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The Relationship Between Gender Difference and the Circadian Rhythm of Renal Function and Histopathology in an Adenine-Induced Kidney Disease Rat Model

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Abstract

Introduction: Chronic kidney disease (CKD) is characterized by progressive kidney damage and decreased renal function. There may be differences in the incidence of CKD between males and females. This study aimed to evaluate the variations in biochemical and pathological parameters, including induced circadian rhythm changes, in male and female rats with CKD.

Methods: 24 male, 24 intact female, and 24 ovariectomized female rats were divided into CKD and control groups, with two subgroups for each main group (n=6). After inducing CKD, urine samples were collected at 12:00 a.m. (midnight) and 12:00 p.m. (noon) to measure urea, creatinine, and protein levels. The right kidneys were evaluated histopathologically. The data were analyzed using a three-way repeated-measures analysis of variance (ANOVA) and Bonferroni correction for post hoc analysis.

Results: CKD increased urine volume, urea, creatinine, urine protein levels, GFR, and histopathological indices compared to the control group at both time points. In addition, male rats exhibited more significant damage in renal function and structure at 12:00 p.m. compared with intact female and ovariectomized rats.

Conclusions: CKD and circadian rhythm both affect the physiological functions of the kidneys. However, sex differences potentially contribute to the development and progression of these changes.

Keywords: Chronic kidney disease, Sex Characteristics, Urine, Circadian rhythm, Rat

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Introduction

Kidney diseases are an important global health problem with numerous complications and substantial economic burdens. Chronic kidney failure is one of the most prevalent of these diseases and a serious problem that affects millions of people worldwide (1, 2). CKD is characterized by a progressive decline in renal function over time in patients (3, 4). The disease often alters glomerular filtration, urea, and creatinine levels, which leads to renal interstitial inflammation, fibrosis, and glomerular damage (5-7).

Circadian rhythms, the body's internal 24-hour clocks, control various necessary physiological functions to maintain physiological homeostasis. These rhythms are synchronized by the suprachiasmatic nucleus (SCN) in response to light cues from the retina (8, 9). In addition to SCN as the central clock, mammalian tissues also have

intrinsic circadian clocks that regulate their function (10-12). The kidney's local clock regulates a wide range of physiological processes, including urine volume, water levels, and solute excretion, during both day and night (13, 14). However, previous studies have demonstrated that chronic kidney disease can disrupt circadian rhythms and impair renal function (15-17).

While most research about CKD has focused on male models, some evidence suggests that sex differences may be important in the development and severity of the disease (18-20). For example, in research involving both genders in CKD rats, glomerular sclerosis was lower in female rats than in male rats (21). In addition, previous studies indicate that renal damage and decreased kidney function were more prominent in male rats than in females (18, 22). Thus, gender may have a role in the physiological and morphological variations in the functional and molecular features of kidneys in males and females (23).

Therefore, considering the possible influence of gender and renal circadian rhythm on chronic kidney disease, this study aimed to investigate the effects of renal circadian rhythm on biochemical parameters and histological changes in male and female CKD rats. This research could provide valuable insights for developing gender-specific treatment strategies for this disease.

Methods

Experimental animals

In total, 72 adult male and female Wistar rats (180–220 grams) were purchased from Kerman University of Medical Sciences. The rats were housed at a temperature of 23 ± 2°C and on 12 12-hour light/dark cycle. 24 males, 24 intact females, and 24 ovariectomized female rats were randomized into CKD and control groups (n = 12 per group). The individuals in each study group were also divided into two subgroups: light (12:00 p.m.) and night (12:00 a.m.) groups. A brief overview of the animal groups is presented in Figure 1.

Study design and Sample Collection

Figure 1 shows that sample collection in all 6 subgroups of control and CKD was done at 12:00 a.m. and 12:00 p.m. Male mice (control and CKD, n = 24) received adenine by gavage from day 1 to 10. On the last day after receiving adenine, half of the mice were placed in the metabolic cage at 12:00 a.m. and the other half at 12:00 p.m. After 24 hours (day 11), urine and kidney tissue samples were collected at 12:00 a.m. and 12:00 p.m., respectively. Female mice were housed together for 14 days to synchronize their cycles. On day 14, adenine was gavaged to the female mice for 10 days (days 14-24). On day 24, half of the animals were placed in the metabolic cage at 12:00 a.m. and the other half at 12:00 p.m., and after 24 hours (day 25), urine and kidney tissue samples were collected at 12:00 a.m. and 12:00 p.m., respectively (Figure 1).

