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Original article

Methadone and the Kidney: Dissecting Gender Differences in Inflammation and Oxidative Stress Responses

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Abstract

Background: This study explored the gender-specific effects of methadone, a synthetic opioid receptor agonist commonly used in opioid addiction treatment, on renal tissue and function. We aimed to elucidate the underlying mechanisms involving inflammatory pathways and redox system activity.

Methods: Forty-two Wistar rats (200-250 g) were allocated into six groups: three males and three females, each comprised of control, and methadone-treated 5 mg/kg and 20 mg/kg. Over eight weeks, animals received either saline or methadone syrup orally. Blood urea nitrogen (BUN) and serum creatinine (sCr) were measured in serum. The inflammatory cytokines and antioxidant enzyme activity were assessed in left kidneys, which were preserved at -80 °C, while histopathological analysis via H&E staining was done on the formalin-fixed right kidneys.

Findings: Methadone administration resulted in renal tissue injury characterized by enhanced glomerular and interstitial inflammation. Notable increases in malondialdehyde (MDA), BUN, sCr, transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), and interleukin 17 (IL-17) were observed in methadone-treated groups, indicating impaired renal function associated with oxidative stress and inflammation, with male rats exhibiting more severe alterations. Conversely, methadone treatment elevated glutathione peroxidase (GPx), and catalase (Cat) activities, predominantly in females.

Conclusion: Prolonged methadone therapy exerts a nephrotoxic effect through the activation of oxidative stress and inflammatory pathways, with male rats displaying greater renal pathology and dysfunction, potentially attributed to diminished antioxidant defenses.

Keywords: Methadone-nephrotoxicity, Oxidative stress, Inflammation, IL-17, Gender difference

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Introduction

Substance abuse is characterized by the extreme and addictive use of drugs, psychotropic substances, and narcotics, leading to both physical and psychological harm to the individual and society. This issue has emerged as a significant global concern.[1] By 2020, the worldwide burden of disease attributed to opioid use was escalating, with an estimated 40.5 million individuals dependent on opioids, resulting in over 100 000 deaths each year.[2]

Methadone maintenance therapy (MMT) is recognized as an effective intervention that mitigates the impacts of opioid addiction by maintaining a stable concentration of the drug in the bloodstream.[3,4]

Methadone, an opioid agonist with a prolonged effect, is frequently used to treat opioid addiction and to alleviate chronic pain.[5,6] Patients undergoing methadone treatment often experience a more balanced and stable lifestyle, thereby improving their overall quality of life.[7] However, the long-term methadone use can lead to several side effects that differ among individuals, influenced by their physical health and conditions. Among the most prevalent adverse effects are liver and kidney disorders.[8]

Methadone overdose or poisoning can result in damage to skeletal muscle cells, a condition known as rhabdomyolysis. The destruction of muscle cells releases toxic substances into the bloodstream, with acute renal failure being a critical complication that can lead to mortality. Myoglobin released during this process has nephrotoxic effects and is implicated in 5% to 40% of cases of acute renal failure.[9] The mortality rate associated with acute renal failure resulting from rhabdomyolysis is approximately 17%.[10] Furthermore, significant disparities in susceptibility to kidney disease have been observed based on race and gender.[11]

Research has indicated that differences in gene expression related to hormonal regulation, renal physiological functions, and kidney disease exist between sexes.[12] Injuries resulting from prolonged methadone therapy also vary by sex, with evidence suggesting that women may be more susceptible than men.[13]

Given the rising prevalence of substance abuse and the use of methadone in addiction treatment clinics, there is a notable lack of comprehensive information regarding the differential effects of long-term methadone use on renal function in men and women, as well as the underlying mechanisms involved. Therefore, this study aimed to investigate gender differences in possible changes in renal tissue and function due to methadone consumption by focusing on the role of the inflammatory pathway and redox system activity as its possible mechanisms.

Material and Methods

Animals

In this study, 42 Wistar rats of both sexes, weighing 200-250 g, were randomly divided into six groups (n = 7): Group 1. consisted of male control rats receiving normal saline orally for eight weeks (MC). Groups 2 and 3 included male rats that were administered methadone syrup (5 mg/mL, Daru Pakhsh Co. Tehran, Iran) at doses equivalent to 5 mg/kg (MM5) and 20 mg/kg (MM20) methadone, respectively, for the same duration. Groups 4-6 comprised female rats treated similarly, designated as FC, FM5, and FM20.

