ISSN: 2980-3063 (online) Original Article

Rats
2023; 1(2): 27-34
Doi: 10.5281/zenodo.10444360
https://ratsjournal.com

Therapeutic effect of pomegranate peel extract on heme oxygen-free 1 (HO-1) and angiotensin-converting enzyme-2 (ACE-2) in the kidney tissue of mice treated with mitomycin

Habibe Gündoğdu[1], Erhan Uluman[2], Sevda Eliş Yıldız[3], Pınar Aksu Kılıçle[4], Arzu Gezer[5*], Ebru Karadağ Sarı[6]

[1]Department of Histology and Embryology, Ataturk University School of Medicine, Erzurum, Türkiye
[2]Department of Biology, Faculty of Arts and Sciences, Kafkas University, Kars, Türkiye
[3]Department of Midwifery, Kafkas University Faculty of Health Sciences, Kars, Türkiye
[4]Department of Biology, Faculty of Arts and Sciences, Kafkas University, Kars, Türkiye
[5]Vocational School of Health Services Ataturk University, Erzurum, Türkiye
[6]Department of Histology, Kafkas University Faculty of Veterinary Medicine, Kars, Türkiye
*Corresponding: a.gezer25@hotmail.com

Recevied: 11.10.2023 Accepted: 08.12.2023 Published: 30.12.2023

Abstract
This study aimed to investigate the effects of pomegranate peel (PP) extract on the damage caused by mitomycin-C (MMC) in kidney tissue. In the study, 32 female Mus musculus albino mice were divided into 4 groups, as; Control Group: distilled water was administered via oral gavage for 15 days. MMC Group: distilled water was administered via oral gavage for 15 days. On day 15, MMC was administered at a dose of 2 mg/kg i.p. MMC+150PP Group: 150 mg/kg of PP extract was administered via oral gavage for 15 days. On day 15, MMC was administered at a dose of 2 mg/kg i.p. MMC+300PP Group: 300 mg/kg of PP extract was administered via oral gavage for 15 days. On day 15, MMC was administered at a dose of 2 mg/kg i.p. Crossman's triple staining method was employed to examine the histological structure of the kidney tissue. Additionally, the Streptavidin-Biotin Peroxidase complex technique was applied to determine the immunoreactivity of heme oxygenase-1 (HO-1) and angiotensin-converting enzyme-2 (ACE-2). The application of MMC caused degeneration in the kidney tissue, but when MMC was used in combination with PP extract, it significantly protective effect the existing degeneration in the kidney tissue. HO-1 immunoreactivity was similar in the control and MMC+300PP groups, but it was higher in the MMC+150PP group compared to the control and MMC+300PP groups, although it was lower than in the MMC group. ACE-2 immunoreactivity was weak in the control and MMC+300PP groups, moderate in the MMC+150PP group, but intense in the MMC group. ACE-2 and HO-1 immunoreactivity were not observed in the medulla and vascular endothelium of the kidney tissues in any of the groups. It was determined that MMC causes damage to kidney tissue, and it was found that PP extract protective effect this damage. Based on these findings, it is believed that PP extract can be used in the prevention and treatment of kidney diseases.
Keywords: *Kidney, mitomycin-C, heme oxygenase-1, angiotensin-converting enzyme-2, pomegranate peel*

1. Introduction

The kidneys are responsible for the disposal of waste products from the body, managing electrolytes, maintaining acid-base balance, eliminating toxins, preserving intravascular volume, regulating renin-angiotensin-aldosterone secretion, and controlling blood pressure. Additionally, they play a role in the reabsorption of electrolytes, amino acids, calcium, phosphate, water, and glucose, as well as in the secretion of hormones calcitriol and erythropoietin.[1,2]

