

Effects of Chitosan on Cisplatin-Induced Hepatorenal Toxicity in an Animal Model

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ABSTRACT

Introduction: We examined the effects of chitosan (CTS) for reducing cisplatin (CIS)-induced hepatorenal toxicity in a rat model.

Methods: A hepatorenal toxicity model was established by administering a single dose of 7 mg/kg CIS intraperitoneally (i.p) to Wistar albino rats. After seven days of once-daily normal saline or CTS (200 mg/kg; po) treatment, liver and kidney tissue samples were obtained from rats and Bcl-2/Bax ratio and caspase-3 expression levels were evaluated by Western blotting method. Histologically, structural damage was measured under a light microscope, and the degree of damage to organs was evaluated by a scoring system.

Results: CIS-induced hepatorenal damage was reversed with CTS treatment in the renal and hepatic tissues ($p < 0.05$). Overall, CIS-induced increases in apoptotic parameters, Bcl-2/Bax ratio and caspase-3 expression, were reversed by CTS treatment ($p < 0.05$).

Conclusion: It was determined that CTS, a biopolymer, may have a protective effect by preventing hepatorenal damage caused by CIS.

Keywords: Chitosan, cisplatin, apoptosis, hepatorenal toxicity

Introduction

Although cancer survival rates have increased from 50% to 70% recently, the need to produce new solutions to protect the quality of life of individuals both during and after chemotherapy continues (1,2). This situation led to the acceleration of studies aimed at mitigating the side effects of drugs that are in clinical use, apart from the studies in which new drugs and combination therapy of cisplatin (CIS) with other cancer drugs that can be used in clinical use have been developed (3). Today, CIS is widely used for treating many types of cancer cases. Although it varies depending on the dose and duration of the treatment, hepatorenal toxicity is one of the most common side effects seen in individuals with cancer during CIS-treatment (4). Hepatorenal injuries with a high risk of mortality may develop in approximately one-third of patients after CIS-treatment. The literature findings emphasize that CIS-treatment is also effective in oxidative stress-induced hepatotoxicity, as well as causing damage to liver and kidney tissue due to apoptosis (5).

Chitosan (CTS), a degradable and non-toxic biopolymer obtained by deacetylation of chitin, in which the basic monomer unit is glucosamine, has many applications in the pharmaceutical field due to its antibacterial, antimicrobial, hemostatic, antioxidant activity and antitumor properties (6-8). The main advantages of CTS are its non-toxicity and free radical

scavenging properties have been reported in previous studies (9). CTS is widely used for various different medical applications such as wound healing materials and drug carriers (10). Because of these advantageous properties of CTS, we planned to investigate the protective effect of CTS on CIS-induced hepatorenal injury in a rat model and performed in vitro biochemical tests, including Bax/Bcl-2 ratio, when lower indicating resistance to apoptosis, and caspase (casp)-3 protein expression, when lower indicating resistance to apoptosis, in liver and kidney tissue as well as light microscopic assessments of hepatorenal histological damage indicating experimental tissue toxicity.

Methods

Drugs and Chemicals

CIS (50 mg/100 mL) (Koçak, İstanbul) was used for animal studies. All antibodies were purchased from Santa Cruz Biotechnology (USA) and other compounds and chemicals were obtained from the Sigma-Aldrich (USA).

Animal Experiments

Experimental procedures were conducted in accordance with the international ethical guidelines for investigations in laboratory animals



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and were approved by the Animal Research Ethics Committee of Near East University (approval number: 2019/101). The animals used in the study were obtained from the Near East University Animal Experiments Unit were fed with standard rat chow without any food and water intake restrictions. Wistar albino rats (male, 250-300 g and 8-10 weeks) were housed with three rats per cage under the conditions at 22 ± 2 °C temperature, $50\pm 10\%$ humidity, and 12:12 h dark/light cycle.

Rats were divided into 4 study groups (n=8): normal saline (NS) group, received NS (0.9% NaCl) 0.01 mL/kg; CTS group, received CTS 200 mg/kg orally for 7 days; CIS group, received a single dose of CIS 7 mg/kg intraperitoneally on day 1; and CIS/CTS group, received a single dose of CIS 7 mg/kg intraperitoneally on day 1 and CTS 200 mg/kg orally for 7 days (11,12).