The rats were housed in a controlled laboratory environment with a temperature of 23 ± 2 °C, relative humidity of 40%-45%, and a 12-hour light/dark cycle.

Study design

Rats were acclimatized in the animal facility for one week before the experiment, and had unrestricted access to food and water throughout the study. Over eight weeks, rats in the methadone groups received daily doses of methadone at 5 or 20 mg/kg body weight via gavage, establishing methadone dependence.[14,15] Normal saline was gavaged for control group animals in an equivalent volume and at the same intervals.[15]

To evaluate withdrawal, naloxone hydrochloride was administered at a dosage of 3 mg/kg body weight intraperitoneally to one male and one female from each methadone groups, and behavioral symptoms were observed.[16] The rats were monitored in transparent containers for signs of methadone withdrawal, including swallowing disorders, rapid and abnormal movements of the jaw, grinding of teeth, trembling of the head and limbs, jumping, diarrhea, ptosis, abnormal postures, and irritability.[17]

Body weights were recorded at the beginning and weekly thereafter until the study's conclusion. For euthanasia, deep anesthesia was induced using a ketamine/xylazine mixture (100:10 mg/kg),[15] followed by cardiac blood sample collection to assess serum urea and creatinine levels. The left kidneys were removed, weighed, and stored at -80 °C for subsequent analysis of oxidative and antioxidant indices and cytokine levels, while the right kidneys were reserved for histological examination.

Tissue staining and histopathology

Left kidney tissues were preserved using 10% buffered formalin, embedded in paraffin, and cut into slices 2 µm thick. Hematoxylin and eosin (H&E) staining was conducted to assess tissue damage. An experienced pathologist, unaware of the group assignments, reviewed the sections with an Olympus microscope (CX41, Tokyo, Japan). For quantitative analysis, six fields from each sample were chosen and scored on a scale of 0 to 4 at 100 × magnification.

The tissue injury parameters included glomerular shrinkage, sclerosis, hypertrophy, tubular epithelial cell degradation (TECD), tubular and interstitial inflammation, cellular and acellular casts, tubular necrosis, widening of Bowman's capsule, vascular congestion, mesangial matrix accumulation, and hemorrhage. The cumulative mean of these parameters was termed the kidney tissue damage score (KTDS). The scores described as: 0 = normal, 1 = damage 10-25%, 2 = damage 25-50%, and 3 = damage 50-75%, 4 = damage more than 75%.[18]

Measurement of antioxidant indices

The activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (Cat) enzymes in kidney tissue were assessed using specific methods. SOD activity was determined via a colorimetric assay based on the enzyme's ability to inhibit the autoxidation of pyrogallol. Kidney tissue (50 mg) was homogenized in a cold buffer and centrifuged at 12 000 rpm for five minutes. SOD activity was measured at 570 nm in the supernatant using a commercial kit, with one unit defined as the enzyme amount that inhibits 50% of the MTT reduction rate.[19]

CAT activity in kidney tissue was measured at 240 nm using a colorimetric method based on the rate of decomposition or disappearance of H_2O_2. The tissue was homogenized on ice, and the extraction buffer contained

50 mM saline phosphate. The homogenate was centrifuged at 4 °C. A reaction buffer was prepared with 50 mM phosphate saline and 10 mM hydrogen peroxide, to which 600 µL was added to 5 µL of the enzyme extract. CAT activity was reported as the number of H_2O_2 macromolecules decomposed per minute per mg of protein.[19]

GPx measurement relies on the enzyme's ability to oxidize glutathione (GSH) to oxidized glutathione (GSSH), which is involved in hydrogen peroxide reduction. Glutathione reductase transforms GSSH into GSH by utilizing nicotinamide adenine dinucleotide phosphate (NADPH). The reduction of NADPH, assessed at 340 nm, acts as a marker for GPx activity.[19]

