

# Effects of Apocynin against Monosodium Glutamate-Induced Oxidative Damage in Rat Kidney

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## ABSTRACT

**Objective:** Monosodium glutamate causes oxidative stress that affects a variety of organ systems, along with the kidney. The aim of this study was to evaluate the protective role of apocynin in kidney degeneration caused by monosodium glutamate using biochemical and histological methods.

**Materials and Methods:** Sprague-Dawley rats (n=32) were used for this study. Four experimental groups were randomly formed: the Control (Cont), Apocynin (APO), Monosodium Glutamate (MSG), and MSG+APO groups. The MSG group received oral administration of MSG (120 mg/kg) for 28 days. The MSG+APO and APO groups received apocynin (25 mg/kg) during the last 5 days of the experiment. Kidney tissue was processed for biochemical and microscopic analyses. Biochemical methods were used to examine the levels of malondialdehyde (MDA) and glutathione (GSH) in the tissue and the activities of myeloperoxidase (MPO) and superoxide dismutase (SOD). Light and electron microscopy were also used to examine for histological changes in kidney tissue.

**Results:** The MSG group was compared with the Cont and APO groups; it was found that MDA and MPO levels were elevated, whereas GSH and SOD activity were decreased. In contrast to the Cont and APO groups, the MSG+APO group showed higher GSH levels, lower MPO activities and increased MDA levels. While apocynin treatment improved renal tissue histology, MSG-induction led to deterioration of renal morphology.

**Conclusion:** The study revealed that MSG increased oxidative damage and renal tissue degeneration. Moreover, apocynin reduced renal damage by modulating the ratio of antioxidants to oxidants.

**Keywords:** Monosodium glutamate, Apocynin, Kidney, Galectin

## INTRODUCTION

The flavor of food is improved by the use of monosodium glutamate (MSG), a common additive in prepared foods. However, various animal studies have shown that consumption of MSG is hazardous to a number of organs, including the liver, brain, thymus, and kidneys (1,2). Previous studies showed an association between renal fibrosis and chronic MSG consumption (3) and

that oxidative stress was the main reason for kidney injury (4). The main factor of oxidative stress is free radicals. The primary causes of a reactive oxygen species (ROS) and oxygen radicals in cells are increased formation or decreased elimination of these radicals (5). Studies showed that the metabolism of nutritional factors, intracellular or extracellular factors, or detoxification processes could be caused by oxidative stress (6). Con-



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sequently, increased glutamate metabolism in the kidneys, as seen with chronic MSG consumption, may contribute to ROS formation. Thus, excessive glutamate metabolism in the kidneys, as seen with chronic MSG consumption, was a source of ROS that could contribute to kidney disease.

The NADPH oxidase (Nox) complex is inhibited by apocynin (APO, 4-hydroxy-3-methoxyasetophenan) isolated from the medicinal plant *Picrorhiza kurroa*. The enzyme Nox is in charge of the production of ROS (7). APO is known as a selective Nox inhibitor in activated human neutrophils. It prevents its activity and the accompanying ROS production (7). In recent years, APO was the subject of experimental studies due to its anti-inflammatory activity. It was observed that APO induced the antioxidant defense system by increasing glutathione (GSH) and reduced cellular stress caused by ischemia (8). APO has become an especially important and widely used experimental agent to affect Nox activity. In addition, recent animal studies demonstrated the protective effects of APO on the kidneys in a model of induced nephrotoxicity (9).

Galectin-3 belongs to a family of soluble beta-galactoside-binding proteins found in many vertebrate epithelial and myoid cell types (10). Because of its ability to bind carbohydrates, it regulates cell growth, differentiation, and inflammation. It also plays a complicated, content-dependent function in the kidneys. During development, it supports the formation of kidney tissue (nephrogenesis). Its expression level is quite high in the ureteric bud and the structures derived from this bud (11). Elevated plasma galectin-3 levels are associated with the risk of recurrent renal dysfunction, chronic kidney disease, infections leading to cardiovascular problems, and renal dysfunction leading to death (11). In a study with rats, Nishiyama et al. showed that galectin-3 expression increased in acute renal failure due to ischemia and toxic damage and emphasized that galectin-3 could be important in acute tubular injury and in the subsequent recovery period (12). The researchers immunohistochemically detected the development of galectin-3 in proximal convoluted tubules two hours after reperfusion. In the later stages of regeneration period, they confirmed the presence of galectin-3 in the proximal and distal tubules (12). Chronic kidney disease is a global health problem, so the use of galectin-3, which is associated with kidney development in many studies and serves as a biomarker in this and related diseases, is important for early diagnosis of the disease (11).

In this study, we investigated the ameliorative effect of APO on kidneys, as a possible antioxidant effect, against MSG-induced kidney degeneration using biochemical, light, fluorescence and electron microscopic methods.

