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The potency of obestatin in improving kidney functions and apoptosis in rats with cisplatin-induced acute kidney injury

Zarife Nigâr ÖZDEMİR KUMRAL[1]*, Alisina BULUT[1]+, Bahadır ÜZÜLMEZ[1]+, Mustafa VEZİRHÜYÜK[1]+, Zafer KÖK [1]+, Naziye ÖZKAN YENAL[2], Berrak Ç. YEĞEN[1], Mehmet KOÇ[1,3]

1 Department of Physiology, Faculty of Medicine, Marmara University, Istanbul, Turkey.
2 Department of Medical Pathology Techniques, Vocational School of Health Services, Marmara University, Istanbul, Turkey
3 Department of Internal Medicine, Division of Nephrology, Faculty of Medicine, Marmara University, Istanbul, Turkey.

Corresponding Author. E-mail: drmkoc@yahoo.com (M.K.); Tel. +00-000-000 00 00.

+ These authors equally participated in the present study.

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ABSTRACT: Cisplatin (CP), which is the most commonly used anticancer agent to treat several solid tumors, may cause acute kidney injury (AKI) as the major limiting factor for its clinical use. Obestatin (OB) is a ghrelin gene-derived peptide produced in several tissues and has shown anti-oxidant, anti-apoptotic, and anti-inflammatory effects in many experimental models. This study investigated the effect of OB treatment on nephrotoxicity induced by CP. Rats were divided into 4 groups as two control (1 ml/kg, saline, intraperitoneal (ip), single dose) and two CP-induced (7 mg/kg, ip, single dose) AKI groups (8 rats in each group). Immediately after the CP injection and the following two days, injections of OB (10 µg/kg, ip) were performed. Rats were decapitated at the end of 72 hours. Blood and kidney tissue samples were taken for biochemical and histopathological measurements. The results of the present study revealed that serum creatinine and BUN levels were significantly increased in the CP-induced AKI group when compared to the control group. Treatment with OB improved kidney functions and ameliorated renal oxidative injury and maintained oxidative balance in the CP-induced AKI model, which was revealed by elevated malondialdehyde and depleted glutathione levels. TUNEL scores also demonstrated that CP increased the apoptotic response, while OB treatment abolished it. CP-induced medullary and cortical injuries were also partially reversed by OB treatment. Thus, our findings show that OB alleviates CP-induced nephrotoxicity in rats through the abolishment of oxidative stress and apoptosis.

KEYWORDS: cisplatin; acute kidney injury; obestatin; rat; apoptosis.

1. INTRODUCTION

Cisplatin (CP) is an effective chemotherapeutic agent widely used to treat a wide variety of malignancies. However, side effects of CP, which include gastrointestinal toxicity, hepatotoxicity, neurotoxicity, and acute kidney injury (AKI) have been commonly reported [1]. AKI is a common kidney disease with a multifactorial clinical syndrome characterized by the loss of renal function and acute tubular necrosis that occur due to renal tubular damage, vascular injury, and inflammation [1,3]. Damage and death of medullary and tubular cells contribute to the pathogenesis of AKI, while apoptosis plays an important role in this process [1,2]. The range of treatment strategies includes avoidance of nephrotoxins, volume control, management of blood pressure, and kidney replacement therapy [1]. After a single dose of nephrotoxic agent CP therapy, approximately 25-35% of patients experience a significant decline in renal function [2,3], which commonly necessitates dose reduction or discontinuation of the treatment. The mechanisms involved in cisplatin-induced AKI appear to be multifactorial, including inflammation, oxidative stress, and apoptosis [1]. Although most agents with deleterious effects on genetic material are generally not highly toxic to non-proliferating cells, CP selectively damages non-dividing renal proximal tubule cells and causes an immediate and typically reversible impairment in kidney function [4]. Generally, necrosis occurs at high doses, while apoptosis can occur with therapeutic doses [1]. In addition, several studies have proven that CP-induced renal tubular damage is also facilitated by oxidative stress, which involves the production of reactive oxygen species (ROS) and the

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depletion of antioxidants [5,6], leading to the peroxidation of membrane lipids, protein denaturation, DNA damage, inflammation, and apoptosis. Several studies have suggested that inflammatory mechanisms play an essential role in the pathogenesis of CP nephrotoxicity, while modulation of the renal inflammatory reaction by free radical scavengers and antioxidants is effective in ameliorating CP-induced nephrotoxicity [7]. However, except for supportive practices that include fluid resuscitation, no specific therapeutic strategies exist to alleviate cisplatin-induced AKI. Therefore, identifying new agents to ameliorate CP-induced AKI would be beneficial for patients in need of CP-based chemotherapy.