Measurement of cytokine levels

To determine the levels of TNF-α, IL-17, and TGF-β in the kidney, 100 mg of frozen kidney tissue was allowed to thaw at room temperature, then homogenized in a chilled phosphate buffer solution and centrifuged at 5000 rpm for five minutes. The supernatant was analyzed using the appropriate ELISA kit (TNF-α: Karmania pars gene Catalog # KPG-RTNF-a, IL-17: Karmania pars gene Catalog # KPG-RIL-17A, TGF-β: PharmPak R&D Systems, Catalog # PMB100B).[20,21]

MDA measurement

Malondialdehyde (MDA), a byproduct of lipid peroxidation, was quantified by homogenizing kidney tissue in a 1.5% KCl solution and centrifuging at 1000 rpm for 10 minutes. The resulting supernatant was analyzed using thiobarbituric acid. A solution was prepared consisting of 15 g of trichloroacetic acid (TCA), 0.375 g of thiobarbituric acid (TBA), and 2 ml of hydrochloric acid. One milliliter of this solution was added to the supernatant and heated for 50 minutes. Absorbance was then recorded at 535 nm, and MDA concentration was calculated in µM/g of kidney tissue using the following formula: $C = A / (1.56 \times 10^5)$.[22,23]

Statistical analysis

Statistical analyses were carried out using SPSS software version 26. The Shapiro-Wilk test was utilized to evaluate the normality of the data. For data that followed a normal distribution, group comparisons were made using two-way analysis of variance, followed by Tukey and student's t-tests for comparisons within male and female groups. Histopathological data were analyzed using the Kruskal-Wallis test, accompanied by post-hoc Mann-Whitney tests. The results are presented as mean ± SEM, with significance defined at $P < 0.05$.

Results

Serum levels of blood urea nitrogen (BUN), creatinine (Cr), and BUN/Cr ratio

Methadone therapy led to a dose-dependent increase in BUN, serum creatinine (sCr) with the highest levels observed in the methadone 20 mg/kg (M20) groups, indicating that long-term methadone use is harmful to the kidneys ($P < 0.001$). Additionally, there was a reduction in the BUN/Cr ratio attributed to a greater elevation in sCr levels compared to BUN levels in M20 animals. Both sexes exhibited similar trends in these parameters, although BUN levels showed a significant difference between males and females in the M20 groups ($P < 0.05$). Also, the BUN/Cr ratio was significantly different in the FM20 group compared to the MM20 group (Figure 1A-C).

Kidney and body weight

During the 8 weeks of study, body weight (BW) gain slowed down due to methadone therapy in M5 groups, while BW was reduced significantly in the M20 groups compared to the controls ($P < 0.001$). In female rats, body weight slightly increased in the FM5 group, while it decreased in the FM20 group compared to the FC. Sex differences were observed in delta weight. The BW in the female control (FC) group was lower than in the male control (MC) group, related to the age-matched animals used in the study. There was a significant difference in their BW, although the BW gain was significantly different in the MM5 and FM5 groups ($P < 0.01$). However, this difference was not observed in methadone-treated animals. The kidney weight-to-body weight (KW/BW) ratio increased with methadone treatment, significantly in the 20 mg/kg doses for both sexes compared to the controls ($P < 0.001$). In females, the KW/BW ratio began to rise at the lower dose, resulting in a significant difference between the FM5 and MM5 groups ($P = 0.03$) (Figure 1D-E).

The redox system activity

The tissue levels of MDA were higher in the methadone-treated groups compared to the control group ($P < 0.001$), and there were no significant differences observed between the sexes ($P = 0.08$) (Figure 2A).

SOD activity in the kidneys showed no significant changes due to methadone treatment, although a notable increase was found in the FM20 group compared to the MM20 group ($P < 0.05$) (Figure 2B).

Renal GPx levels were slightly reduced in the M5 group but significantly increased in the M20 group compared to controls and M5 animals ($P < 0.001$), with a significant difference indicating greater GPx increases in females (FM20 vs. MM20) (Figure 2C).

Methadone therapy also promoted renal CAT activity in a dose-dependent manner ($P < 0.001$), with a steeper increase observed in females, leading to a significant difference between FM20 and MM20 ($P < 0.05$) (Figure 2D).