Heme oxygenase (HO) was first discovered as a microsomal enzyme in 1968 by Tenhunen et al. HO is responsible for the breakdown of heme-containing proteins such as hemoglobin, myoglobin, and cytochromes, leading to the production of products like bilirubin.[3,4] HO plays an important role in regulating body homeostasis through its antiinflammatory, antioxidant, and antiapoptotic properties, making it significant in the treatment of diseases such as diabetes, obesity, pulmonary disease, gastrointestinal disorders, kidney dysfunction, dermatitis, and others.[5] HO is found in 3 different forms in the tissues and organs of living organisms, which are HO-1, HO-2, and HO-3. The HO-1 isoenzyme is particularly prevalent in the spleen, liver, and kidneys.[6] HO-1 is not produced as a result of the activity of other enzymes, such as lipooxygenase, cyclooxygenase, xanthine oxidase, or myeloperoxidase. HO-1 is an enzyme responsible for breaking down heme molecules and converting them into products like bilirubin. HO functions by breaking down heme, a compound, to eliminate excess heme molecules in the body, which can be harmful. During this process, it contributes to the production of protective substances like bilirubin. Therefore, the activity of HO-1 is not directly associated with other enzymes.[7] HO-1 plays an important role in cellular defense against oxidative stress, which is a subtype of oxidative stress. One of the main functions of HO-1 is to reduce oxidati-

How to cite this article: Gündoğdu H, Uluman E, Eliş Yıldız S, Aksu Kılıçle P, Gezer A, Karadağ Sarı E. Therapeutic effect of pomegranate peel extract on heme oxygen-free 1 (HO-1) and angiotensin-converting enzyme-2 (ACE-2) in the kidney tissue of mice treated with mitomycin. *Rats.* 2023; 1(2): 27-34 Doi: 10.5281/zenodo.10444360

ve stress within cells and provide a protective effect to cells. Additionally, as part of the HO system, it can assist in preventing apoptosis, which is programmed cell death. Therefore, the activation of HO-1 plays a significant role in cellular defense and combating oxidative stress.[4] HO-1 plays an important role in diagnosing metabolic and vascular diseases of the kidney and is critically important for regulating the physiological functions of the kidney.[8] A deficiency in HO-1 can result in kidney oxygen deprivation, leading to the inability to perform filtration and absorption functions, exposing the kidney to harmful substances.[9]

Angiotensin-converting enzyme-2 (ACE-2) is a type I integral membrane glycoprotein with a molecular weight of approximately 120 kDa. It consists of 1277 amino acids and attaches to the cell membrane through a hydrophobic region located at the C-terminal end.[10,11] ACE-2 is found in various tissues throughout the body, including Leydig cells in the testes, epithelial cells in alveoli and the small intestine, endothelial cells in arterial and venous blood vessels, the epidermal basal cell layer of the skin, and nasal and oral mucosa. Additionally, ACE-2 is commonly present in the distal and proximal tubules of the kidneys, glomerular epithelial cells, and urothelial cells in the bladder.[12] ACE-2 modulates the effects of angiotensin II (Ang-II) within the renin-angiotensin system. Ang-II is a peptide that causes blood vessels to constrict, it raises blood pressure, and leads to water and salt retention in the body. ACE-2 breaks down Ang-II and instead produces the more protective angiotensin 1-7 (Ang 1-7). Therefore, ACE-2 indirectly plays a role in regulating blood pressure and body salt balance.[13]

The pomegranate, belonging to the Punicaceae family, is known as a fruit rich in bioactive compounds. Additionally, it possesses strong antioxidant properties. The pomegranate tree is a tropical plant that takes the form of a shrub and can range from 2 to 5 m in height. Pomegranate juice, peel, and seeds contain numerous beneficial components for health, making it a popular fruit in terms of nutrition and health.[14] The pomegranate, with its origins in Southeast Asia, is also grown in Turkey, Iran, the Middle East, the Mediterranean, the United States, and Arab countries. Pomegranate peel (PP), methanol, and water extracts containing anthocyanins, punicalagin, gallic acid, ellagic acid, and other polyphenols are used in the treatment of various diseases such as cancer, diabetes, AIDS, Alzheimer's, male infertility, inflammation, cardiovascular diseases, acute kidney failure, kidney diseases, and aging due to their antimicrobial effects.[15,16] Pomegranate helps lower blood pressure in cardiovascular diseases by inhibiting angiotensin-converting enzymes.[17]

Mitomycin-C (MMC) was first discovered in Japan in 1967 as an antibiotic derived from the culture of a bacterium called *Streptomyces caespitosus*. MMC is a drug with sitostatic and cytotoxic effects, and it has been used for antineoplastic purposes. It is commonly used in cancer treatment and can help control tumors by inhibiting or killing cancer cells. MMC is used alone or in combination with other chemotherapeutic agents in the treatment of stomach, pancreas, lung, bladder, cervical cancers, and chronic leukemia. It induces selective fibroblast apoptosis through the caspase pathway, reducing the formation of fibrous tissue, scar tissue, and granulation. Additionally, MMC can lead to lung fibrosis and kidney damage.[18,19]

In this study, the therapeutic/preventive effects of PP extract on the damage caused by MMC in the kidney tissue of mice were investigated.