Obestatin (OB), a peptide consisting of 23 amino acids, was originally isolated from rat stomach as a post-translational product of preproghrelin [8]. Apart from the gastric oxyntic mucosa, OB is also produced in the duodenum, jejunum, pancreas, spleen, mammary gland, breast milk, blood plasma, Leydig cells, and salivary glands [9-12]; however, its specific receptor is not defined yet [13]. In humans, OB has been shown to play an important role in cardiac function under both physiological and pathological conditions. When compared to healthy control subjects, salivary OB level was higher in overweight patients with ischemic heart disease [14], while serum OB was lower in type 2 diabetic and obese subjects [15]. Likewise, in spontaneously hypertensive rats, plasma OB levels were elevated and altered ghrelin/OB ratios were detected [15]. In another study, OB improved myocardial function in isolated rat hearts by reducing cell death and apoptosis of cardiomyocytes injured by ischemia/reperfusion [16]. On the other hand, peripheral administration of OB has been demonstrated to exert potent neuroprotective effects by improving inflammation and oxidative damage in rats induced with subarachnoid hemorrhage [17]. It has also been shown that OB, via its possible anti-inflammatory and anti-apoptotic properties, has attenuated renal ischemia/reperfusion injury (I/R) [18]. Thus, based on the aforementioned observations, it was aimed to elucidate the possible protective effects of OB administration on CP-induced AKI and the underlying mechanisms in its renoprotective activity.

2. RESULTS

2.1. OB protected against CP-induced AKI

At the 72nd h of CP-induced AKI, serum levels of creatinine (Cr) and urea nitrogen (BUN) were significantly increased when compared to the control group ($p<0.05$, Figure 1), verifying the impairment in renal function. On the other hand, the elevations in serum Cr and BUN levels were significantly decreased in the OB-treated CP group ($p<0.05$ and $p<0.01$). The significant body weight loss that occurred within 72 h of AKI induction ($p<0.01$) was reduced when the rats received OB treatment ($p<0.05$, Figure 1C).

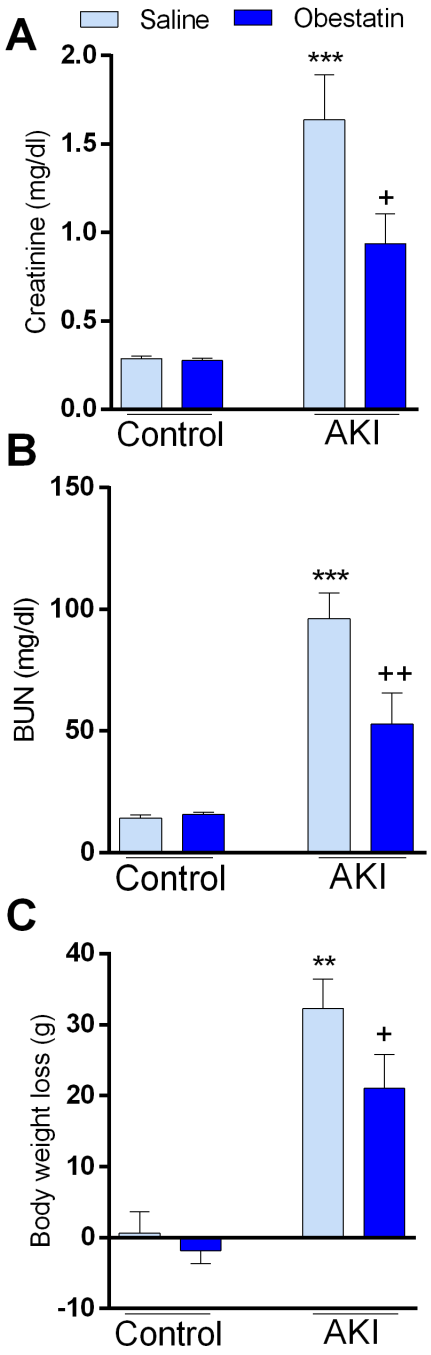

Figure 1. Serum levels of A) creatinine, B) blood nitrogen urea (BUN), and C) body weight loss. Data are mean ± SEM; **p<0.01, ***p<0.001 compared to saline-treated control group, +p<0.05, ++p<0.01 compared to saline-treated AKI group (n=8 in each group).