Ovary removal surgery (Ovariectomy)

Female rats were anesthetized with a mixture of ketamine and xylene (60 and 10 mg/kg) before bilateral ovariectomy. First, a 2.5 cm incision was made in the ventral midline

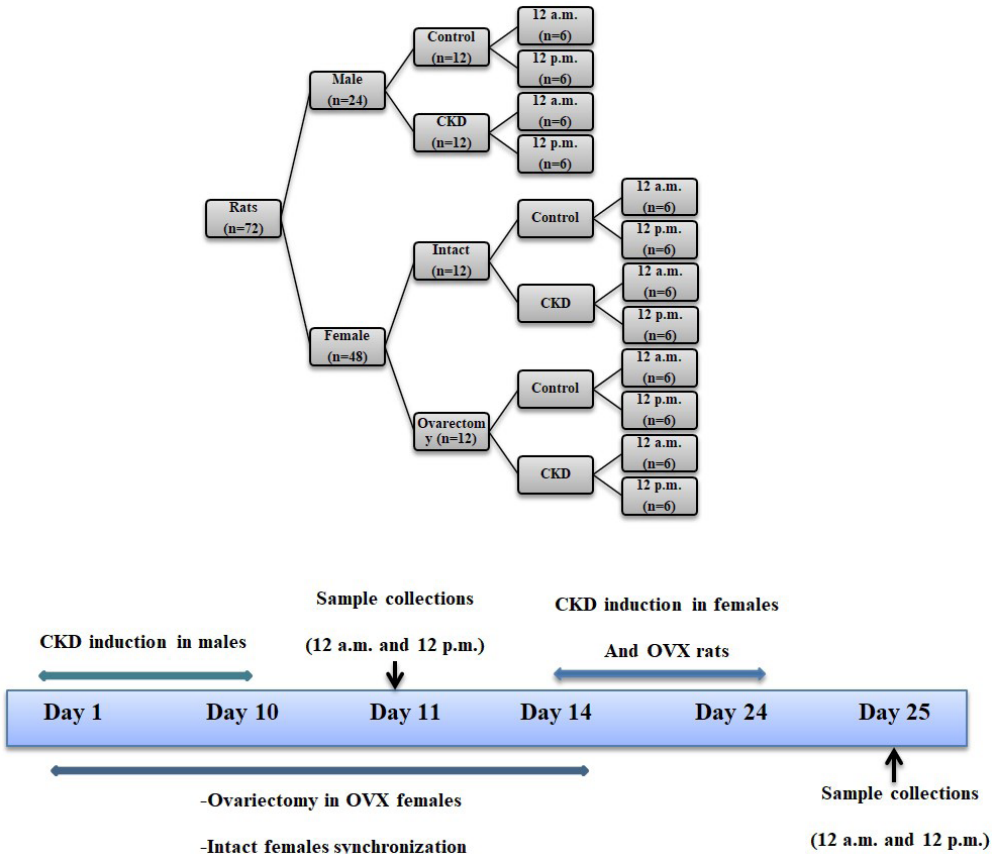

Figure 1. An overview of the animal groups and experiment design

between the vagina and umbilicus to provide access to the reproductive organs for the surgical procedure. The ovaries were carefully excised from the surrounding tissues and vasculature and were removed from the abdominal area. The fallopian tubes were knotted below the ovaries using catgut thread.

CKD induction

For CKD induction, adenine solution (200 mg/kg body weight of adenine in 1% (w/v) gum acacia solution) was gavaged every day for 10 days (24). To verify renal impairment, urea and creatinine plasma levels were measured one day after the last day of CKD induction (12:00 a.m. or 12:00 p.m.).

Urinary indicator evaluations

Urine samples were collected in a metabolic cage. The urea, creatinine, and protein levels were measured by standard kits (Pars Azmoon, Tehran, Iran) using an autoanalyzer (Selectra-XL, Vital Science; Netherlands) in a standard laboratory setting.

Evaluation of glomerular filtration rate (GFR)

The following formula was used to determine the glomerular filtration rate (24), which was based on creatinine clearance measured in milliliters per minute:

The creatinine clearance rate in ml/min was calculated as follows: (urinary creatinine concentration in mg/dl * urine volume per minute)/creatinine plasma concentration in mg/dl.