2. Material and methods

The study involved the use of 32 female *Mus Musculus* albino mice, aged 8 weeks, and weighing between 20 and 30 g. The mice, with 8 in each cage, were housed under laboratory conditions with a relative humidity level of 50%, at 20 ± 2°C, following a light/dark photoperiod of 12:12. They were provided ad libitum access to food. Routine observations were conducted on a daily basis. The doses of the substances to be used in the study were prepared according to the daily weights of the mice and administered via oral gavage after being dissolved in distilled water. The randomly selected mice were grouped as follows:

1. Control Group (*n = 8*): distilled water was administered via oral gavage for 15 days.

2. MMC Group (*n = 8*): distilled water was administered via oral gavage for 15 days. On day 15, MMC (Sigma cat. No: M0440, with the chemical formula C15H18N4O5) was administered at a dose of 2 mg/kg intraperitoneally (i.p.). [20,22]

3. MMC+150PP Group (*n = 8*): 150 mg/kg of PP extract was administered via oral gavage for 15 days. On day 15, MMC was administered at a dose of 2 mg/kg i.p. [20,22]

4. MMC+300PP Group (*n = 8*): 300 mg/kg of PP extract was administered via oral gavage for 15 days. On day 15, MMC was administered at a dose of 2 mg/kg i.p.[20,22]

2.1. Preparation of the pomegranate (P. granatum L.) peel extract

The Pomegranate (*P. granatum* L.) used in the study was obtained from the Mediterranean region. The peels of the fruit were cleaned, dried in an area with adequate air circulation and no direct sunlight, and then ground. The ground PP, weighing 50 g, was placed into a 500 mL Soxhlet extractor after being washed with ethanol as the extraction solvent. A boiling flask containing 650 mL of ethanol solvent was used, and the extraction process was carried out for approximately 10 h, siphoning every 10–15 min, until the solvent became clear. After 10 h, the obtained liquid extracts were passed through a blue-ribbon filter paper to separate the particles, and the separated pomegranate extract was kept in a desiccator for 12 h at 35–45°C, where it was separated from the PP extract using a rotary evaporator. The PP extract, completely free of the solvent, was weighed with a sensitivity of 0.1 mg and placed in an extract box, and then stored at +4°C.[23]

2.2. Histological examination

After fixation in a 10% formaldehyde solution, the kidney tissues were dehydrated through a series of graded alcohols and xylene, and then embedded in paraffin blocks. Sections measuring 5–7 µm thick were cut from the blocks and subjected to Crossman's triple staining

technique to reveal the general structure of the kidney tissue.[1]

2.3. Immunohistochemical examination

The Streptavidin-Biotin Peroxidase Complex technique was applied to examine the immunolocalization of HO-1 and ACE-2 in the sections[24]. The sections, which had undergone deparaffinization and dehydration processes to prevent endogenous peroxidase activity, were incubated in a 3% hydrogen peroxide solution in phosphate-buffered solution (PBS) at pH 7.4 for 15 min. After passing through the PBS, the sections were placed in a 600-W microwave oven at for 10 min in sodium citrate buffer (pH 6.0) to expose the antigenic receptors. The sections were incubated in Blocking Solution A (Histostain-Plus IHC Kit, HRP, broad spectrum Ref.) for 10 min, after which the primary antibodies HO-1 (sc-390991; Santa Cruz, diluted at a ratio of 1/500) and ACE-2 (sc-390851; Santa Cruz, diluted at a ratio of 1/500) were applied. Subsequently, the broad-spectrum antibody was applied onto the sections and incubated at room temperature for 20 min. After washing with PBS, horse radish peroxidase (HRP) streptavidin was incubated at room temperature for 20 min. For chromogen application, 3,3'-diaminobenzidine (DAB) substrate solution was added. Then, Gill's 3 hematoxylin was used for counterstaining. To determine whether the immunoreactivity of the HO-1 and ACE-2 primary antibodies was specific, the sections held in PBS without the addition of primary antibodies (negative control) underwent the same procedures. Preparations prepared for the histological and immunohistochemical examinations were evaluated under a light microscope (Olympus Bx53; Olympus Scientific Solutions, Shinjuku, Tokyo, Japan), and photographs were taken. In all of the groups, semi-quantitative analyses were conducted to examine the immunoreactivity levels of HO-1 and ACE-2 at the cellular level. In the semiquantitative analysis, scoring was performed based on the staining intensity of the cells. The data were graded as follows: mild (+), moderate (++), and intense (+++).[25,26]