## MATERIALS AND METHODS

### Animals

Sprague-Dawley rats (n:32, 250-300 g) were housed in wire-bottomed individual cages (at 22±2°C) and relative humidity and fed pelleted laboratory chow on a 12-h/12-h light-dark cycle. The Animal Care and Use Committee of Acibadem University,

Istanbul, Turkey (ACU HADYEK -2021/63) approved the experimental protocols. The animals were randomly divided into four groups: (1) The Cont group was administered 120 mg/kg distilled water for 28 days with oral gavage, (2) the APO group was administered 120 mg/kg distilled water for 23 days, and APO 25 mg/kg was administered for 24 to 28 days. (3) The MSG group, which received MSG (120 mg/kg/day) for 28 days. (4) The MSG + APO group received MSG (120 mg/kg) during the experiment and APO (25 mg/kg) was given during the last 5 days of the experiment. All treatments continued for 28 days at the same hour (at 10 o'clock). At the end of the experiment, animals were immediately sacrificed, and kidney tissues were removed. For biochemical analysis, tissue samples were homogenized with ice-cold 150 mM KCl for the determination of MDA, MPO, SOD and GSH levels. Kidney tissues were also prepared for histological, immunohistochemical, and transmission electron microscopical analyzes to evaluate the morphological and ultrastructural changes.

### Biochemical Analysis

#### Measurements of Kidney Tissue MDA Level

A commercially available kit (E-BC-K025-M, Elabscience, Houston, TX, USA) was used to determine the MDA levels. Thiobarbituric acid (TBA), one of the degradation products of lipid peroxidation, was used in the procedure to measure the intensity of the pink complex formed by MDA at a wavelength of 532 nm. The MDA levels in tissue were calculated in nmol/g.

#### Measurements of Kidney Tissue of MPO Activity

The MPO activity was determined using a commercially available kit (MBS704859, MyBioSource, CA, USA) Standard solutions were prepared according to the kit instructions. The solution was added after each application, and the plate was measured at 450 nm with a spectrophotometer (13).

#### Measurements of Kidney Tissue GSH Activity

GSH plays a role as an antioxidant molecule in the structural and functional protection of the integrity of cells, tissue, and organ systems. GSH analysis in testicular tissues was performed according to the Beutler method (13). The principle of the method is based on the fact that the GSH in the analysis tube reacts with 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) to give a yellowish color and the light intensity of this color was read in the spectrophotometer at a wavelength of 410 nm. Tissue homogenates were centrifuged, and 10% TCA solution was added to the obtained supernatant, mixed, and centrifuged again to allow the proteins to precipitate. Light-colored supernatant samples were used for GSH analysis. The intensity of the color formed in the samples kept at room temperature for 5 minutes was read at 410 nm in the spectrophotometer and GSH levels in µmol/g tissue were found by using the glutathione standard graph.

#### Measurements of Kidney Tissue SOD Activity

SOD activity was determined with the Sigma SOD Determination Kit (E-BC-K019-M, Elabscience, Houston, TX, USA). The absorbance values were read at 450 nm after the SOD activity was incubated using an enzyme working solution.

## Microscopical Analysis

### Light Microscopical Preparation

Kidney tissue samples were fixed in a 10% neutral buffered formalin solution for 72 hours. After fixation, tissues were dehydrated with ethyl alcohol series (70%, 90%, 96%, 100%) and cleared with xylene. The tissues were embedded in paraffin. Sections (at 5  $\mu$ m) were then stained with hematoxylin-eosin (H&E) for general histopathologic assessment of renal tissue and with Periodic Acid-Schiff Reaction (PAS) to assess changes in the basement membrane and tubule brush border (14). The H&E-stained sections were semi-quantitatively scored for proximal and distal tubule damage, inflammatory cell infiltration, and vascular congestion (15). Sections were scored between 0 and 3 (0:none, 1:mild, 2:moderate, 3:severe) for each criterion. The total score was 9.

### Galectin-3 Immunofluorescence Analysis

Paraffin tissue sections were taken on positively charged slides. The sections were rehydrated with decreasing ethyl alcohol (100%, 96%, 90%, 70%) series. Then the slides were placed in citrate buffer and kept in the microwave. The sections were then cooled, rinsed with phosphate-buffered saline (PBS), and treated with 5% bovine serum albumin (BSA). After rinsing with PBS, the slides were treated with goat anti-rabbit galectin-3 primary antibody (1:200, Cedarlane Laboratories Burlington, ON, Canada). The sections were stored (at +4°C, overnight) in the dark. The sections were washed with PBS (containing %1 BSA) and then treated with the secondary antibody conjugated

with Alexa Flour 488 (1:1000, Thermo Fisher Scientific, USA) for 1 hour and then washed with PBS (at room temperature) (16). Subsequently, the sections were incubated with 4'-6-diamidino-2-phenylindole (DAPI) in the dark and examined with a confocal microscope (Zeiss LSM 700).