Histopathological evaluations

For histopathological studies, the animals were perfused with formaldehyde solution. Subsequently, the kidneys were extracted and immediately washed with saline. The right kidney was fixed in 10% formalin and subjected to H&E staining analysis. An experienced pathologist performed a random histopathological evaluation based on tubular dilatation, degeneration and necrosis, degree of inflammation and interstitial fibrosis, and degree of glomerulosclerosis.

Statistical analysis

The data were analyzed by three-way repeated-measures ANOVA (group × sex × time) and Bonferroni's correction for post-hoc tests in the SPSS software package (version 20). A *P*-value less than 0.05 was considered statistically significant. The data are presented as the mean ± SEM.

Results

Gender Differences and Circadian Rhythms' Effect on Urinary Parameters in CKD Rats at 12:00 a.m. and 12:00 p.m.

Urine volume

According to the statistical analysis, CKD significantly increased urine volume in both male and female rats compared to controls during both times ($P < 0.001$) (Figure 2a). Figure 2b shows that male rats and OVX rats with CKD (respectively) displayed a greater increase in urine volume compared to female rats with CKD ($P < 0.001$ and $P < 0.01$, respectively).

Urine urea level

The results demonstrated that CKD significantly decreased urine urea levels in all groups, regardless of sex or ovariectomy status, at both 12:00 a.m. and 12:00 p.m. ($P < 0.001$) (Figure 3a). Furthermore, no significant differences in urine urea levels were observed among the CKD groups at either 12:00 a.m. or 12:00 p.m. (Figure 3b). In addition, the urine urea level in the control female rats was greater than that in the OVX and male rats at both time points ($P < 0.001$). However, the urine urea level in OVX rats was significantly higher at 12:00 p.m. ($P < 0.001$) and lower at 12:00 a.m. ($P < 0.05$) compared to male rats (Figure 3b).

Urine creatinine level

Urinary creatinine levels decreased in all groups after CKD induction at both times, as shown in Figure 4a. Furthermore, intact female rats in the control group exhibited higher urinary creatinine levels ($P < 0.001$ and $P < 0.05$) than male rats at both times. Additionally, OVX female control rats displayed lower urinary creatinine levels than intact females at 12:00 a.m. ($P < 0.001$) (Figure 4b). These findings suggest that both CKD and ovarian hormones may influence urinary creatinine levels in female rats.

Urine protein level

Figure 5a shows that urine protein levels increased in all animals after CKD induction at both time periods

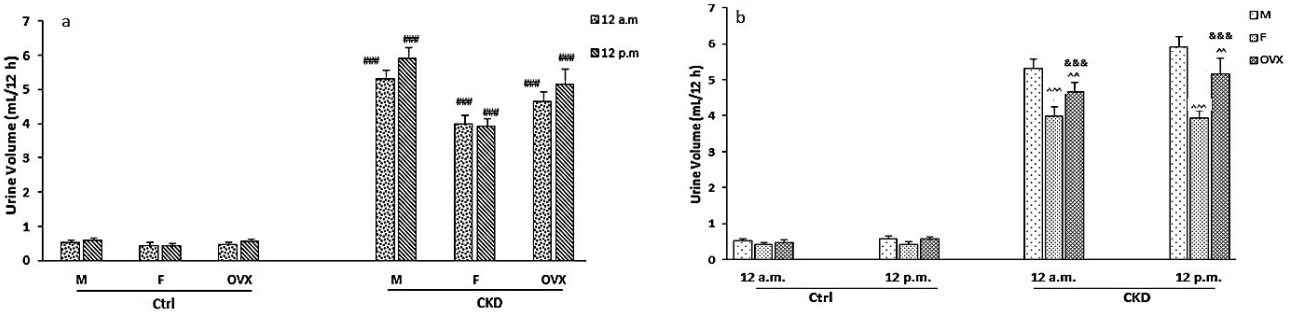

Figure 2. Comparison of the Urine volume (ml/12 h) based on sex (A) and time (B) in control and CKD rats (n = 6). The data are shown as the mean ± SEM. Ctrl: control, CKD: chronic kidney disease, M: male, F: intact female, OVX: ovariectomized. $^{###}P < 0.001$ vs. the control group, $^{\wedge\wedge\wedge}P < 0.001$ and $^{\wedge\wedge}P < 0.01$ vs. the male group; $^{\&\&\&}P < 0.001$ vs. the intact female group