Levels of pro-inflammatory cytokines

Methadone administration decreased renal TNF-α level at

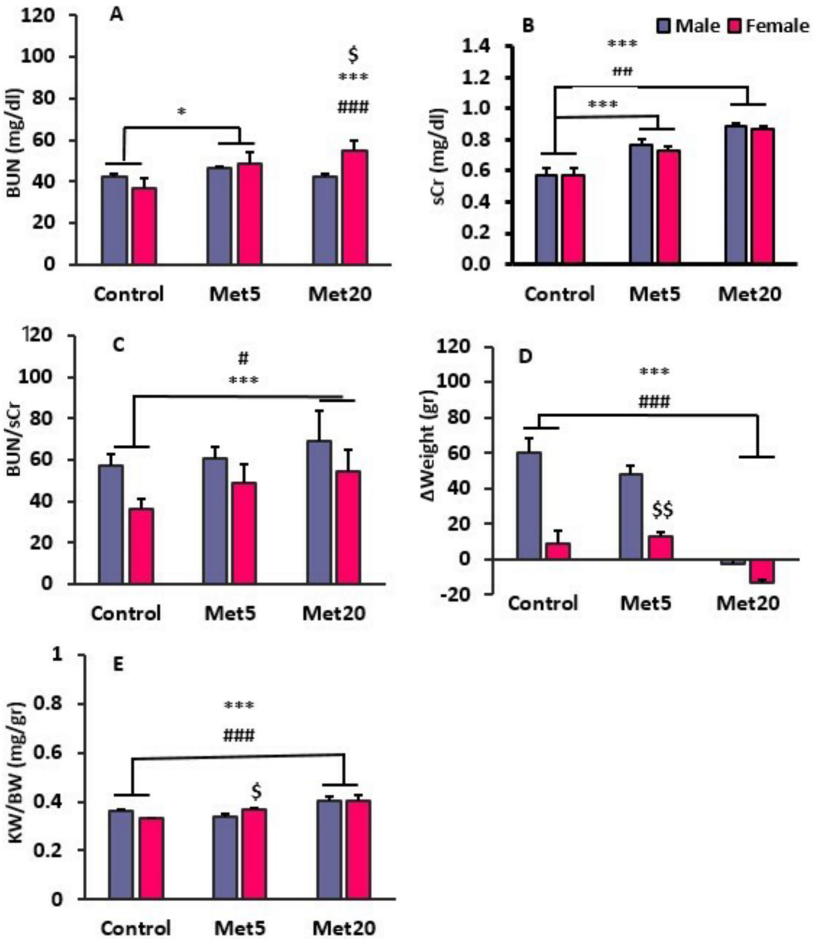

Figure 1. Effects of methadone on serum parameters of renal function, body weight change, and kidney weight to body weight ratio. Data are represented as mean ± SE. n = 6; * $P < 0.05$, *** $P < 0.001$, vs control, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs Met5, $ $P < 0.05$, $$ $P < 0.01$ female vs male groups

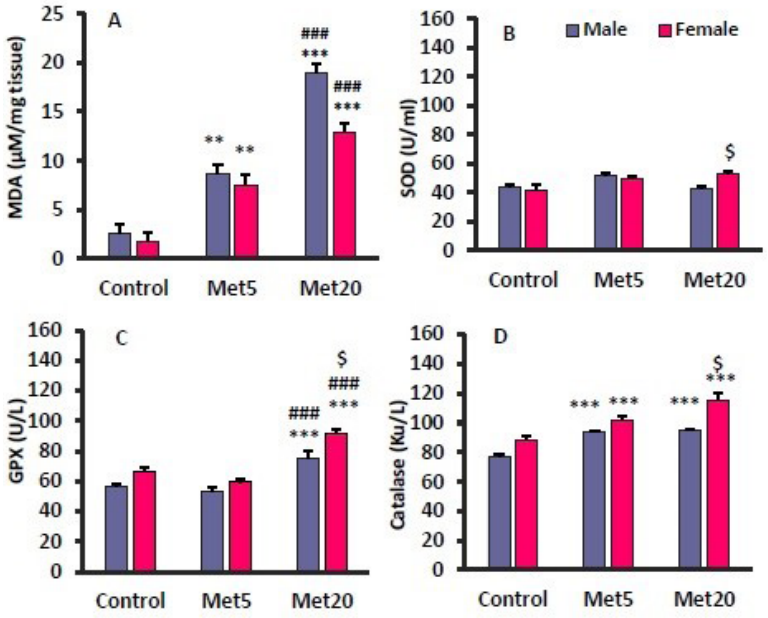

Figure 2. Effects of Methadone on Kidney Redox Activity Status. Data are represented as mean ± SE. n = 6; * $P < 0.05$, *** $P < 0.001$ vs control, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$vs Met5, $ $P < 0.05$, $$ $P < 0.01$ female vs male groups