2.2. OB prevented oxidative damage in CP-induced AKI

When compared to that of the saline-treated control group, renal level of MDA, a product of lipid peroxidation, was significantly increased in the saline-treated AKI rats (p<0.05, Figure 2A), while OB treatment significantly depressed tissue MDA (p<0.01). Despite that the antioxidant GSH content of the kidney tissue was not altered by CP-induced AKI, OB treatment elevated GSH levels in AKI rats (p<0.05, Figure 2B).

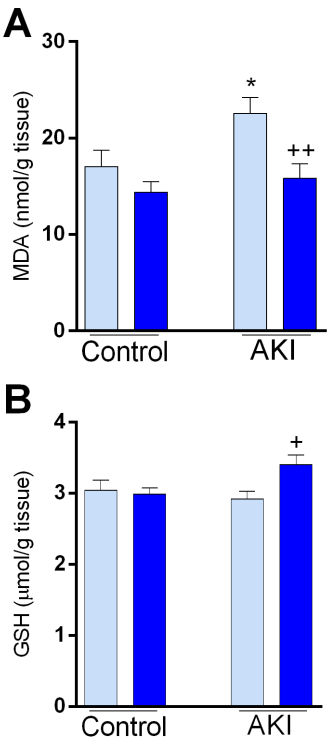

Figure 2. Markers of antioxidant; glutathione (GSH) status and oxidative stress parameter; malondialdehyde (MDA) levels in rats. Data are mean ± SEM; *p<0.05, ***p<0.001 compared to saline-treated control group, +p<0.05, ++p<0.01 compared to saline-treated AKI group (n=8 in each group).

2.3. OB preserved kidney morphology after the CP administration.

No morphological changes were observed in the renal cortical or medullar areas of saline- or OB-treated control groups (Figures 3 and 4). However, extensive acute tubular damage, including tubular epithelial cell swelling, tubular dilatation, necrosis, and tubular epithelial degeneration were observed in the saline-treated AKI group (Figure 3), but the histopathological damage scores of the cortex (Figure 4A) were not different among the groups. On the other hand, elevated scores verified the presence of CP-induced medullary damage (p<0.01), while OB treatment had no significant impact on medullary damage. The number of TUNEL-positive cells in the kidney sections of saline-treated AKI, showing epithelial cell necrosis, was significantly increased as compared to the control group (p<0.01). TUNEL-positive cells were slightly (p=0.071) reduced in the OB-treated AKI group, but were still higher as compared to the control group (p<0.01).

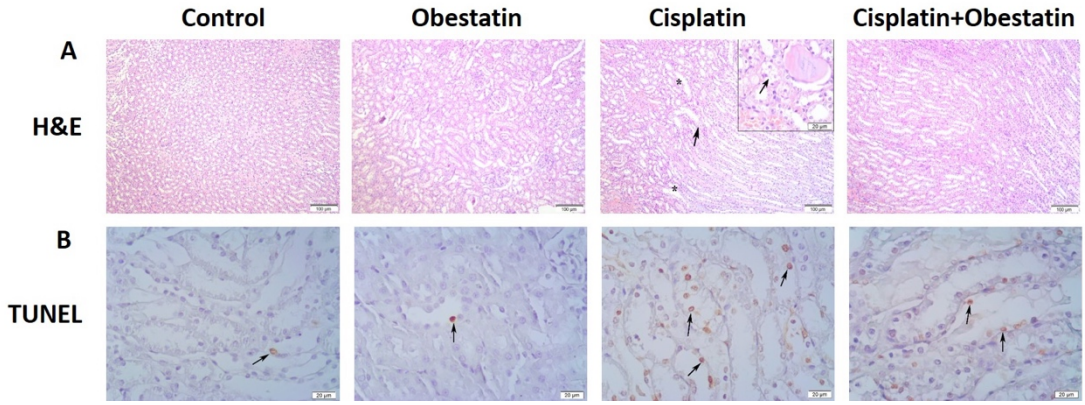

Figure 3. Representative H&E stained images of saline or obestatin-treated control and AKI groups (A). CP caused a marked pathological response to tubular damage including tubular epithelial cell swelling and dilatation (*), necrosis,

tubular epithelial degeneration (arrow) extensive cell injury in the cortex compared to saline-treated controls. Rats in the CP+OB group displayed significantly less severe damage pathology compared to the CP group. Representative photomicrographs of kidney tissue sections for TUNEL staining (B) to apoptosis detection. (Arrows) Brown nuclear stain indicates TUNEL-positive cells. Scale bars; for TUNEL: 20 µm, for H&E: 100 µm (inset 20 µm).