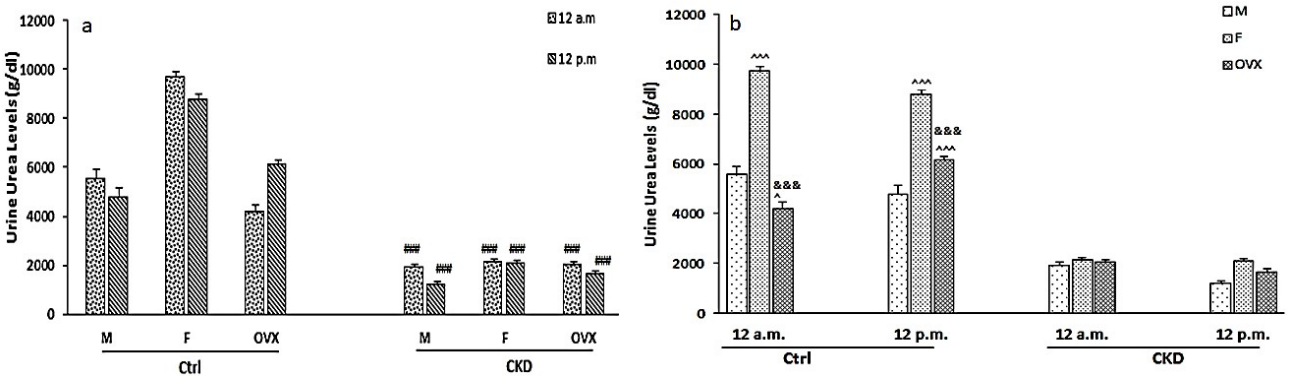

Figure 3. Comparison of the Urinary urea level (ml/12 h) based on sex (A) and time (B) in control and CKD rats (n = 6). The data are shown as the mean ± SEM. Ctrl: control, CKD: chronic kidney disease, M: male, F: intact female, OVX: ovariectomized. $^{\#\#\#}P < 0.001$ vs. the control group, $^{\wedge\wedge\wedge}P < 0.001$, and $^{\wedge}P < 0.05$ vs. the male group, $^{\&\&\&}P < 0.001$ vs. the intact female group

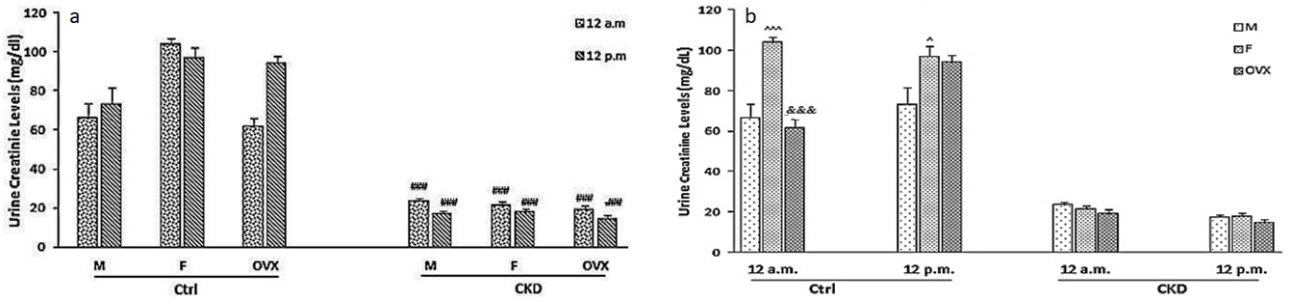

Figure 4. Comparison of the urinary creatinine levels (ml/12 h) based on sex (A) and time (B) in control and CKD rats (n = 6). The data are shown as the mean ± SEM. Ctrl: control, CKD: chronic kidney disease, M: male, F: intact female, OVX: ovariectomized. $^{\#\#\#}P < 0.001$ vs. the control group, $^{\wedge\wedge\wedge}P < 0.001$ and $^{\wedge}P < 0.05$ vs. the male group, $^{\&\&\&}P < 0.001$ vs. the intact female group

