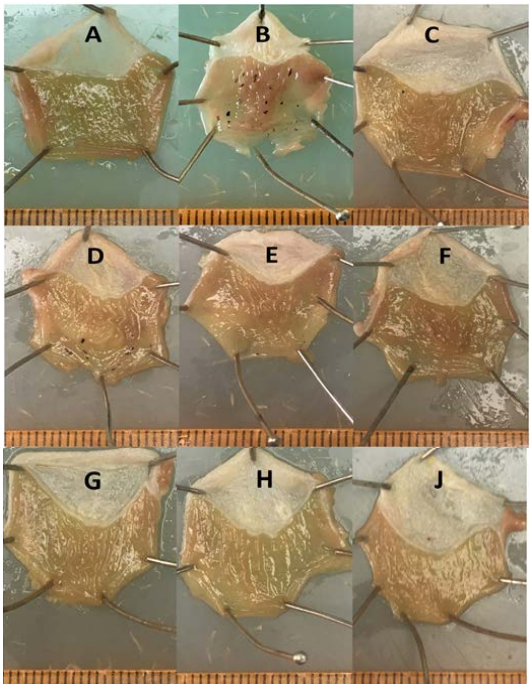

Figure. 1. Ulcer areas of stomach tissues. A: Healthy, B: INDO, C:INDO+FAMO, D:INDO+PH1, E:INDO+PH2, F:Healthy+PH2, G:INDO+PZ1, H:INDO+PZ2, J:Healthy+PZ2.

INDO:Indomethacine (25 mg/kg), FAM:Famotidine (40 mg/kg), PH1:Phloretin (50 mg/kg), PH2:Phloretin (100 mg/kg), PZ1:Phloridzin (50 mg/kg), PZ2:Phloridzin (100 mg/kg).

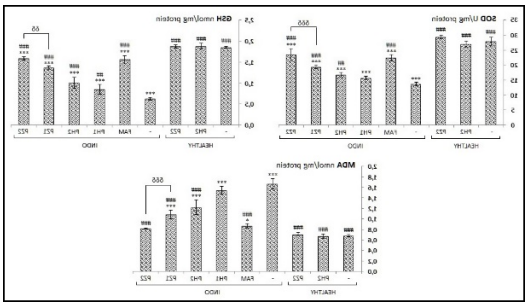

Figure. 2. Biochemical results of Phloretin and Phloridzin administration in stomach tissues.

GSH: Glutathione levels, MDA: Malondialdehyde levels, SOD: Superoxide dismutase activities. INDO:Indomethacine (25 mg/kg), FAM:Famotidine (40 mg/kg), PH1:Phloretin (50 mg/kg), PH2:Phloretin (100 mg/kg), PZ1:Phloridzin (50 mg/kg), PZ2:Phloridzin (100 mg/kg).

In the present study, SOD activities, GSH and MDA

levels were analyzed in the indomethacin induced peptic ulcer model in mice. The GSH levels and SOD activities were found to significantly decrease and the MDA levels were found to significantly increase in the stomachs of the INDO group compared to Healthy group as shown in Fig. 2 ($p<0.001$). As a positive control drug FAM treatment significantly increased SOD activities and GSH levels, while significantly decreased MDA levels compared to INDO group ($p<0.001$). Looking at the PH and PZ treated groups in Healthy mice, it was shown that PH and PZ did not significantly changed SOD, GSH and MDA levels when compared with Healthy group.

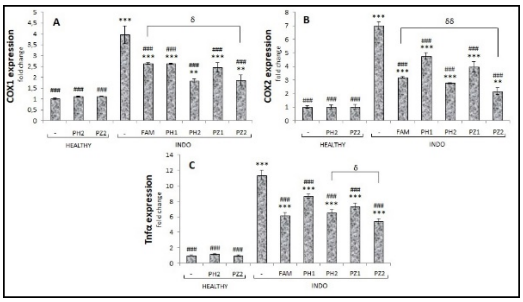

Figure. 3. Relative mRNA expression levels of Cox1, Cox2 and Tnfα in the stomach tissues.