3. Results

3.1. Histopathological findings

Histopathological evaluation was performed on the kidney tissues from all of the groups, focusing on the glomeruli, tubules, interstitium, and blood vessels. No signs of degeneration were observed in the kidney tissues of the control group (Figure 1a). In the MMC group, the kidney tissue showed a reduction in the size of the renal corpuscles in the cortex, leading to the loss of the parietal and visceral layers surrounding Bowman's capsule, ultimately resulting in the disappearance of Bowman's space. Additionally, vacuolization occurred in the structure of the glomerulus and in the nuclei of tubulus proximalis and tubulus distalis, leading to degeneration in the tubular structure (Figure 1b). The glomerular structure, The Bowman's capsule, and structures of the proximal and distal tubules in the MMC+150PP group were similar to those in the kidney tissue of the control group. It was observed that the administration of PP in conjunction with MMC reduced MMC-induced degeneration in the kidneys of the mice (Figure 1c). In the MMC+300PP, there was improvement in the glomerular, proximal, and distal tubular structures in the kidneys due to the MMC, and a reduction in degeneration (Figure 1d).

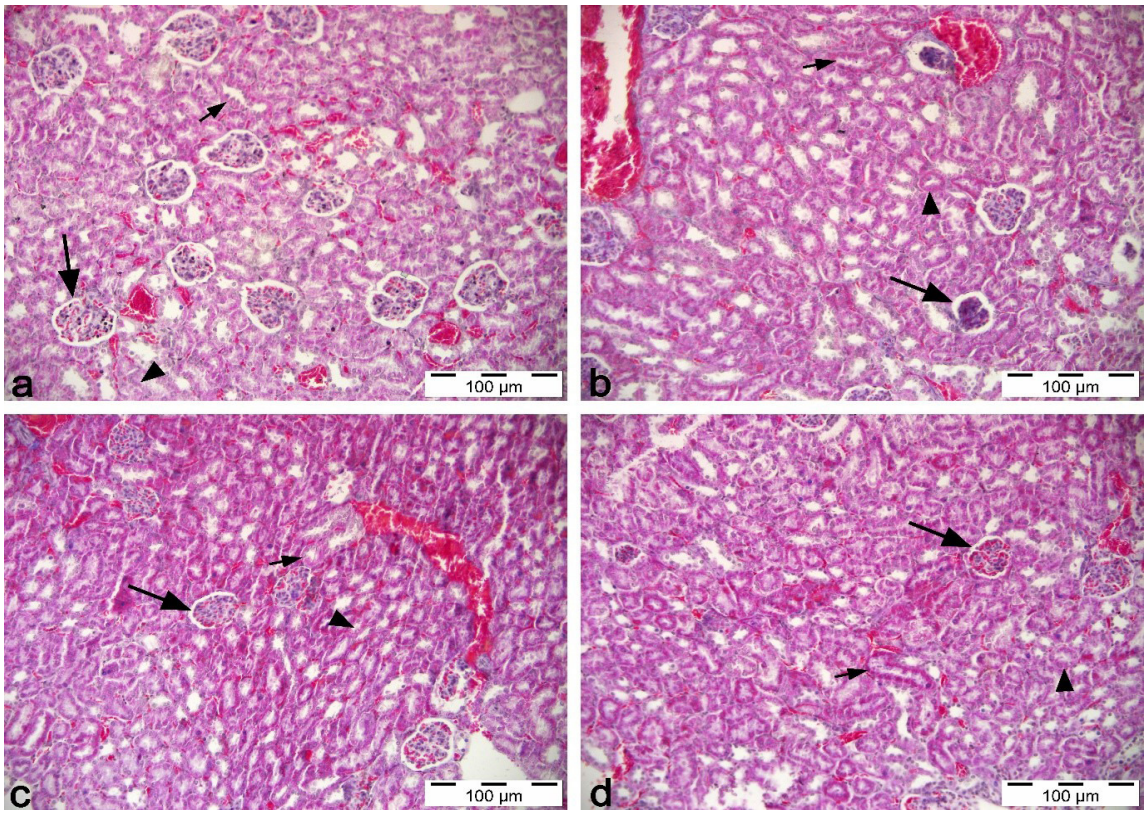

Figure 1. Mouse kidney tissue. a) Control Group, b) MMC Group, c) MMC+150PP Group, d) MMC+300PP Group. Arrow: Glomerulus, Arrow head: Distal Tubule, Small arrow: Proximal Tubule. Triple Staining

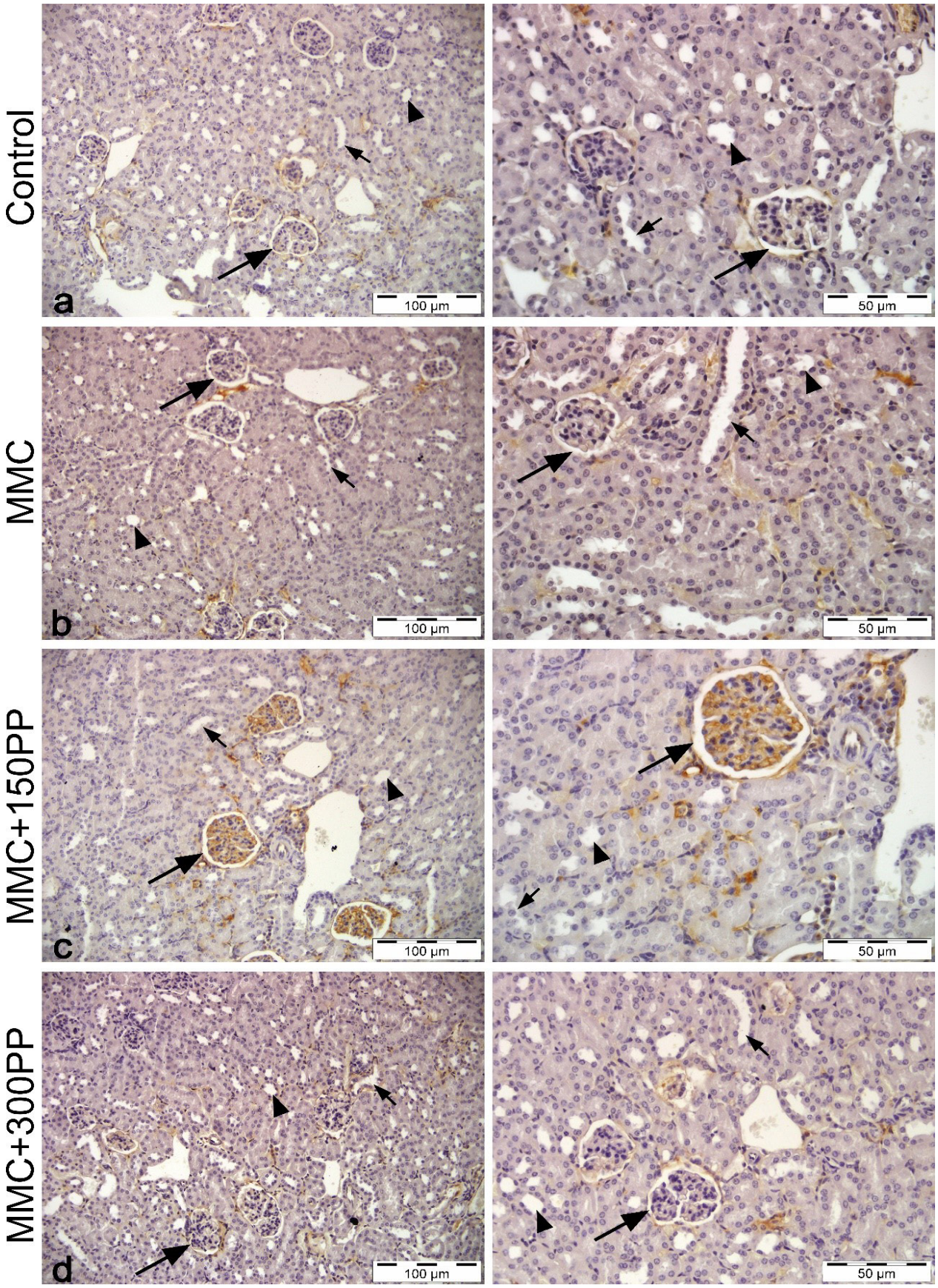

Figure 2. Mouse kidney tissue. a) Control Group, b) MMC Group, c) MMC+150PP Group, d) MMC+300PP Group. Arrow: Glomerulus, Arrow head: Distal Tubule, Small arrow: Proximal Tubule. Triple Staining