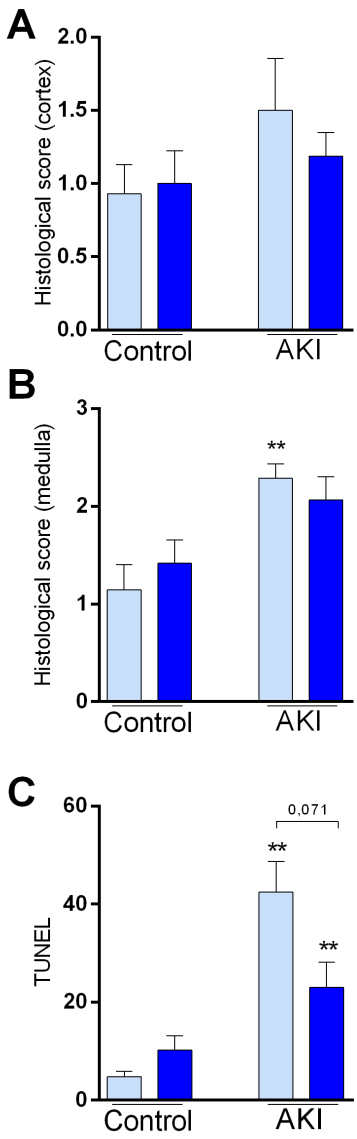

Figure 4. Histological damage scores of A) cortex and B) medulla. C) Semiquantitative assessment of apoptosis score. **p<0.01 versus the saline-treated control group. The data are expressed as mean ± SEM.

3. DISCUSSION

Cellular toxicity develops when using most chemotherapeutic agents, which aim to prevent the growth and proliferation of malignant cells with their cytotoxic effects [21]. Metabolites of these drugs, mostly excreted by the kidneys, cause nephrotoxicity, and AKI is triggered by damage to the glomeruli, tubules and vessels. The results of the present study revealed that pre-treatment with OB improved kidney functions and ameliorated renal oxidative AKI due to CP, by supporting the balance of oxidant/antioxidant systems. TUNEL scores also revealed that CP increased the apoptotic response while OB treatment abolished it. CP-induced medullary

and cortical injuries were also partially reversed by OB treatment in this model. Thus, our findings show that OB ameliorates CP-induced nephrotoxicity in rats through the abolishment of oxidative stress and apoptosis.

It has been shown that a low concentration of CP triggers apoptosis [22], while its high concentration causes necrosis in proximal tubular cells [20]. The complex in-vivo mechanisms that include oxidative stress are involved in the pathogenesis of CP-induced AKI, and kidney functions are seriously impaired by the upregulation of the oxidative system and reduction in antioxidant capacity [23,24]. Ramesh et al. [37] have shown that the blockade of TNF-α action prevented the upregulation of other cytokines and ameliorated CP nephrotoxicity, demonstrating an important role for TNF-α in CP nephrotoxicity. Cytokines released during the course of CP-induced AKI (e.g. TNF- α) are responsible for apoptosis. ROS either directly or by generating signal transduction molecules, causes cellular damage, and thereby has a major role in the development of renal apoptosis [25]. In the present study, OB alleviated CP-induced apoptosis as well as oxidative damage, while other studies have shown that OB inhibits the release of cytokines from infiltrating cells [17, 26]. Moreover, OB was demonstrated to prevent apoptosis in both rodent/human pancreatic islet cells and cardiomyocytes by binding the specific OB receptors through the activation of PI3K/AKT and ERK ½ pathways [27,28].