At the end of the experiment, all rats were sacrificed and dissected to obtain their liver and kidney tissue. Half of the liver and kidney tissue were transferred into 10% buffered formaldehyde for histopathological examinations, while the remaining tissues were stored at -80 °C for western blotting.

Histopathologic Assays

The fixation of the tissues was provided, tissue tracing procedures (Leica TP1020, Leica Biosystems Nussloch GmbH, Germany) were completed, and then were embedded in paraffin (Leica EG1150 H, Leica Biosystems Nussloch GmbH, Germany). Sections of 5 µm thickness were taken with a microtome (Leica RM2255, Leica Biosystems Nussloch GmbH, Germany) and hematoxylin and eosin staining was performed (Bancroft and Gamble 2008). Histomorphology examination of the sections was performed using a Leica DM500 light microscope combined with the Leica Microsystem Framework integrated digital imaging analysis system (Leica Application Suit ver. 3.0 series 38132019, Leica ICC50 HD, Leica Biosystems Nussloch GmbH, Germany).

Liver tissue was evaluated in terms of eosinophilic cytoplasm, necrosis, congestion and mononuclear cell infiltration criteria, and kidney tissue was evaluated in terms of tubular damage, glomerular damage and mononuclear cell infiltration criteria. For each tissue section, these criteria were evaluated in 10 different fields (13,14).

Immunoblotting Assays

Bax, Bcl-2, and casp-3 expression levels in the kidney and liver tissue were determined using western blot analyses. To determine the protein

content of tissues, the Lowry method was used after the dissected tissues were centrifuged (15). Samples containing 100 µg protein were prepared and carried out in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins in the obtained gels were transferred to the membranes (Schleicher and Schuell, 0.45 µm, Germany) (16). Membranes were incubated with polyclonal primary antibodies for 12 h at +4 °C at a dilution of 1:200. Densitometric analysis of membranes was performed using Bio Rad Molecular Analyst software (www.totallab.com).

Statistical Analysis

Data are presented as mean with standard error of mean. At first, normality test in groups was performed using the Shapiro-Wilk test. Histopathological and molecular data were analysed using GraphPad Prism ver. 5.03 (GraphPad, San Diego, CA, USA) by Kruskal-Wallis ANOVA test followed by post-hoc Dunn's test. A p-value of 0.05 or less was required to confirm statistical significance.

Results

CIS-Induced Hepatorenal Damage was Reversed with CTS Treatment

The CIS treatment significantly increased the scores of tubular damages in kidney tissue compared with the NS and CTS treatments ($p<0.05$). The CTS treatment decreased tubular damage induced by the CIS treatment ($p<0.05$). The CIS treatment significantly increased both glomerular damage and mononuclear cell inflation in kidney tissue compared with the NS and CTS treatments ($p<0.05$). The CTS treatment significantly reduced both glomerular injury and mononuclear cell inflammation induced by the CIS treatment ($p<0.05$) (Table 1).

Microscopic examination revealed that the renal cortex and medullary structures were normal in the NS group (Figure 1a, b). In the CIS group, tubule structures were disrupted and Bowman's spaces in the glomeruli were very narrowed, as well as enlargements in the cortex and tubules (Figure 1c, d). In the CIS group, degeneration was also detected in the straight parts of the proximal and distal tubules in the outer medulla. The presence enlargement of the tubules was seen in the CIS/CTS group, which was less than the enlargement seen in the CIS group. In the CTS group, neither degeneration of the tubules in the the cortex and outer medulla nor narrowing of Bowman's spaces was detected (Figure 1e, f).

When the hepatocytes with eosinophilic cytoplasm and necrosis in the liver tissue were evaluated, most of the damage was observed in the CIS group. The number of hepatocytes with eosinophilic cytoplasm

Table 1. Histomorphological measurement scores of liver and kidney tissues

		NS (n=8)	CTS (n=8)	CIS (n=8)	CIS/CTS (n=8)
Liver	Eosinophilic cytoplasm	0.01±0.01	0.03±0.02 ⁺	0.35±0.09 [*]	0.1±0.05 ⁺
	Necrose	0.02±0.01	0.02±0.02 ⁺	0.42±0.06 [*]	0.18±0.04 ^{*,+}
	Congestion	0.02±0.02	0.03±0.02 ⁺	0.32±0.04 [*]	0.22±0.07 [*]
	Mononuclear cell infiltration	0.02±0.02	0.05±0.02 ⁺	0.35±0.02 [*]	0.22±0.04 ^{*,+}
Kidney	Tubular damage	0.05±0.02	0.03±0.02 ⁺	0.82±0.1 [*]	0.43±0.1 ⁺
	Glomerular damage	0.01±0.01	0.05±0.02 ⁺	0.62±0.1 [*]	0.18±0.08 ⁺
	Mononuclear cell infiltration	0.02±0.02	0.03±0.02 ⁺	0.4±0.04 [*]	0.2±0.09 ⁺