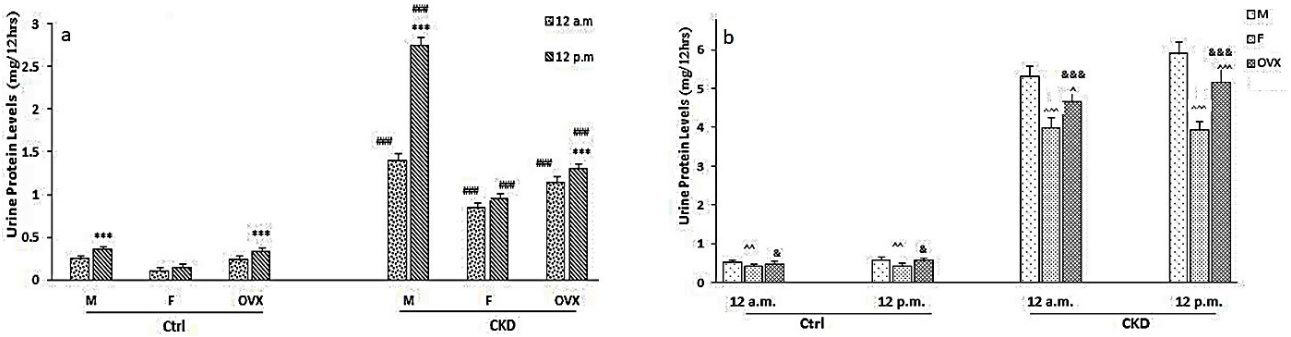

Figure 5. Comparison of the urinary protein levels (ml/12 h) based on sex (A) and time (B) in control and CKD rats (n = 6). The data are shown as the mean ± SEM. Ctrl: control, CKD: chronic kidney disease, M: male, F: intact female, OVX: ovariectomized. $^{***}P < 0.001$ vs. 12:00 a.m., $^{\#\#\#}P < 0.001$ vs. the control group, $^{\wedge\wedge\wedge}P < 0.001$, $^{\wedge\wedge}P < 0.01$ and $^{\wedge}P < 0.05$ vs. the male group, $^{\&\&\&}P < 0.001$ and $^{\&}P < 0.05$ vs. the intact female group

($P < 0.001$). However, the increase was more pronounced in the male and OVX groups at 12:00 p.m. compared to 12:00 a.m. ($P < 0.001$, Figure 5a). In addition, intact female rats had lower urine protein levels than both male ($P < 0.01$ and $P < 0.001$) and OVX female rats ($P < 0.001$ and $P < 0.05$) at both time points. Furthermore, the urine protein levels in the control and CKD OVX groups were significantly lower than the male groups at both times ($P < 0.001$ and $P < 0.05$) (Figure 5b).

Gender Differences and Circadian Rhythms' Effect on the GFR in CKD Rats at 12:00 a.m. and 12:00 p.m.

According to the statistical analysis, CKD significantly reduced the GFR in all the rats at both time points ($P < 0.001$). The decrease in GFR was more significant at 12:00 p.m. than at 12:00 a.m. (Figure 6a). Although GFR showed a significant difference between the control OVX group and the control intact female group at 12 a.m. ($P < 0.05$), there was no significant difference in GFR between the other groups at either time point (Figure 6b).

Gender Differences and Circadian Rhythms' Effect on the renal histopathology and Tubular Damage in CKD Rats at 12:00 a.m. and 12:00 p.m.

Tubular dilatation, degeneration, and necrosis

Figure 7 shows that CKD led to a significant increase in tubular dilatation, degeneration, and necrosis in all groups of rats (male, intact female, and ovariectomized female) ($P < 0.001$). These changes were more pronounced at 12:00 p.m. compared to 12:00 a.m. (Figure 7a, c, and e, respectively). Additionally, the male and OVX animals displayed higher levels of tubular damage than intact