### Transmission Electron Microscopical Preparation

Kidney tissue samples were fixed with 2.5% glutaraldehyde solution and then postfixed in 1% osmium tetroxide. They were then processed for routine electron microscopic analysis (17). Uranyl acetate and lead citrate were used to contrast sections before being evaluated with a transmission electron microscope (Thermo Scientific TALOS L 120 C, The Netherlands).

### Statistical Analysis

GraphPad Prism 4.0 was used for statistical analysis (GraphPad Software, San Diego, CA, USA). All data were expressed as means $\pm$ SD. An analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to compare the data sets. It was stated that  $p < 0.05$  was statistically significant.

## RESULTS

### Biochemical Results

MDA concentration in kidneys was significantly ( $p < 0.01$ ) higher in the MSG group (41.52 $\pm$ 12.9) than in the Cont group (18.75 $\pm$ 2.59) and the APO group (14 $\pm$ 7.08). The APO treatment tended to reduce the MDA concentration in the MSG+APO group (16.79 $\pm$ 7.57) compared with the MSG group (Figure 1a).

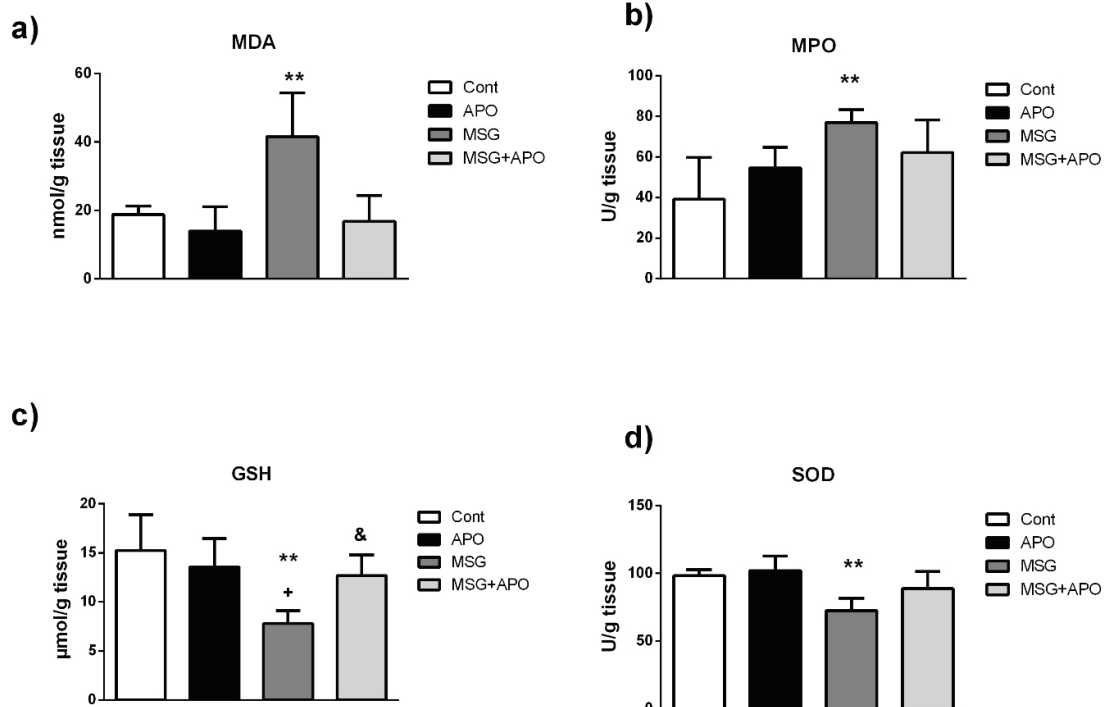


Figure 1. (a) Malondialdehyde (MDA) levels, (b) myeloperoxidase (MPO) activities, (c) glutathione (GSH) levels and (d) superoxide dismutase (SOD) activities in the kidney tissues. Values are represented as mean $\pm$ SD. \*\* $p < 0.01$  versus control group; + $p < 0.001$  versus APO, & $p < 0.05$  versus MSG group.

It was found that MPO activity was significantly ( $p < 0.01$ ) increased in the MSG group ( $76.97 \pm 6.35$ ) as compared to the Cont ( $39.21 \pm 20.52$ ) and APO ( $54.53 \pm 10.18$ ) groups, while the APO treatment tended to increase in the MSG+APO group ( $62.17 \pm 16.19$ ), but this increase was not statistically significant. (Figure 1b).

GSH levels in the MSG group ( $7.80 \pm 1.30$ ) renal tissue showed a significant ( $p < 0.01$ ,  $p < 0.001$ , respectively) decrease compared to the Cont ( $15.26 \pm 3.62$ ) and APO ( $13.58 \pm 2.87$ ) groups, while the MSG+APO ( $12.68 \pm 2.12$ ) group showed increased GSH levels ( $p < 0.05$ ) compared to the MSG group (Figure 1c).