Our team has previously reported that peripheral administration of OB exerts its attenuating effects in renal I/R injury through its possible anti-inflammatory properties, such as suppression of neutrophil accumulation, limitation of lipid peroxidation, and triggering antioxidant capacity [18], as well as that it has an anti-apoptotic effect in intestinal I/R injury [29]. The present study demonstrated that CP application increased MDA levels, while OB treatment had a significant effect in reducing the increased MDA levels. In contrary to previous studies showing that CP causes depletion of GSH and other antioxidants [30,31], our results showed that GSH was not depleted due to CP induction, but OB treatment provided elevated GSH levels in the presence of AKI. By comparing OB-treated and saline-treated rats with CP-induced AKI model, we showed that OB has a partial therapeutic effect on CP-induced AKI for the first time. As compared to the control group, the CP-administrated rats significantly lost body weight, which is in parallel with the earlier studies showing that CP causes weight loss [32,33]. OB treatment significantly reduced renal dysfunction and tubular epithelial cell necrosis caused by CP. In previous studies, it was shown that OB treatment reversed the increased inflammatory response in many oxidative stress models in several tissues [17,18,29,34,35]. Apoptosis is one of the role players in the pathogenesis of CP-induced nephrotoxicity, and it was suggested that the mechanism of CP nephrotoxicity includes a robust inflammatory response and apoptotic process [1]. In the present study, CP administration resulted in nephrological impairment that was accompanied by increased MDA levels and medullar histological scores. On the other hand, OB improved the nephrological state of CP-induced rats and alleviated both the apoptotic and oxidative activities in the kidney tissue. OB is an amidated peptide of 23 amino acids identified in different species using bioinformatics analyzes of the preproghrelin genomic sequence that is derived from the C-terminal portion of the preproghrelin precursor but does not activate orphan G protein-coupled receptor GPR39 [8,36]. Current findings indicate that OB treatment may be an option to alleviate AKI caused by chemotherapeutics, including CP.

Numerous studies have shown that proinflammatory cytokines play a critical role in the pathogenesis of CP-induced AKI. It has been shown that gene and protein levels of TNF-α, an inflammatory cytokine [37], are increased after the CP injection and that TNF-α-deficient mice are resistant to CP-induced AKI [38-41]. This study found that OB partially suppressed the increased number of infiltrating macrophages in kidney tissues induced by CP. OB, which is encoded by the same gene that encodes ghrelin, has the opposite effect of ghrelin in some parameters, such as growth hormone secretion and increased appetite [42]. Multiple apoptosis pathways are activated in CP-induced AKI, including both extrinsic and intrinsic pathways of apoptosis [43]. CP or its metabolites can be absorbed by renal tubular cells via organic cation transporters located on the basolateral side of tubular cells, leading to tubular cell death and AKI [44]. Since tubular cell death is a precipitating factor for CP-induced AKI in both patients and animal models [44,46], protecting tubular epithelial cells from death should effectively stop the onset and progression of CP-induced nephrotoxicity [1,47]. In the extrinsic pathway of apoptosis, TNF-α or other cell death receptor ligands can lead to apoptosis by activating death receptors on the proximal tubule cell surface, leading to activation of caspase-8 and triggering of downstream effector caspases such as caspase 3 [48]. In a recent study, which was shown to prevent apoptosis by activating PI3K/Akt and ERK1/2 pathways in rodent cells and human pancreatic cells, OB attenuated apoptosis as evidenced by the TUNEL test [49]. In the present study, CP administration led to tubular epithelial cell necrosis and prominent histological changes in the kidneys of the rats with AKI. These

morphological changes are associated with renal dysfunction and are supported by an increase in serum Cr and BUN levels.

Besides these pieces of information, it is important to note that the case of cisplatin-induced AKI occurs over a period of days in both rodents and humans depending on the regiments of the doses and application numbers. Further, in the case of a single application, the clinical and histological changes in the kidney develop faster than in multiple-dose treatments. In the present study, we used a single high nephrotoxic dose (e.g; 3-8 mg/kg in rats) which shows 1-2 days after administration only a few morphological changes within 5 days after the CP administration. In this single high dose CP-induced AKI model, first signs of structural regeneration were observed 7 days after the CP injection which return to the baseline levels within 14 days [49]. Chronic repeated pretreatment with OB in the CP-induced nephrotoxicity model can be used to clarify the underline mechanisms triggered by OB.

In clinical practice, the pathophysiology of CP-induced nephrotoxicity is not well defined. Repeated administration of cisplatin results in a time-related increase of many parameters. However, the time course of the disease depends on the dosage, frequency of cisplatin injection, and cumulative dose of cisplatin. When using a single high nephrotoxic dose only a few minimal changes can be found at 1-2 days after its administration [50,51], while morphological changes are usually seen not earlier than 3 days after the CP administration. Future studies will be needed to elucidate more details of the molecular mechanisms of OB's suppressive effects on long-term administration of CP-induced nephrotoxicity.

Very little was found in the literature on the question of whether OB improves renal damage besides our previous study indicating that it attenuates renal I/R injury. Recent results provide further support for the hypothesis that OB treatment alleviates kidney damage due to CP administration, reduces TUNEL-positive apoptotic cells, and improves renal function tests.