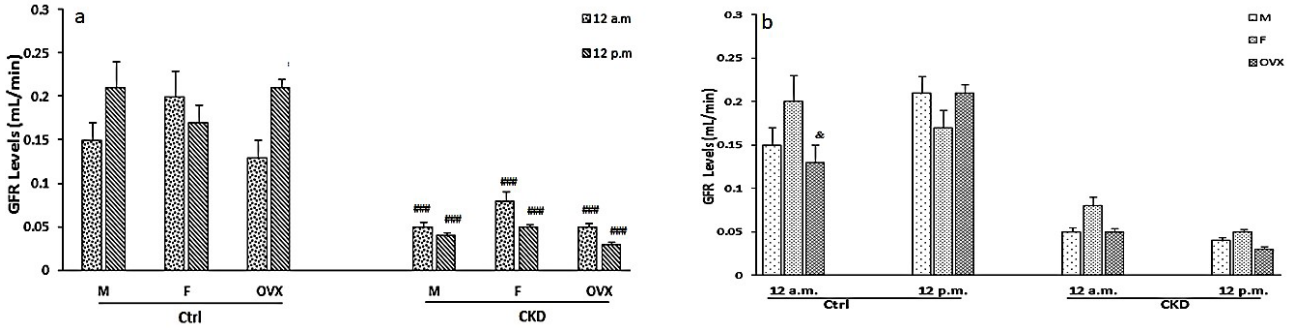

Figure 6. Comparison of renal glomerular filtration rate (ml/min) based on sex (A) and time (B) in control and CKD rats (n = 6). The data are shown as the mean ± SEM. Ctrl: control, CKD: chronic kidney disease, M: male, F: intact female, OVX: ovariectomized. $^{\#\#\#}P < 0.001$ vs. control group, $^{\&}P < 0.05$ vs. the intact female group