SOD activity was significantly ( $p < 0.01$ ) decreased with MSG administration in the MSG group ( $72.75 \pm 9.03$ ) as compared to the Cont ( $98.32 \pm 4.60$ ) group. The SOD activity also decreased in the APO ( $102 \pm 10.94$ ) group. Additionally, the MSG+APO group ( $88.766 \pm 12.52$ ) tended to increase, but this was not statistically significant (Figure 1d).

### Light Microscopical Results

Normal kidney morphology was observed in the Cont and APO groups. Light micrographs showed that the ingestion of MSG affected the morphology of the nephrons. Thus, in the MSG group, a relative increase in Bowman's spaces, congestion of

glomerular capillaries, and thickening of the basement membrane were observed. In the APO group, an improvement in morphology was observed. In the MSG+APO group, there was less vascular congestion, tubular damage, and a reduction in Bowman's space observed (Figure 2). PAS positivity was seen in the proximal and distal tubules. PAS positivity was higher in the Cont and APO groups, but this positivity was decreased in the MSG group (Figure 3). Proximal and distal tubule PAS positivity was higher in the MSG+APO group than the MSG group (Figure 3).

### Galectin-3 Immunofluorescence Results

Galectin-3 (Gal-3) immunoreactivity (IR) was observed predominantly in the proximal tubules of renal tissue in the experimental groups. The cytoplasm of proximal tubule cells had significantly higher IR, but glomerular Gal-3 expression was very low in the experimental groups. Gal-3 IR was similar in the Cont and APO groups, but Gal-3 IR was higher in the MSG group than in the other experimental groups. Gal-3 IR in the MSG+APO group was lower than in the MSG group (Figure 4). The fluorescence intensity of Gal-3 was highest in the MSG group as compared with the other experimental groups. The fluorescence intensity of the MSG+APO group was lower as compared to the MSG group (Figure 5).

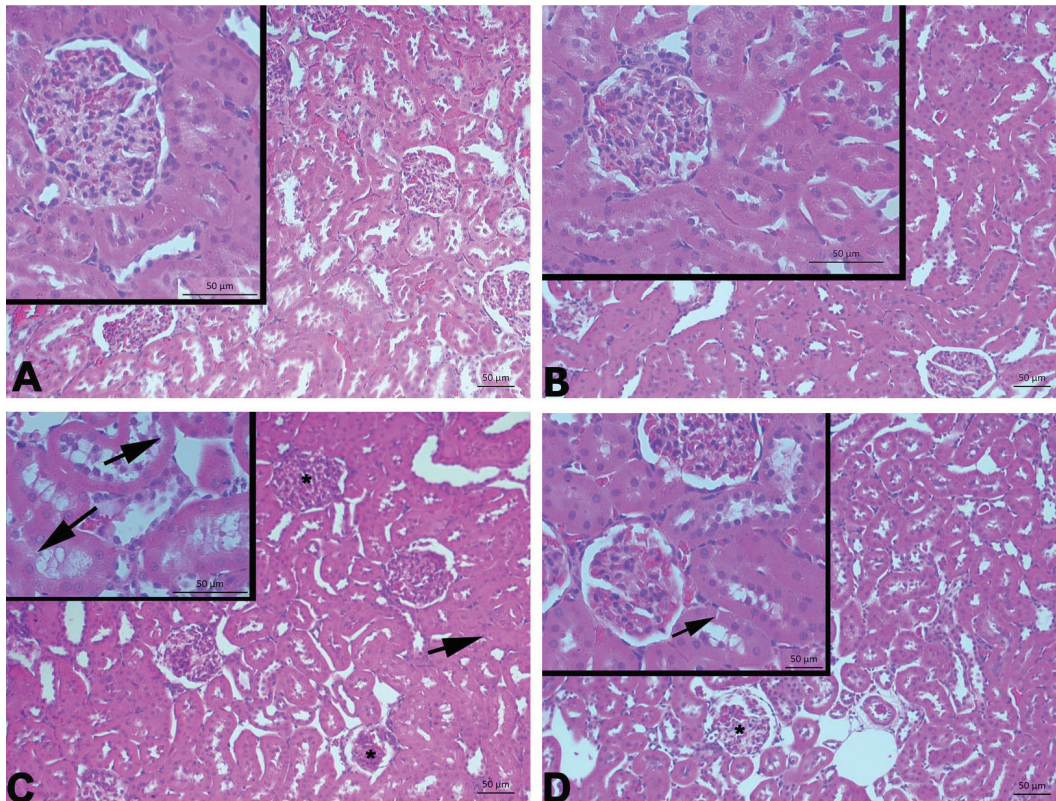


Figure 2. Normal kidney morphology in the Cont (A) and APO (B) groups. Severe tubular (arrow) and glomerular (\*) degenerations were observed in the MSG group (C) and mild tubular (arrow) and glomerular (\*) degenerations in the MSG+APO group (D). H&E staining.