the 5 mg/kg dose ($P < 0.05$) but increased it at the 20 mg/kg dose compared to controls ($P < 0.05$), with a significant difference between the M20 and M5 groups ($P < 0.001$). No gender differences were noted (Figure 3A).

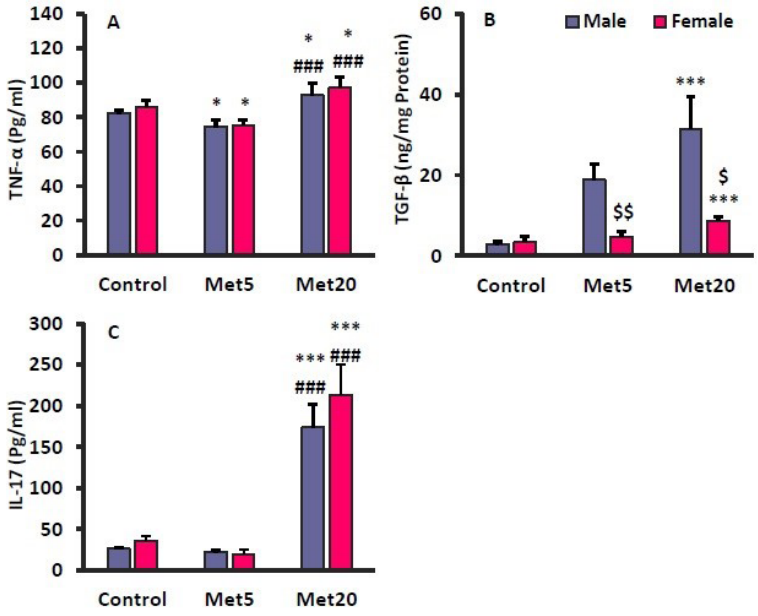

Figure 3. Effects of methadone on kidney inflammatory cytokines. Data are represented as mean ± SE. n = 6; * $P < 0.05$, *** $P < 0.001$ vs control, ### $P < 0.001$ vs Met5, $ $P < 0.05$ female vs male groups

Renal TGF-β levels rose in a dose-dependent manner for both sexes, although the increase was more pronounced in males than females ($P < 0.001$), with this gender difference observed at both doses (Figure 3B).

Methadone therapy increased IL-17 levels in the M20 groups for both sexes ($P < 0.001$) (Figure 3C).

Histopathologic study

Evaluation of kidney sections by H&E staining revealed that methadone administration resulted in a dose-dependent increase in the mean of the KTDS, which is the cumulative mean of pathologic parameters. Glomerular shrinkage in the male group is significantly more than control group ($P < 0.05$). Eight weeks of methadone gavage did not affect glomerular sclerosis, Bowman's capsule dilatation, mesangial matrix accumulation, and glomerular hypertrophy alone ($P > 0.05$). Notably, significant differences were observed in several parameters as tubular damage and interstitial inflammation between male and female groups and also between methadone-treated and control groups, as illustrated in Figure 4. Overall, male animals exhibited greater kidney tissue damage at the high dose of methadone ($P < 0.05$).

Discussion

This study was conducted to investigate possible gender differences in kidney damage resulting from long-term methadone therapy. The alterations in redox system activity and inflammatory pathways were evaluated as possible mechanisms. The main findings include: 1. Methadone caused renal dysfunction and tissue damage in a dose- and gender-dependent manner; 2. Oxidative stress and the redox system contributed to the effects of methadone; and 3. Inflammation also played a significant role in methadone's impact on the kidneys.