NS: Physiological saline solution, CIS: Cisplatin, CTS: Chitosan, CIS/CTS: Cisplatin and chitosan. Data were analysed using Kruskal-Wallis ANOVA test followed by post-hoc Dunn's test. ^{*}p<0.05 vs. NS; ⁺p<0.05 vs CIS

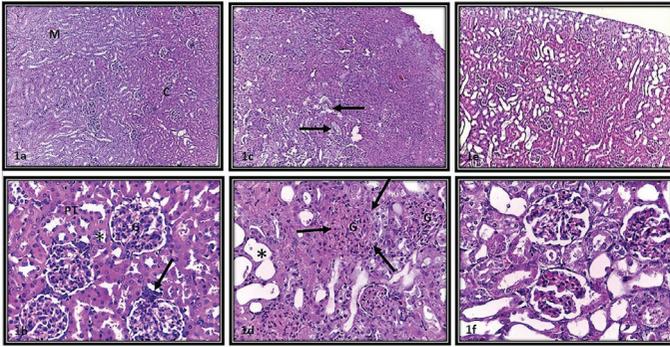


Figure 1. After normal saline (NS) exposure, a) the cortex (C) and medulla (M) (x10), b) glomeruli (G), proximal tubule (PT), distal tubule (*) and macula densa (→) structures of the kidney tissue were normal (x40). After cisplatin (CIS) exposure, c) glomerular structures could not be distinguished, and degeneration (→) was seen in tubules in the outer medulla (x10). d) Moreover, dilatation in tubular structures (*), closure of Bowman's space in glomerular structures, and tubule structures cannot be distinguished (x40). After CIS exposure followed by chitosan (CTS) treatment, e) tubular dilatation in the kidney tissue was reduced compared to the cisplatin exposure, and the degeneration in the tubules in the outer medulla region was very minimal (x10). f) The statuses of tubular degenerations were similar between the NS and CTS exposures (x40)

and necrosis occurring in the CIS group was significantly higher than those of the NS and CTS groups ($p < 0.05$). The damage to hepatocytes with eosinophilic cytoplasm in liver tissue due to CIS treatment was significantly reduced by CTS treatment ($p < 0.05$). Moreover, the necrosis caused by CIS in the liver tissue decreased with the addition of CTS treatment ($p < 0.05$). The congestion and mononuclear cell infiltration in the CIS group significantly increased compared to those of the NS and CTS groups ($p < 0.05$). The CTS treatment applied to the CIS-induced hepatorenal toxicity group reduced mononuclear cell infiltration and congestion in the liver tissue induced by CIS ($p < 0.05$) (Table 1).

By H&E staining of the cytoplasm of hepatocytes from the NS group, bile duct in the portal area, vessels and sinusoidal structures in the liver tissue was determined to be normal (Figure 2a, b). In the CIS group, the cytoplasm of hepatocytes around the v. centralis stained very lightly and their nuclei were heterochromatic and small, while the cytoplasm of hepatocytes around the portal area was stained darker (Figure 2c, d). The number of dark-stained hepatocytes around the v. centralis and portal area was increased in the CTS group compared in the CIS group (Figure 2e, f). While enlargement of the sinusoids, focal necrotic areas, and vacuolization in hepatocytes were determined in the CIS group (Figure 3a, b), necrotic areas and vacuolization were significantly reduced in the CIS/CTS group.