4. CONCLUSION

The present study, which investigated possible reno-protective effects of OB on CP-induced experimental AKI model, demonstrated significant reductions in the serum levels of Cr and BUN, as well as in renal MDA levels along with an elevation in the renal antioxidant GSH level, indicating that OB alleviated oxidative renal injury and improved renal function. Histological analysis further showed that OB was effective in reducing injury scores and apoptosis of the renal tissue. In conclusion, our findings link OB with decreased oxidative damage and apoptosis, suggesting OB may provide a potential target for AKI treatment.

5. MATERIALS AND METHODS

Female (n=32) Sprague-Dawley rats (250±20 g), obtained from Marmara University Experimental Animals Application and Research Center, were housed under conditions maintained at room temperature (22±1°C) with a 12/12 hour light/dark cycle and 65-70% humidity. Rats with free access to water and food were fed with standard rat pellets. The study protocol has obtained ethical approval from Marmara University Animal Experiments Local Ethics Committee (97.2010.mar).

Rats were randomly divided into 4 groups as two control and two CP-induced AKI groups with 8 rats in each group. AKI was induced with a single intraperitoneal (ip) injection of cisplatinum (CP Ebewe; 7 mg/kg), and vehicle (saline) was injected immediately after the CP administration and the following two days. Another CP group received ip OB (10 µg/kg; Alexis Biochemicals, San Diego, CA) immediately after the CP injection for the following two days. The control group was treated only with saline (1 ml/kg, ip), while the OB-treated control group received ip OB starting immediately after AKI induction and the following 2 days. The reason for the chosen dose of OB was based on a previous study showing its renoprotective, anti-apoptotic, and anti-inflammatory effects in ischemia/reperfusion-induced renal injury [18].

The rats were decapitated at the 72nd hour of the experimental procedure. Trunk blood samples were collected, and kidney tissues were gently separated from adjacent tissues and removed. Samples were incubated at 1 h at room temperature and then centrifuged at 3500 RPM for 15 min, and serum was retained. Samples were stored at -80°C until urea nitrogen (BUN) and creatinine (Cr) levels were measured with an autoanalyzer (Cobas 8000, Roche Diagnostics, Basel, Switzerland). Kidney tissues were kept at -80°C until the measurements of malondialdehyde (MDA), and glutathione (GSH) levels were performed. For the determination of

apoptosis, kidney tissues were assessed using the in situ DNA nick end labeling (TUNEL), while the tissues were fixed in 4% formaldehyde for histopathological scoring.

5.1. Malondialdehyde and glutathione levels

Kidney samples were homogenized with iced cold 150 mM KCl and subjected to two different modified protocols for kidneys to determine MDA levels, which is one of the lipid peroxidation products, and secondly, GSH, which indicates the intracellular antioxidant status [18]. Spectrophotometric readings for MDA levels were expressed as nanomoles of MDA per gram of tissue. GSH results were determined by a spectrophotometric method based on Ellman's reagent and were expressed as µmol GSH per gram of tissue [19].

5.3. Histological analyses

The kidneys were sectioned and fixed in 10% formalin, dehydrated, and embedded in paraffin. Tissues were sectioned at 3 Am and stained with hematoxylin and eosin (H&E), azan (Heidenhain), and periodic acid-chiff (PAS). The histological slides of kidneys were evaluated for semiquantitative analysis without knowledge of the treatment protocol, as described previously [19]. The changes seen were limited to the tubulointerstitial areas and graded as follows: 0, normal; I, areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving 25% of cortical tubules; II, similar changes involving 25 – 50% of cortical tubules; III, similar changes involving 50 – 75% of cortical tubules; IV, similar changes involving 75% of cortical tubules [52].

5.4. Apoptotic index

Apoptosis in tubular cells was assessed using the in situ DNA nick end labeling (TUNEL) technique [21]. Detection of DNA fragmentation was performed using ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, MA, USA, cat no: S7101). A quantitative analysis was performed by counting TUNEL-positive cells per one field at ×400 magnification in OSOM. The mean number of stained cells in 20 randomly selected fields in each kidney was expressed as the number of TUNEL-positive cells.

5.5. Statistical analyses

All values described in the text and figures are expressed as mean ± standard error of the mean (SEM). Statistical analyses were carried out using GraphPad Prism, 8 (GraphPad Software, San Diego, CA, USA). Data were analyzed using one-way ANOVA followed by Bonferroni's post hoc test or Mann Whitney U tests. P-value of less than 0.05 was considered to be significant.

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