High prevalence of drug abuse, especially opioids, is a social problem worldwide. Methadone is the most commonly prescribed medication for opioid addiction, with 40% of its usage in the U.S. attributed to women.[6] MMT offers several benefits, including oral administration and reduced risk of disease transmission, such as hepatitis and AIDS.[24] However, like other medications, long-term methadone administration can lead to adverse effects, posing significant concerns for researchers. Our study observed increases in BUN, sCr, and KW/BW, along with morphological changes such as glomerular shrinkage, sclerosis, hypertrophy, TECD, and inflammation, confirming the harmful effects of methadone on renal tissue. Previous research has linked renal dysfunction to the release of skeletal muscle myoglobin, decreased glomerular filtration rates, and renal inflammation.[25,26]

In animals receiving the 20 mg/kg methadone dose, a decrease in body weight was noted, particularly in females. This finding aligns with those of Tahergorabi et al, who reported similar results in methadone-treated rats.[15] Ling et al also reported weight loss in Sprague-Dawley rats subjected to a 72-hour intravenous infusion of methadone.[27] Conversely, Fenn et al noted a 10% weight gain in patients after six months of methadone therapy.[28] KW/BW ratio increased in both male and female groups receiving the higher methadone dose compared to controls and the lower dose group. Other studies have similarly reported increased kidney weight in models of glycerol- and cisplatin-induced renal toxicity.[26,29]

Histological assessments in our study revealed glomerular and interstitial inflammation, alongside