INDO: Indomethacine (25 mg/kg), FAM: Famotidine (40 mg/kg), PH1:Phloretin (50 mg/kg), PH2:Phloretin (100 mg/kg), PZ1:Phloridzin (50 mg/kg), PZ2:Phloridzin (100 mg/kg). The expression of mRNAs was detected using quantitative Real-Time PCR analysis. β-actin was used as the reference gene. Results are expressed as relative fold compared with healthy animals. Each bar expressed as mean value ± SD. Significant differences were detected between all groups, compared to Healthy group (**p<0.01, ***p<0.001), compared to INDO group (###p<0.001) and compared to INDO+PZ2 group (δp<0.05, δδp<0.01) by one-way ANOVA followed by Tukey test.

As shown in Fig. 2 both doses of PH and PZ treatments ameliorated SOD, GSH and MDA parameters in dose dependent manner. It was found that both doses of PZ treatment decreased MDA levels while increased SOD and GSH levels compared to PH1 and PH2 treated groups. It was also shown in Fig. 2 that PZ2 treatment significantly decreased MDA levels ($p<0.001$) and significantly increased SOD activity ($p<0.001$) and GSH levels ($p<0.01$) when compared to PZ1 treatment.

COX1, COX2 and TNF-α gene expressions were analyzed with RT-PCR. As shown in Fig 3A and B, INDO administration caused a significant up-regulation of COX1 and COX2 mRNA expressions compared to the healthy group (p). Looking at the INDO treated groups; it was shown that FAM administration significantly decreased COX1 and COX2 mRNA expressions when compared with INDO group (p). It was also shown that PH and PZ, dose dependently inhibited the mRNA expressions of COX1 and COX2. Similarly to these results, INDO administration significantly increased TNF-α expression while FAM, PH and PZ administrations decreased the expression as shown in Fig 3C.

DISCUSSION

NSAIDs are known as the most preferred drug group in the world due to their analgesic and anti-inflammatory activities. However these drugs have some side effects as gastric mucosal damage[5]. The gastric mucosal damage is one of the important indicators of peptic ulcer. Nowadays, some drugs are used on the treatment and protection of the ulcer disease but lots of them have also serious side effects[31]. Accumulating evidence showed that natural compounds may be useful on the protection or treatment of ulcer disease with their less toxicity[32]. In this study we explained the anti-ulcerative effects of phloretin and phloridzin in peptic ulcer.

Phloretin and phloridzin are natural compounds and members of bicyclic flavonoids[13]. Antioxidant effects of phloretin and phloridzin have shown before in animal models[16,17]. Antioxidant molecules regulate the oxidative stress and help the tissue regeneration during treatment[26,33]. Oxidative stress is one of the most important etiological factors of the ulcer[34]. Reactive oxygen species (ROS) are continuously produced during healthy metabolic condition and are balanced by antioxidant defense systems. Whereas, the increase in ROS production exceeds the antioxidant capacity, oxidative stress increases, resulting in epithelial damage and cell death[34]. For this reason antioxidant treatment may help the protection and treatment of gastric mucosal damage.

It has been reported that non-steroidal anti-inflammatory drugs cause damage to the stomach tissue, increase the level of oxidative stress by causing an increase in lipid peroxidation and thus, cause the development of pathological mechanisms[35]. Indomethacin, a NSAID group drug, has been demonstrated by studies that increased stomach tissue MDA levels[21,36]. It has been found that indomethacin caused a significant increase in stomach tissue MDA levels[37]. In similar studies, it has been reported that stomach tissue MDA levels

increase with indomethacin, and antioxidants given in different doses for protective purposes to reduce MDA levels partially or completely[32,38]. It was shown that phloretin treatment reduces MDA levels in arthritis induced mice study[39]. In another study phloridzin also decreased MDA levels[40]. Similar to these results, we showed that phloretin and phloridzin decreased the MDA levels in peptic ulcer.