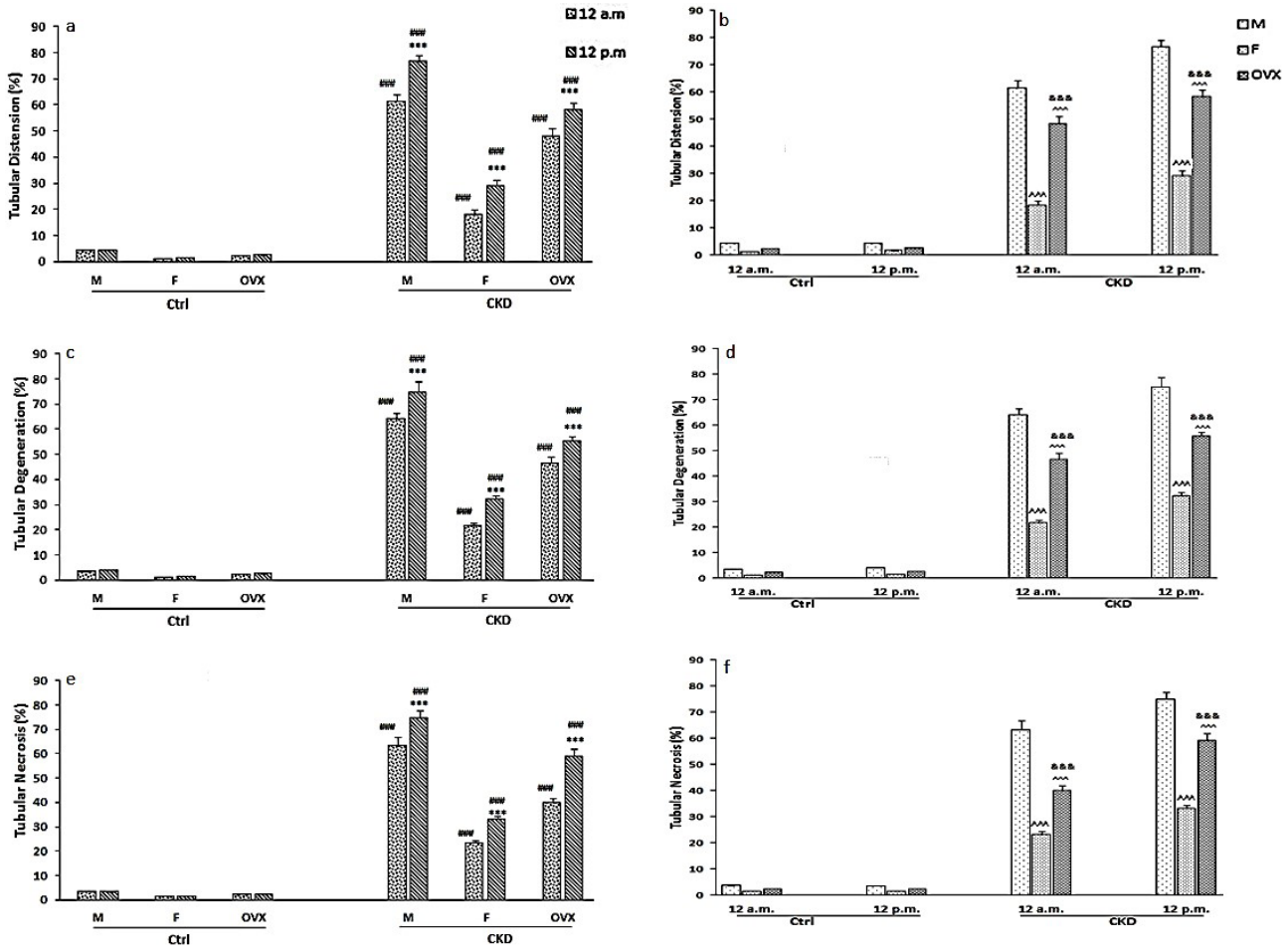

Figure 7. Comparison of the renal tubular dilatation, degeneration, and necrosis based on sex (A) and time (B) in control and CKD rats (n = 6). The data are shown as the mean ± SEM. Ctrl: control, CKD: chronic kidney disease, M: male, F: intact female, OVX: ovariectomized. $^{***}P < 0.001$ vs. 12:00 a.m., $^{\#\#\#}P < 0.001$ vs. the control group, $^{\wedge\wedge\wedge}P < 0.001$ vs. the male group, $^{\&\&\&}P < 0.001$ vs. the intact female group

female rats at both time points ($P < 0.001$) (Figure 7b, d, and f, respectively).

Inflammation and interstitial fibrosis

The data reveal that both inflammation and interstitial fibrosis increased significantly in rats with CKD compared to control rats at both time points ($P < 0.001$) (Figure 8a and c, respectively). This increase was more significant at 12:00 p.m. ($P < 0.001$) in CKD groups. In addition, the changes observed in Figure 8b and d were significantly decreased in intact female and OVX rats compared to male rats ($P < 0.001$). Furthermore, inflammation and interstitial fibrosis were more pronounced in the OVX females than in intact females at both time points ($P < 0.001$) (Figure 8b and d).

Glomerulosclerosis

Glomerulosclerosis, a marker of kidney damage, increased significantly in all rats following the induction of CDK ($P < 0.001$). This increase was observed at both 12:00 a.m. and 12:00 p.m. (Figure 9a). As shown in Figure 9b, male and OVX female rats exhibited higher levels of glomerulosclerosis than intact female rats, particularly at 12:00 p.m. ($P < 0.001$). However, ovariectomized female