CIS-Induced Increase in Apoptotic Parameters Was Reversed by CTS-Treatment

The Bax/Bcl-2 ratios of liver and kidney tissue increased in the CIS group compared to the NS group ($p < 0.05$) (Figure 4a, b). In the CIS/CTS group, Bax/Bcl-2 ratio significantly decreased in liver and kidney tissue compared to the CIS group ($p < 0.05$). The casp-3 expression levels of liver and kidney tissue increased in the CIS group compared to the NS groups ($p < 0.05$) (Figure 4c, d). The casp-3 expression level in the liver tissue increased in the CIS group compared to the CST group ($p < 0.05$, Figure 4c). CTS administered after CIS treatment decreased the

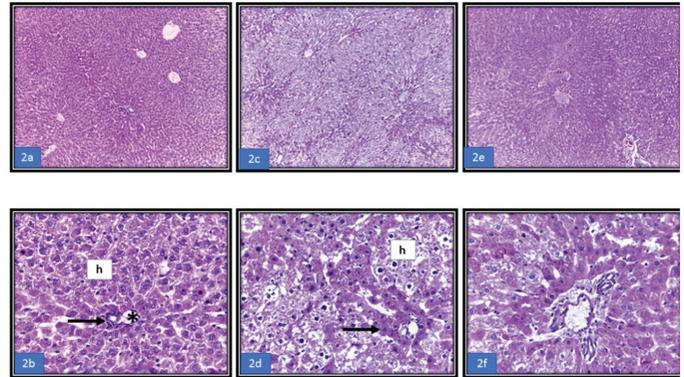


Figure 2. In the liver tissue of the normal saline group, a) hepatocyte cell cords and sinusoids (x10); b) bile duct, v. Porta branch (*) and hepatocytes (h) were seen to be in normal structure in the portal area (→) (x40). c) In the cisplatin (CIS)-exposed liver tissue, areas where hepatocytes dark and light stained cytoplasm were found (x10), d) hepatocytes with heterochromatic nuclei and light stained cytoplasm (h), hepatocytes with normal structure (arrows) are seen around the portal area (x40). In the hepatic tissue that is chitosan-treated after CIS exposure, compared to the only CIS exposure, e) the light-stained areas were significantly reduced (x10), and f) hepatocytes with normal structures around the portal area were more common (x40)

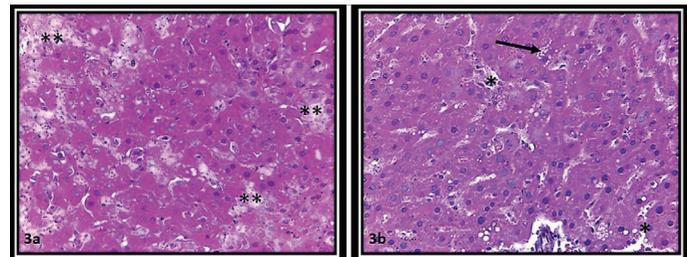


Figure 3. (a) Focal necrotic areas (**), (b) enlargement of sinusoids (*) and vacuolization of hepatocytes (→) (x40) were observed in the cisplatin-exposed liver tissue with H&E staining

casp-3 expression level in the liver tissue compared to the CIS ($p < 0.05$). However, CTS administered after CIS treatment did not cause any change in the casp-3 expression level in the kidney tissue. Representative images of the membranes obtained from western blotting are given in Figure 5.

Discussion

CIS is used to treat solid tumors; however, its clinical use is limited due to dose-related side effects such as nephrotoxicity, hepatotoxicity (17,18). Due to its many properties, such as anti-apoptotic and anti-inflammatory, CTS is widely used in different biomedical applications from tissue engineering to obesity treatment (19,20). Therefore, we evaluated the toxic effect of CIS on rats' liver and kidney tissue and the effectiveness of CTS treatment in alleviating CIS-induced toxicity.

The administration of CTS decreased the tubular damage induced by administering CIS. The administration of CTS significantly reduced both glomerular injury and mononuclear cell inflammation induced by administering CIS. On CIS treatment with microscopic examination, tubule structures were found to be disrupted and degeneration was detected in the straight parts of the proximal and distal tubules in the outer medulla. No degeneration of tubules in the cortex and outer medulla and narrowing of Bowman's spaces were detected with CTS treatment.