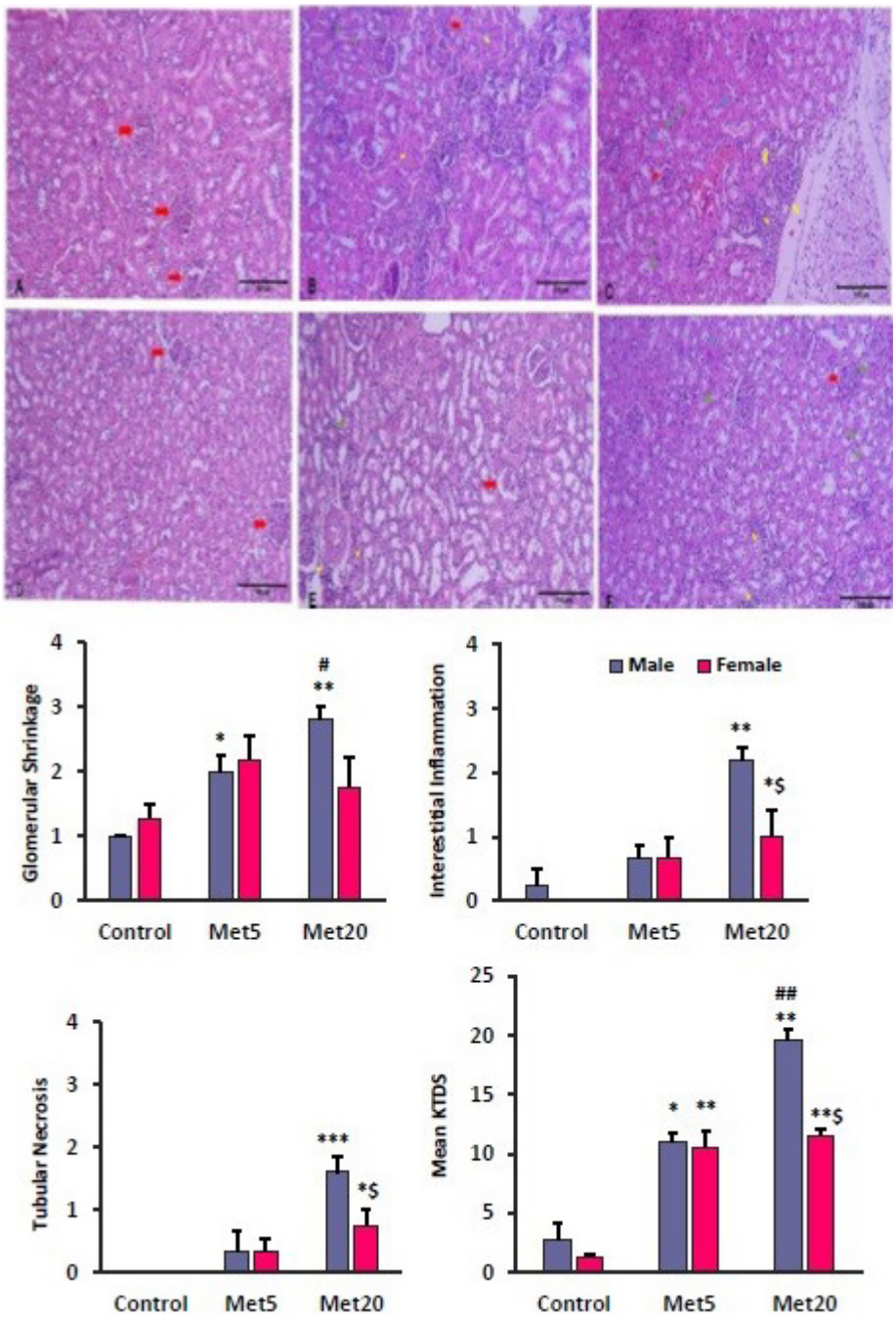

Figure 4. Effects of methadone on histopathologic parameters. Data are represented as mean ± SE. n = 6; Scale bar = 100μm, magnitude × 100. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs control, # $P<0.05$, ## $P<0.01$ vs Met5, $ $P<0.05$ female vs male groups. Kidney tissue damage score (KTDS)

elevated levels of pro-inflammatory cytokines such as TNF-α, TGF-β, and IL-17 in renal tissue, indicating an activated inflammatory response to methadone, which was more pronounced in males, as pathologic observations confirm this gender difference. Inflammation is a key feature of rhabdomyolysis-induced acute kidney injury.[30] Chronic inflammation has been documented in MMT-received addicted patients. In these patients, higher levels of IL-1β, IL-6, and IL-8 compared to healthy controls were reported. Additionally, serum levels of TNF-α and IL-1β correlated with daily methadone dosage, while IL-1β levels were associated with the duration of treatment.[31] IL-17 has been suggested to play a pivotal role in inflammation by regulating the secretion of other cytokines and exhibiting a synergistic effect with TNF-α.[32] Our results indicated elevation of IL-17 levels in the M20-treated groups. Although IL-17 levels did not differ by gender, TGF-β levels were notably higher in males across both dosages.

We observed increased levels of MDA, GPx, and Cat in the kidneys of methadone-treated animals, with variations based on dose and gender. MDA levels

were insignificantly higher in males ($P=0.052$), while antioxidant enzymes activities were more remarkable in females. These findings are consistent with previous reports of antioxidant defense activation in rats treated with tramadol and tapentadol in both renal and liver tissues.[33] Female mice have also exhibited higher GPx and Cat activities in kidney tissue, as well as increased SOD activation in brain and lung tissues compared to males.[34] Also, female mice exhibited lower isoline-induced liver injury, attributed to elevated glutamate-cysteine ligase and GPx activities.[35] In our study, female rats demonstrated less renal injury from methadone therapy, likely due to their enhanced antioxidant defenses. In a time-course study of acute kidney injury transitioning to chronic kidney disease (CKD), female rats indicated lower progression to CKD due to the early increase of GPx beyond TGF-β, epithelial nitric oxide synthase, and hypoxia-inducible factor 1 elevation. While, transition of ovariectomized rats to CKD compared to intact females was faster, the authors suggested that sex hormones can be involved in the gender difference observed in renal injury.[36] While Amraei et al found that doses of 5, 20, and 40 mg/kg of methadone were tolerated by rats over eight weeks,[15] while in our pilot study, animals receiving 40 mg/kg died within several days. Consequently, we restricted our study to 5 and 20 mg/kg doses. The literature indicates an LD50 of 178 mg/kg for methadone in B6C3F1 mice,[37] though specific LD50 data for rats is not reported. Based on our observations, the 40 mg/Kg dose appears close to the lethal dose, suggesting that further dose-response studies are warranted.

In conclusion, methadone caused renal damage by activating oxidative stress and inflammatory pathways. Female rats exhibit greater resilience to methadone nephrotoxicity, potentially due to higher antioxidant enzyme activity.

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Competing Interests
The authors declare no conflict of interest.

Data Availability Statement
The data supporting this study's findings are available upon request from the corresponding author, subject to ethical and ownership considerations.

Ethical Approval
The study protocol was approved by the Ethics Committee of Kerman University of Medical Sciences (Ethical code: IR.KMU.AH.REC.1399.172).

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