One of the important parameter is glutathione (GSH) and superoxide dismutase (SOD) regulation in response to ROS production. GSH directly or indirectly inhibit lipid peroxidation as a result of antioxidant defense[41]. The protection of the cellular proteins is main role of the GSH, which acts as an anti-oxidative barrier in the stomach and intestinal mucosa[21]. SOD enzyme cause the reduction of super oxide radicals which are produce as a results of tissue damage[42]. SOD is the main enzyme, which neutralizes ROS production and causes protection for gastric mucosal damage[42]. GSH and SOD levels were shown to decrease in ulcer in response to gastric damage[43,44]. It has been shown that phloretin increase GSH level and SOD activity in response oxidative stress[45]. Similar effects were shown for phloridzin[40]. In this study we also showed the same effects of phloridzin and phloretin on SOD and GSH levels. Phloretin and phloridzin increased these levels in stomach tissues. Near the oxidative stress, another factor effecting peptic ulcer formation is local severe stomach inflammation. TNF-α, which is the main actor of the cell apoptosis in acute and chronic inflammation, has some role on the pathophysiology of diseases[46]. TNF-α levels are evaluated as important criteria in determining the severity of stomach inflammation[21]. It has been reported that TNF-α levels increase in indomethacin induced ulcer[47]. It has been demonstrated in previous studies that flavonoids are effective in decreasing TNF-α levels[48,49]. In our study, we showed that TNF-α level was decreased by phloretin and phloridzin treatment in ulcer via their potential anti-inflammatory effects.

Indomethacin cause ulcer because of its inhibitory effects on the COX enzymes[21]. COX1 and COX2 produce the prostaglandin E2 and protect the gastric mucosa by increasing mucus secretion, maintaining blood flow and reducing hydrochloric acid secretion[21]. In previous studies it was shown that indomethacin induce peptic ulcer by inhibiting COX1 and COX2[21,36,50]. Similar to these studies, we showed that indomethacin administration caused peptic ulcer. However we showed that indomethacin increased mRNA expressions of COX1 and COX2. This increase can be due to the negative-feedback effects in response to inhibited COX enzymes activity. In this study we showed that phloretin and phloridzin treatments decreased the COX expressions in indomethacin administrated groups. In previous studies it has been reported that phloretin and phloredzin inhibited the COX2 enzymes[51,52]. This data made us to think that flavonoids have potential protective effects on gastric mucosa. Our last result was ulcerative areas of stomach tissues. Ulcerative area directly shows the degeneration levels of the tissue. In some studies ulcerative area analyses was used to show ulcer levels and treatments[21,37]. In the present study we showed that indomethacin caused ulcerative lesions and phloretin and phloridzin decreased these areas and treated lesions.

Our study has two limitations that need to be considered. First, we could not provide sodium-glucose linked transporter 1 and 2 (SGLT1/2) protein levels or gene expression because it is not secreted in stomach tissues. Phloretin is an important SGLT1 and SGLT2 inhibitor, for this reason it could be better if we may examine the SGLT protein levels in stomach. Second limitation is to determine of gastric juice pH of mice. We could not collect gastric liquids from the mice. However further work may be done to better understand the gastric PH levels after a pylorus ligation ulcer model.

Finally this study demonstrated anti-ulcerative effects of natural flavonoids, phloretin and phloridzin. Our results showed that phloretin and phloridzin may be an alternative treatment for peptic ulcer disease due to their potential regulatory effects against oxidative stress and inflammation.

Yazar Katkıları: Çalışma konsepti/Tasarımı: HU, RAU; Veri toplama: HU, RAU; Veri analizi ve yorumlama: HU, RAU; Yazı taslağı: HU; İçeriğin eleştirel incelenmesi: RAU; Son onay ve sorumluluk: HU, RAU; Teknik ve malzeme desteği: HU; Süpervizyon: HU; Fon sağlama (mevcut ise): yok.
Etik Onay: Bu çalışma için Atatürk Üniversitesi Deney Hayvanları Laboratuvarı. Atatürk Üniversitesi Kurumsal Hayvan Bakım ve Kullanım Etik Kurulunda 28.03.2019 tarih ve 2019-4/72 kararı ile onaylanmıştır.
Hakem Değerlendirmesi: Dış bağımsız.
Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemişlerdir.
Finansal Destek: Yazarlar finansal destek beyan etmemişlerdir.

Author Contributions: Concept/Design : HU, RAU; Data acquisition: HU, RAU; Data analysis and interpretation: HU, RAU; Drafting manuscript: HU; Critical revision of manuscript: RAU; Final approval and accountability: HU, RAU; Technical or material support: HU; Supervision: HU; Securing funding (if available): n/a.
Ethical Approval: Ataturk University Experimental Animal Laboratory. The Institutional Animal Care and Use Ethics Committee of Ataturk University approved the study on 28.03.2019, which was conducted in accordance with protocol number 2019-4/72.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: Authors declared no financial support

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