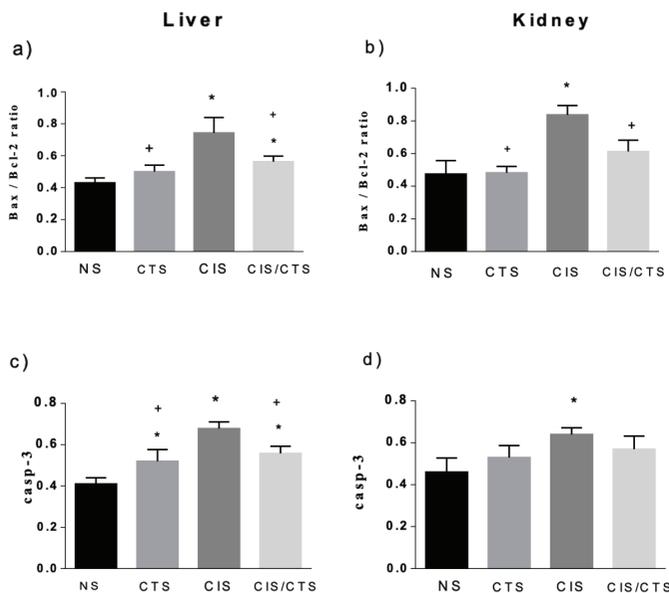


Figure 4. Bax/Bcl-2 ratio and casp-3 expression in CIS-induced hepatorenal toxicity
 β -actin expression was used for the normalizations of all Bax, Bcl-2 and casp-3 expressions (n=4 for each group). *p<0.05 vs. NS; +p<0.05 vs. CIS. NS: normal saline; CIS: cisplatin, CTS: chitosan, CIS/CTS: cisplatin and chitosan, Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma, casp-3: caspase-3

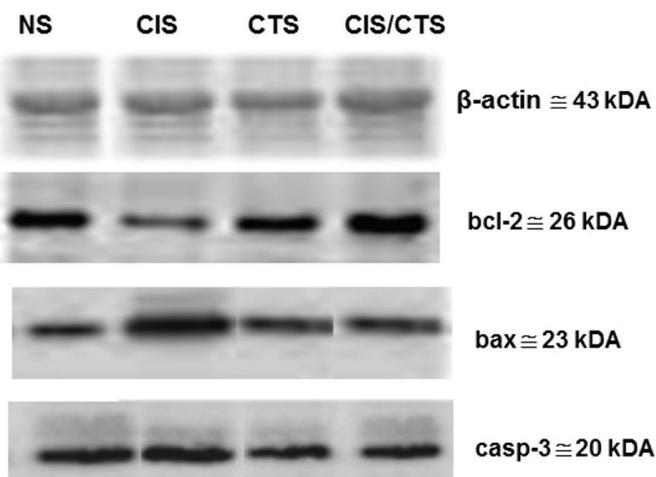
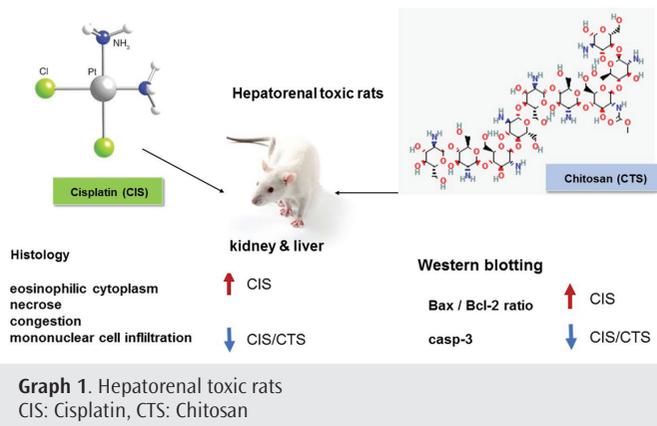


Figure 5. Representative images of expressions of studied proteins measured with western blot analysis
 NS: Normal saline, CTS: Chitosan, CIS: Cisplatin and CIS/CTS: Cisplatin and chitosan

The damage to hepatocytes with eosinophilic cytoplasm in liver tissue due to CIS treatment was significantly reduced by CTS treatment. CIS-induced necrosis of liver tissue was reduced in the CIS/CTS group. The number of dark-stained hepatocytes around the v. centralis and portal area was increased in with CTS treatment compared to the CIS treatment. While enlargement of the sinusoids, focal necrotic areas, and vacuolization in hepatocytes were determined in the CIS treatment, necrotic areas and vacuolization were significantly reduced in the CIS/CTS group.