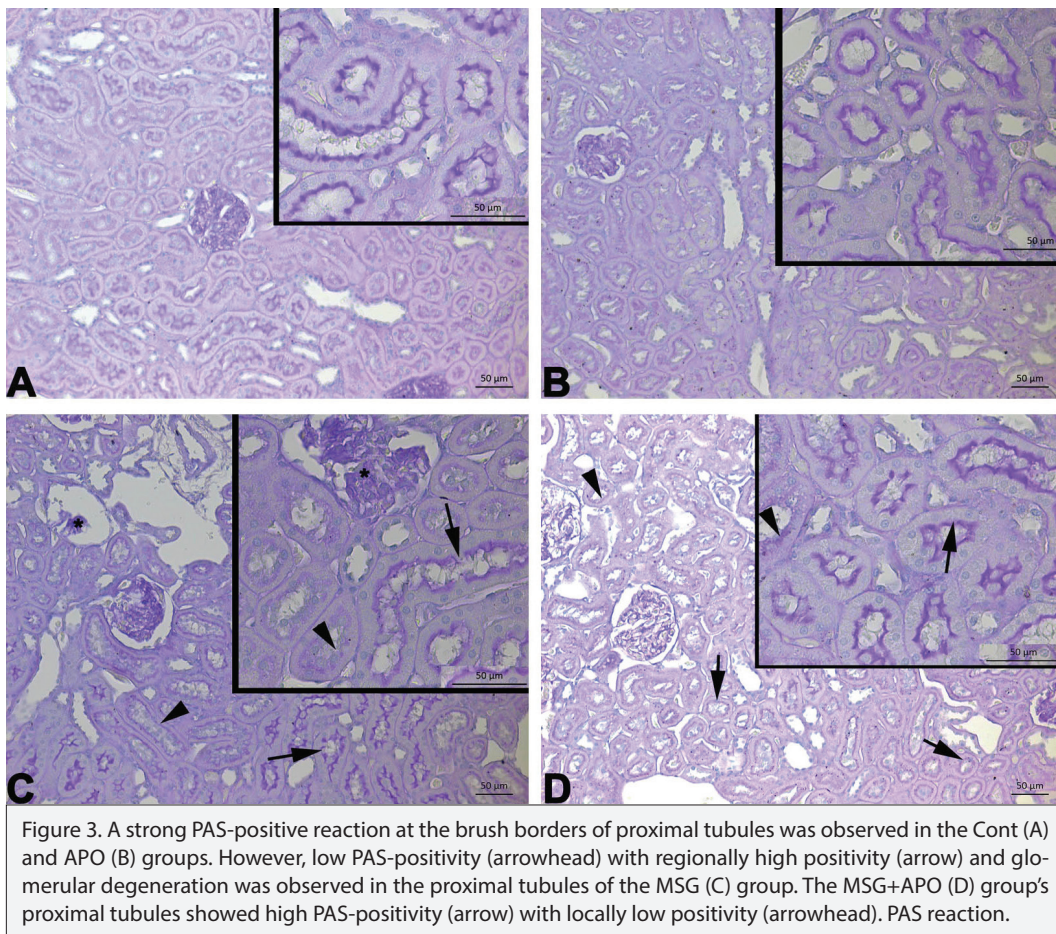


Figure 3. A strong PAS-positive reaction at the brush borders of proximal tubules was observed in the Cont (A) and APO (B) groups. However, low PAS-positivity (arrowhead) with regionally high positivity (arrow) and glomerular degeneration was observed in the proximal tubules of the MSG (C) group. The MSG+APO (D) group's proximal tubules showed high PAS-positivity (arrow) with locally low positivity (arrowhead). PAS reaction.

### Electron Microscopy Results

Normal glomerulus and proximal tubule morphology were observed in the Cont and APO groups (Figures 6 a and b). Degeneration of podocyte structure, vacuoles and lipid droplets in the cytoplasm of proximal tubules were seen in the MSG group (Figure 6c). Ameliorated glomerular and tubular morphology were seen in the MSG+APO group (Figure 6d).

### DISCUSSION

This study showed that MSG leads to oxidative stress by altering biochemical parameters. In the MSG group, both MDA levels and MPO activities increased, while GSH levels and SOD activities decreased. As for histopathological changes, degeneration of proximal and distal tubules, inflammatory cell infiltration, and vascular edema in renal tissue were also detected in the MSG given group. The APO treatment enhanced this biochemical and histopathological degeneration.