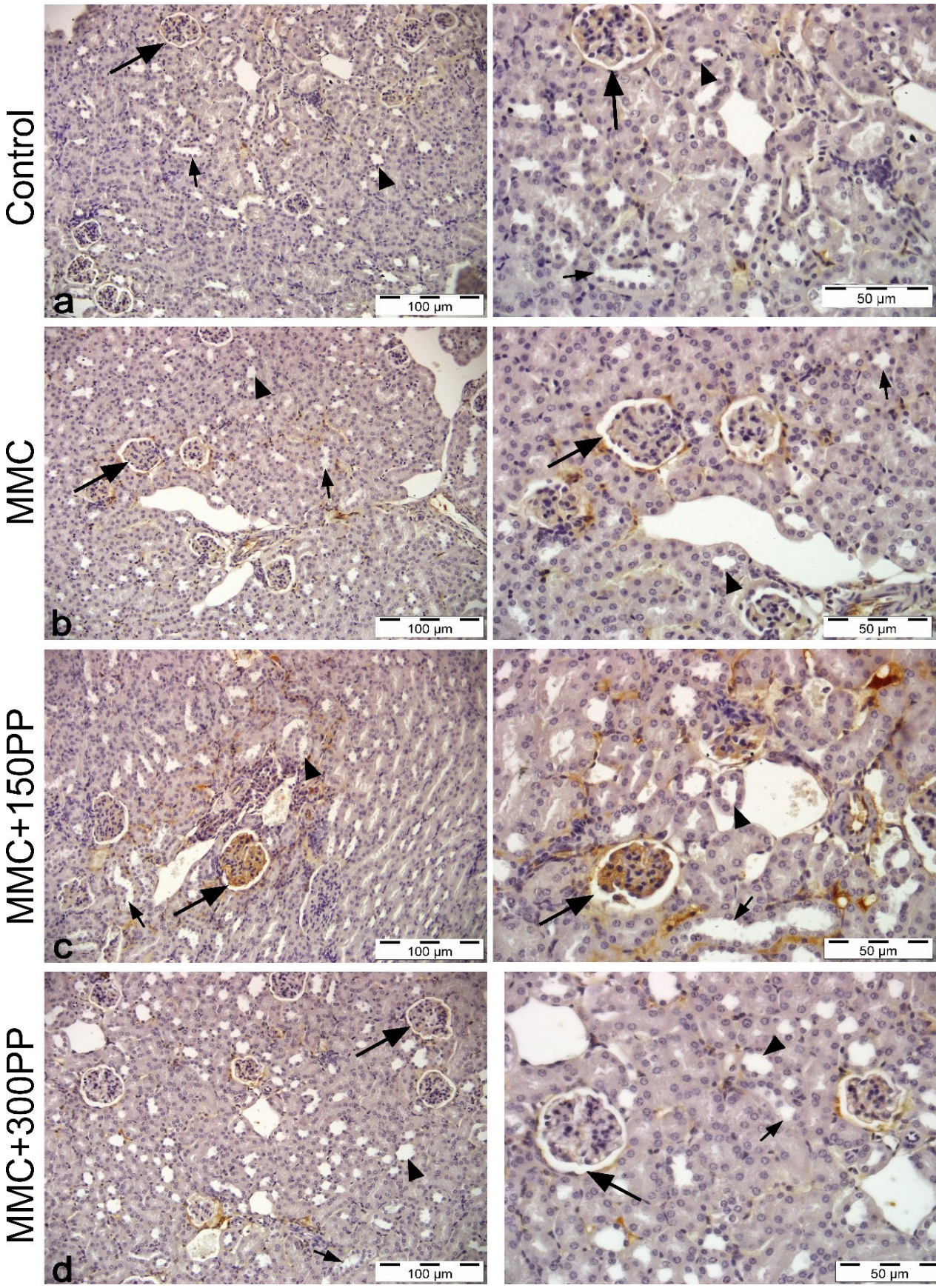

Figure 3. HO-1 immunoreactivity in mouse kidney tissue. a) Control Group, b) MMC Group, c) MMC+150PP Group, d) MMC+300PP Group. Arrow: Glomerulus, Arrow head: Distal Tubule, Small arrow: Proximal Tubule. IHC Staining.