In the CIS/CTS group, Bax/Bcl-2 levels were significantly reduced in liver and kidney tissue compared with CIS treatment. It was determined that



the CIS/CTS group increased the casp-3 expression level in the liver tissue compared to with CIS treatment. However, CIS/CTS group did not cause any change in the casp-3 expression level in the kidney tissue. Apoptosis is a normal cell death process controlled by physiological stimuli. Anti-apoptotic Bcl-2 and pro-apoptotic Bax, members of the Bcl-2 protein family, control many important steps in the apoptosis process (21). Casp-3, one of the apoptosis markers, are responsible for the activation of the casp cascade. The effect of CIS treatment on apoptotic proteins was evaluated in this study. Consistent with the literature, in our study results, high Bax and casp-3 levels and low Bcl-2 levels were observed in the liver and kidney tissue of rats with CIS-induced hepatorenal toxicity. Faubel et al. (22) showed that CIS treatment to mice increased casp-3 activity in the kidney tissue, and Fatima et al. (23) showed that CIS increased casp-3 activation in the liver tissue. Similarly, other studies have shown that the Bax/Bcl-2 ratio increased in both liver and kidney tissue with CIS treatment. In our study, it was determined that Bax/Bcl-2 ratio decreased and casp-3 activation decreased in liver and kidney tissue with CTS treatment. It has been reported that CTS causes apoptosis in cancer cells (8,24,25), but increases the Bax/Bcl-2 ratio by increasing casp-3 activation in damaged inflamed cells. Therefore, it is suggested that CTS has a protective effect owing to its anti-apoptotic property. This suggests that CTS plays an active role in cancer treatment, especially due to its different effects on apoptosis in cancer and inflamed cells. In addition to highlighting once again the well-known profound effects of CIS on the regulation Bax and casp-3 levels and downregulating Bcl-2 expression, our study determined that CTS treatment modifies these adverse effects: Bax and casp-3 were down-regulated and Bcl-2 expression was up-regulated.

Molecular changes in liver and kidney tissue were accompanied by histological changes in CIS-treated rats. As reported in previous studies, portal inflammation, sinusoidal dilatation and granuloma formation in the liver; tubular atrophy, vacuolization and apoptosis were observed in the kidney (26-29). In the study by Işeri et al. (27) they determined severe degeneration in the proximal and distal tubules and glomeruli because of histological evaluation of the kidney of rats treated with CIS. Histological examinations of liver tissue in the same study revealed that CIS treatment caused acute activation of Kupffer cells, degenerated hepatocytes, and moderate enlargement of sinusoids (27). In their study, Palipoch and Punsawad emphasized that CIS administration is effective in pathologies such as the separation of tubular cells from the basement

membrane and tubular necrosis (30). CIS treatment both altered the overall tissue structure and significantly increased the histology score compared with normal rats in this study. Changes were reduced with CTS, as seen in other *in vivo* studies of histology score, CIS-induced liver and kidney injury. The anticancer effect of CIS is manifested by inducing apoptosis, which is programmed cell death. The cytotoxic effects of CIS have been demonstrated in many *in vitro* and fewer *in vivo* studies using different cell types. Both our histological and molecular findings emphasized the role of apoptotic mechanisms in hepatorenal damage caused by CIS, and it was determined that CTS therapy used against damage caused by CIS can be a protective agent, as reported in other studies.

Study Limitations

The most important limitation of this study was that the entire apoptotic pathway was not evaluated. In this context, the inability to evaluate the effective parameters in the apoptotic pathway by western blot or immunohistochemistry analyses is among the limitations of our study.

Conclusions

The preference of CTS usage for several purposes in experimental studies is related to its biodegradable polymer structure having no toxic effect. The findings of the current study support the that CTS has a potential for use to reduce the CIS-induced hepatorenal toxicity in a rat model. This hepatorenal protective ability of CTS may be linked with its anti-apoptotic and anti-inflammatory abilities.

Ethics Committee Approval: The Near East University Local Animal Experiments Ethics Committee granted approval for this study (approval number: 2019/101), date: 21.11.2019).

Informed Consent: Patient approval has not been obtained as it is performed on animals.

Peer-review: Externally peer-reviewed.

Authorship Contributions: Concept - A.A., A.Ö.Ş.; Design - A.A., A.Ö.Ş.; Data Collection or Processing - A.A., H.Ş., A.K., S.S., A.Ö.Ş.; Analysis or Interpretation - A.A., H.Ş., A.K., S.S., A.Ö.Ş.; Literature Search - A.A., H.Ş., A.K., S.S., A.Ö.Ş.; Writing - A.A., H.Ş., A.K., S.S., A.Ö.Ş.

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

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