As well as ischemia and other toxic insults, chemicals can greatly damage the kidneys. Therefore, both direct and indirect disruptions of renal cell energy metabolism can lead to cell damage and acute renal insufficiency (15). MPO can also alter inflammatory responses by affecting multiple signaling pathways involved in cell signaling and cell-cell interactions (18). As

a result, MSG induced renal degeneration and oxidative stress could be related to an increase in MPO activity (19). In addition, APO as a Nox inhibitor requires activation by MPO (20). A significant factor in the nephrotoxic consequences was the exposure of the kidney to MSG, which led to the formation of ROS (21,22). By consuming MSG, ROS levels are elevated, leading to a reduction in antioxidant enzymes while an increase in oxidative stress, which results in tubulointerstitial fibrosis, thus giving rise to structural damage to kidneys.

The administration of MSG either intravenously or orally has been shown to affect both lipid peroxidation by products and renal function in rats (23). Following administration of MSG to rats, Paul et al. reported decreased SOD, catalase, glutathione-S-transferase, and GSH activities in the kidney (23). MDA and conjugated dienes, which are markers for lipid peroxidation, were also found to be increased in renal tissue treated with MSG. Furthermore, MSG treatment resulted in an increase in heat shock cognate 70, which is an indication of oxidative stress, and a reduction in glutathione-S-transferase (4). A few studies demonstrated the ameliorative effects of vitamin C, E, and quercetin on kidneys treated with MSG, however, the mechanisms of how these antioxidants work remain obscured (2). These antioxidants appeared to play a significant role in reducing renal

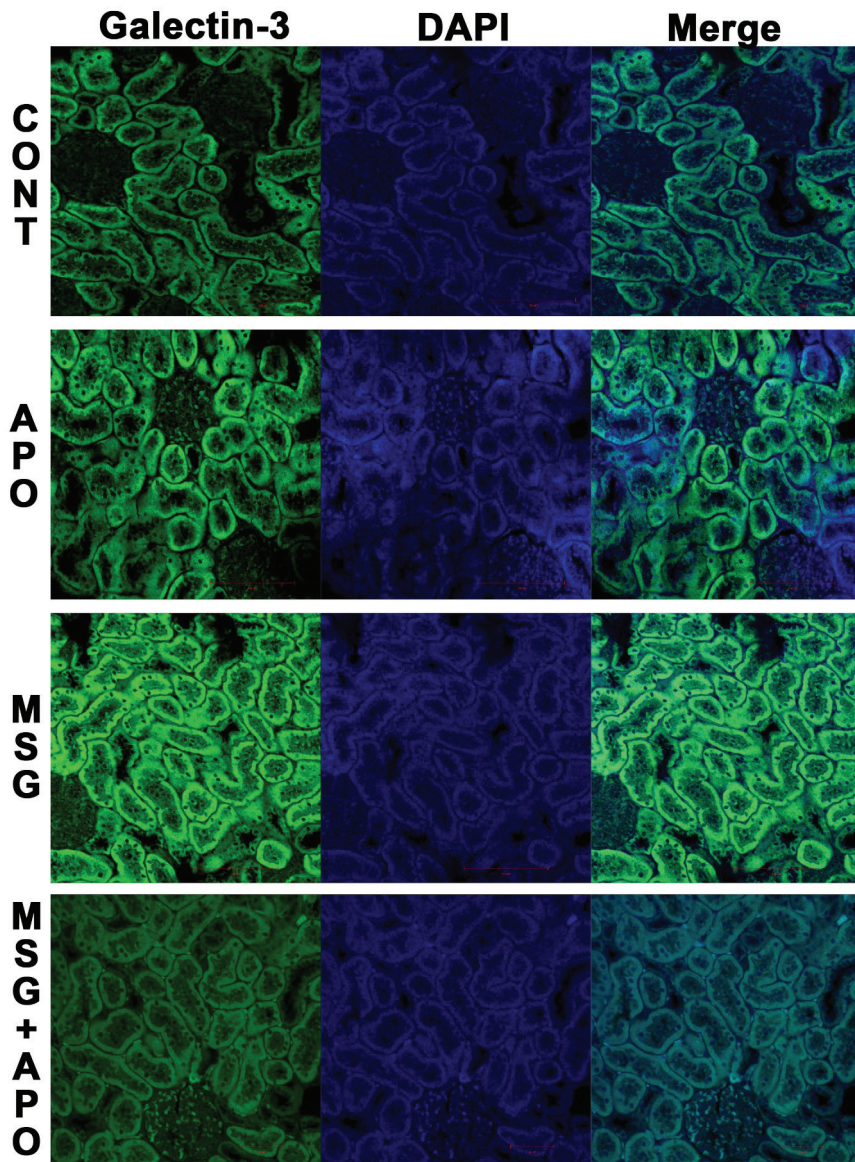


Figure 4. Galectin-3 (Gal-3) immunofluorescence staining in the experimental groups. Immunoreactivity (IR) of Gal-3 was similar in the Cont and APO groups. Gal-3 IR higher in the MSG group. Gal-3 IR in the MSG +APO group was lower than in the MSG group.