3.2. Immunohistochemical findings

The kidney tissues of all of the mice subjected to MMC and PP were evaluated in terms of HO-1 and ACE-2 immunoreactivity. In the control group, there was a weak HO-1 immunoreactivity reaction in the glomerular, proximal tubular, and distal tubular cells (Figure 2a). The HO-1 immunoreactivity in the glomerular, proximal tubular, and distal tubular cells was moderate in the MMC group (Figure 2b). However, the HO-1 immunoreactivity in the glomerular, proximal tubular, and distal tubular cells in the MMC+150PP group was intense (Figure 2c). In the kidneys tissues in the MMC+300PP group, the HO-1 immunoreactivity in the glomerular, proximal tubular, and distal tubular cells was a weak intense (Figure 2d). HO-1 immunoreactivity was not observed in the medullary regions or vascular endothelia of the kidney tissues in any of the groups (Table 1).

When the kidney tissue was evaluated for ACE-2, there was weak immunoreactivity in the glomerulus, proximal tubules, and distal tubules of the control (Figure 3a) and MMC+300PP groups (Figure 3d), intense immunoreactivity in the MMC+150PP group (Figure 3c), and moderate immunoreactivity in the MMC group (Figure 3b). ACE-2 immunoreactivity was not detected in the medulla of the kidney tissues in any of the groups or in the vascular endothelia (Table 2).

Table 1. *Semi-quantitative analysis results of HO-1* immunoreactivity *among groups*

Cell type	Groups			
	Control	MMC	MMC+150PP	MMC+300PP
Glomerulus	+	++	+++	+
Proximal tubule	+	++	+++	+
Distal tubule	+	++	+++	+
Medulla	-	-	-	-

**MMC: Mitomycin-C, *PP: Pomegranate Peel*

Table 2. *Semi-quantitative analysis results of ACE-2* immunoreactivity *among groups*

Cell type	Groups			
	Control	MMC	MMC+150PP	MMC+300PP
Glomerulus	+	++	+++	+
Proximal tubule	+	++	+++	+
Distal tubule	+	++	+++	+
Medulla	-	-	-	-

**MMC: Mitomycin-C, *PP: Pomegranate Peel*

4. Discussion

MMC is known as an important cytotoxic (cytostatic) agent used in the treatment of many tumors and cancers. This medication works through a mechanism that inhibits the growth and proliferation of cancer cells. However, in addition to its clinical benefits, it also has the potential to cause harmful effects on various tissues and organs, such as the heart, kidneys, and liver.[27]

Pomegranate is a significant source of vitamin C, playing a crucial role in maintaining health and enhancing quality of life. Additionally, it has been noted to have an important role in preventing, treating, and slowing down the progression of many diseases, including chronic conditions.[28]

Studies have been conducted indicating that pomegranate juice, PP, and pomegranate itself reduce tissue damage.[29-32] A study conducted on 28 male Wistar albino rats over a period of 10 days found that sisplatin caused kidney damage. However, when the sisplatin was administered along with pomegranate juice, the tissue damage in the kidney decreased.[33] When the kidney tissue of Wistar albino rats treated with gentamicin for 15 days was examined, tubular necrosis and interstitial fibrosis were observed. However, in rats given gentamicin and PP together, a significant reduction in tubular degeneration, tubular necrosis, and glomerular degeneration was observed in the kidney tissue.[30] Following the administration of carbon tetrachloride (CCl4) and glycerol, it was reported that the structure of the glomerulus, Bowman's capsule, and tubular cells was disrupted, occasionally leading to bleeding and vacuolization with necrosis in kidney cells. According to the results of 2 different studies in which 400 mg/kg of PP was used against CCl4-induced kidney damage and pomegranate blossom extract (125 and 250 mg/kg) was used against glycerol-induced kidney damage, it was indicated that the pomegranate husk and flower extracts significantly reduced the abnormal changes in kidney tissue.[29,32] It was found that PP extract improved the kidney tissue in rats with streptozotocin-induced diabetes. Furthermore, it was stated that tissue damage resulting from diabetes was alleviated by PP extract, leading to the improvement of the glomerular structure, recovery in renal tubules and tubular interstitial epithelial cells, and a reduction in the glomerular capillary wall and basal membrane thickness.[33] A similar 14-day study revealed that the administration of cadmium chloride (CdCl2) in mice led to degeneration in the kidney glomerular structure, vascular blockages, the presence of pyknotic nuclei in the kidney tubules and epithelial cells, as well as vacuolization and infiltration of the leukocyte cells in the intertubular spaces. However, the coadministration of $CdCl_2$ and PP significantly reduced kidney tissue damage in mice.[34]