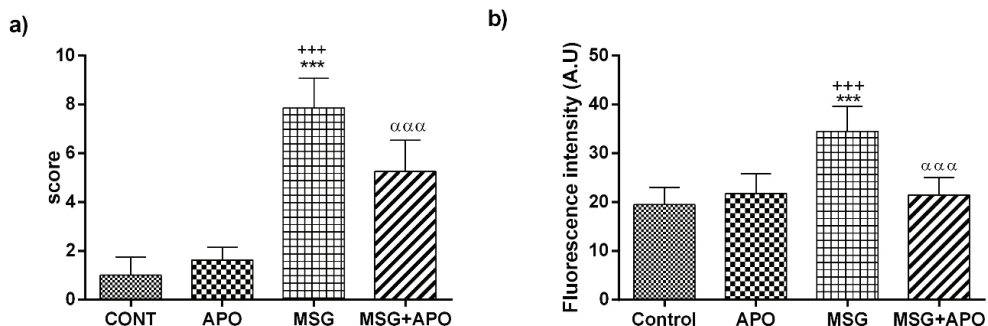


Figure 5. Histopathological score (a) and anti-Galectin-3 fluorescence intensity (b) graphs. Values are represented as mean±SD. \*\*\*p<0.001 versus control group; +++p<0.001 versus APO group, αααp<0.001 versus MSG group.