In the current study, changes in the kidney tissue of mice administered MMC were examined. In the mice administered MMC, there was disruption in the kidney glomerular structure, basal membrane, and distal and proximal tubule structure. Additionally, a decrease in the Bowman's space and the presence of vacuolization were identified. In the MMC+150PP group, the PP reduced the harmful effects of MMC, reduced kidney tissue damage, improved the glomerular structure, and maintained a Bowman's space similar to the control group. Additionally, vacuolization in the distal and proximal tubules decreased. Furthermore, in the MMC+300PP group, the kidney tissue degeneration showed greater improvement compared to the degeneration observed in the MMC+150PP group. In an experimental diabetes study, intense HO-1 immunoreactivity was detected in the renal cortex of both the diabetes and diabetes+basil groups, whereas moderate immunoreactivity was observed in the renal cortex of the basil, sham, and control groups. Although there was excessive immunoreactivity in the proximal tubule and distal tubule cells in the diabetes and diabetes+basil groups, moderate HO-1 immunoreactivity was observed in the basil, control, and sham groups. No HO-1 immunoreactivity was found in the medulla, glomeruli, or vascular endothelium of the kidney tissues in any of the groups. In the same study, ACE-2 immunoreactivity in the kidney tissues was evaluated. The diabetes group had intense ACE-2 immunoreactivity in the renal cortex, while the diabetes+basil group showed moderate ACE-2 immunoreactivity. The diabetes group exhibited intense immunoreactivity in the proximal tubule and distal tubule cells, whereas the diabetes+basil, basil, sham, and control groups displayed moderate immunoreactivity. Moreover, ACE-2 immunoreactivity was absent in the glomeruli, medulla, and vascular endothelium of the kidney tissues in all of the groups.[35]

In the present study, investigating the localization of HO-1 and ACE-2, the control group exhibited weak HO-1 immunoreactivity in the glomerulus, proximal tubule, and distal tubule cells. In the control group, there was a weak HO-1 immunoreactivity reaction in the glomerular, proximal tubular, and distal tubular cells. The HO-1 immunoreactivity in the glomerular, proximal tubular, and distal tubular cells was moderate in the MMC group. However, the HO-1 immunoreactivity in the glomerular, proximal tubular, and distal tubular cells in the MMC+150PP group was intense. In the kidneys tissues in the MMC+300PP group, the HO-1 immunoreactivity in the glomerular, proximal tubular, and distal tubular cells was a weak intense). When evaluating ACE-2 immunoreactivity in the kidney tissues, it was observed that immunoreactivity in the glomerulus, proximal tubule, and distal tubule cells in the renal cortex was weak in the control group and MMC+300PP group, intense in the MMC+PP150 group, and moderate in the MMC group.

5. Conclusion

In conclusion, this research demonstrated that PP reduced the adverse effects of MMC on the kidneys and had the potential to promote tissue healing. It is our belief that PP extract could contribute to the treatment of kidney diseases and the prevention of their complications through further research.

Ethical approval

The approval for the study was obtained from the Local Ethics Committee for Experimental Animals of Kafkas University on 03.10.2023 with the ethical committee number 2023/107.

Authors contribution

HG, EU, SEY, PAK, AG, EKS: Research, planning, article scanning, writing-original draft & review.

Conflict of interest

There are no conflicts of interest associated with this research publication, according to the authors.

Data availability

Data will be made available on request

Acknowledgments

None

Funding statement

None

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