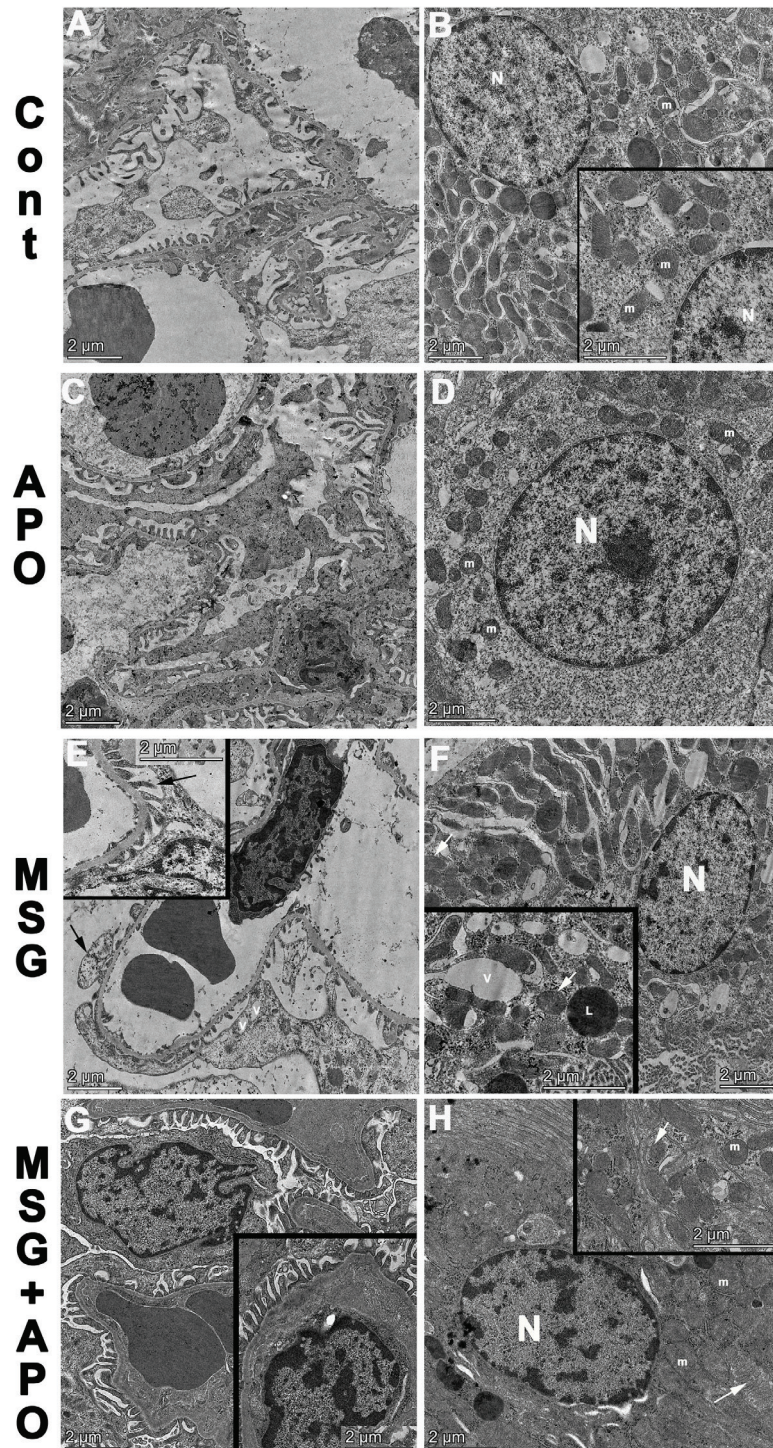


Figure 6. Electron micrographs of the experimental groups. Glomerulus (A, C) and proximal tubule (B, D) morphology is normal in the control and APO groups. Degenerated podocytes (black arrow) in the glomerulus (E), vacuoles (v), lipid droplets (L), degenerated mitochondria (white arrow) in the cytoplasm of proximal tubules were observed in the MSG group (F). Improved morphology of glomerulus (G), slightly degenerated morphology of proximal tubules with normal morphology of mitochondria (m) and degenerated mitochondria (white arrow) were observed in the MSG+APO group (H). Nucleus: N, electron micrographs contrasted with uranyl-acetate-lead citrate.

inflammatory responses through a reduction of inflammatory enzyme expressions and cytokine production (24) or inhibiting Nuclear Factor kappa B activity (25). Similar to previous studies, we observed that MDA level and MPO activity were higher in the MSG group than in the control and APO groups. In addition, GSH levels and SOD activity were lower in the MSG group than in the other experimental groups. APO treatment had a positive effect on these markers of oxidative stress in renal tissue.

Recent experimental studies showed that consumption of MSG altered kidney histopathology. Thus, swollen tubular morphology and infiltration of inflammatory cells were observed in rat kidneys (23,26). Experimental studies revealed that MSG-induced kidney deterioration was accompanied by cytoplasmic and nuclear degenerations of the proximal tubules (27). The studies also showed that several cells exhibited splitting and thickening of the basement membrane, partial loss of the brush border, and loss of the majority of cytoplasmic organelles. The nuclei of podocytes and endothelial cells typically exhibited chromatin condensation adjacent to the nuclear membrane, giving them an atypical appearance (28). The development of vascular problems linked to renal impairments is significantly influenced by oxidative stress, and the protective benefits of antioxidants could be explained by an increase in ROS degradation (29). APO is a Nox inhibitor and one of its positive effects could be a decrease in oxidative stress (30,31). Although the specific process of inhibition is not known, APO appears to be related to the ability of the cytosolic Nox complex component p47phox to translocate the membrane (20). In this study, renal pathophysiology was improved by APO treatment. Thus, it is thought that APO simultaneously improves kidney function. This effect is probably related to the different modes of action of the antioxidant used (32). In this study, renal morphology was degenerated in the MSG group. However, APO improved renal degeneration in the MSG+APO group. This effect might be related to the inhibition of Nox. Thus, renal morphology could be improved by the antioxidant effect of APO.

A member of the galectin family of beta-galactoside-binding lectins, galectin-3 has a carbohydrate recognition domain and is between 32 and 35 kDa in size. It controls cell proliferation, differentiation, and inflammation by binding to carbohydrates (11). The expression of galectin-3 was significantly increased in both ischemia and toxicity, suggesting that it may be crucial for acute tubular injury and subsequent regeneration (11). The kidneys of galectin-3 knockout mice were examined morphologically, and the results showed severe glomerular sclerosis, PAS-positive deposits in the mesangium, thickening of the glomerular basement membrane, and Bowman's capsule (33). Galectin-3 is connected with the onset of renal fibrosis in animal models (34). In this study, it was found that galectin-3 IR was higher in the MSG group. Thus, the toxicity of MSG could cause an increase in this immunoreactivity. APO treatment could also lead to a decrease in this immunoreactivity. The expression of galectin-3 could be useful for the evaluation of morphological degeneration parameters.

## CONCLUSION

In the last decade, it has become clear that continuous intake of MSG has the potential to negatively affect peripheral organs, including the kidneys. In addition, antioxidants such as APO could be helpful in renal injury. Our biochemical and histological results suggest that APO could ameliorate the kidney injury induced by MSG through inhibition of Nox activity by improved biochemical and histopathological degenerations.

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**Ethics Committee Approval:** This study was approved by the ethics committee of Acibadem Mehmet Ali Aydinlar University 06.10.2021, 21/63.

**Informed Consent:** Written consent was obtained from the participants.

**Peer Review:** Externally peer-reviewed.

**Author Contributions:** Conception/Design of Study- M.A.E.; Data Acquisition- M.A.E., O.B.O., G.O.; Data Analysis/Interpretation- M.A.E., O.B.O., Z.G.; Drafting Manuscript- M.A.E., Z.G.; Critical Revision of Manuscript- M.A.E., S.A.; Final Approval and Accountability- M.A.E., S.A., G.O., O.B., Z.G.

**Conflict of Interest:** Authors declared no conflict of interest.

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