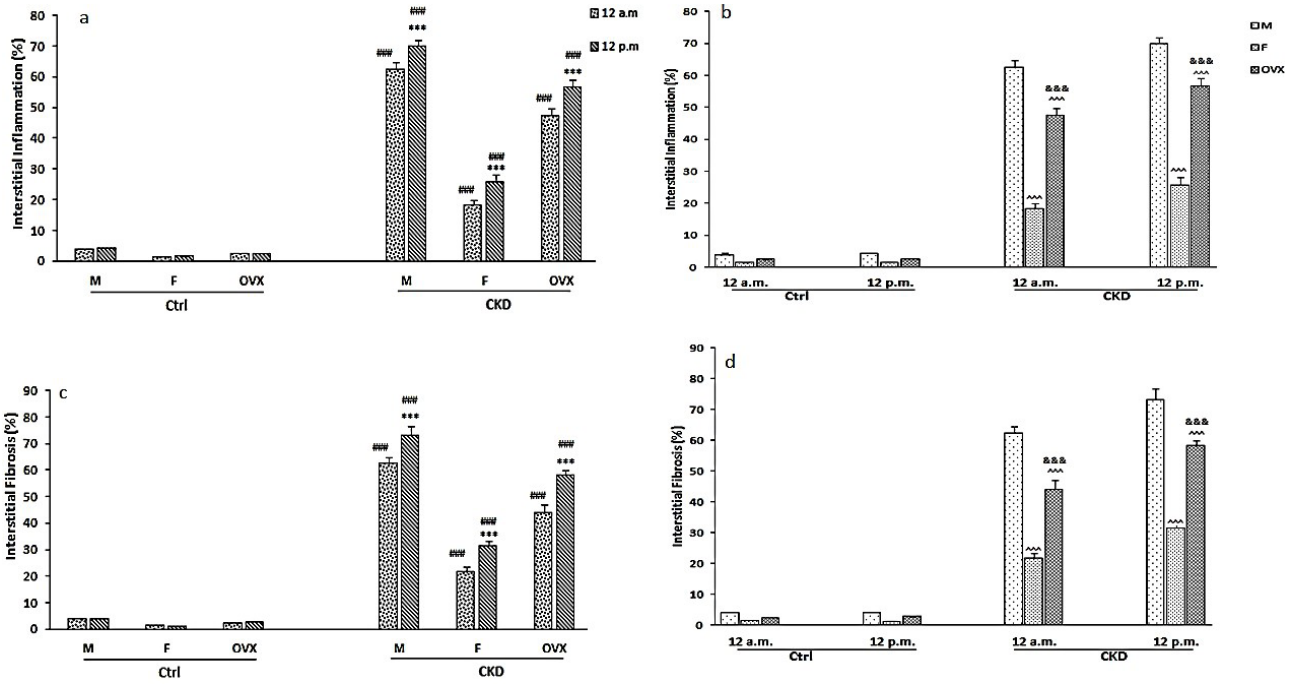

Figure 8. Comparison of the renal interstitial inflammation and fibrosis based on sex (A) and time (B) in control and CKD rats (n = 6). The data are shown as the mean ± SEM. Ctrl: control, CKD: chronic kidney disease, M: male, F: intact female, OVX: ovariectomized. $^{***}P<0.001$ vs. 12:00 a.m., $^{\#\#\#}P<0.001$ vs. the control group, $^{\wedge\wedge\wedge}P<0.001$ vs. the male group, $^{\&\&\&}P<0.001$ vs. the intact female group

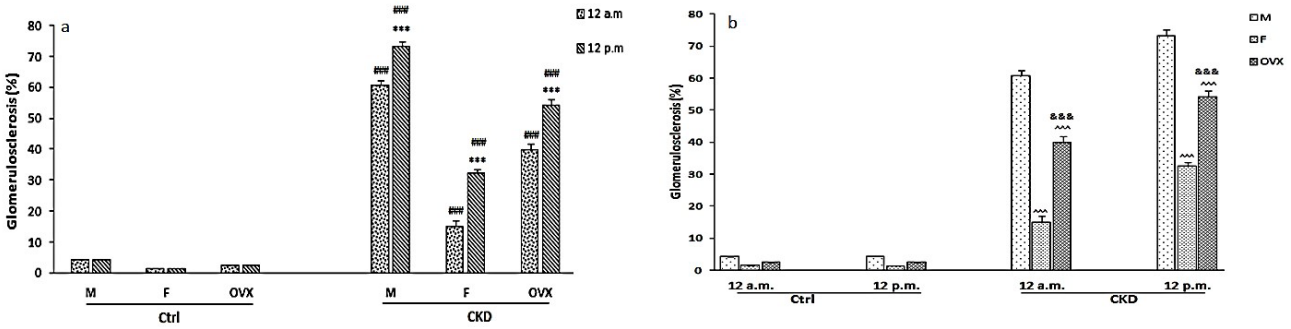

Figure 9. Comparison of the glomerulosclerosis based on sex (A) and time (B) in control and CKD rats (n = 6). The data are shown as the mean ± SEM. Ctrl: control, CKD: chronic kidney disease, M: male, F: intact female, OVX: ovariectomized. $^{***}P<0.001$ vs. 12:00 a.m., $^{\#\#\#}P<0.001$ vs. the control group, $^{\wedge\wedge\wedge}P<0.001$ vs. the male group, $^{\&\&\&}P<0.001$ vs. the intact female group

rats showed a less noticeable increase in glomerulosclerosis compared to male rats ($P<0.001$) (Figure 9b).

Histopathological evaluations

Figure 10 shows the histological sections of kidney tissue from male, intact female, and ovariectomized female CKD and normal (control) rats at 12:00 a.m. and 12:00 p.m. The normal glomerulus and tubules were compared to glomerulosclerosis, tubular necrosis, interstitial inflammation, interstitial fibrosis, and tubular dilatation in CKD groups.

Discussion

In the present study, no difference was found in urea and creatinine levels in urine between night and day, as confirmed by other studies (25, 26). However, some human studies have indicated lower creatinine levels at night (27). CKD reduced urinary urea and creatinine, but no significant daily variations were observed. Previous studies have indicated that the kidney injury type may affect urinary urea and creatinine levels, but findings on diurnal variations have been inconsistent (3, 28, 29). In addition, our results revealed that intact females generally exhibited higher urinary urea and creatinine levels compared to ovariectomized females and males. However, the literature presents conflicting findings on sex-based differences in urinary urea and creatinine levels in humans and animals (30, 31). It seems that sex hormones impact kidney function and circadian rhythms. For example, estrogen has been shown to improve kidney function (32). However, the variability in findings may be attributed to factors such as kidney injury type, animal model, sex, hormones, and treatments.

Our study found no significant difference in urine volume between day and night or between males and females under normal conditions. This is consistent with previous research on daily urine volume patterns in animal (30, 33) and human models (26, 34). However, some studies have reported a decrease in nocturnal urine volume, especially in females (31, 35, 36). This may be

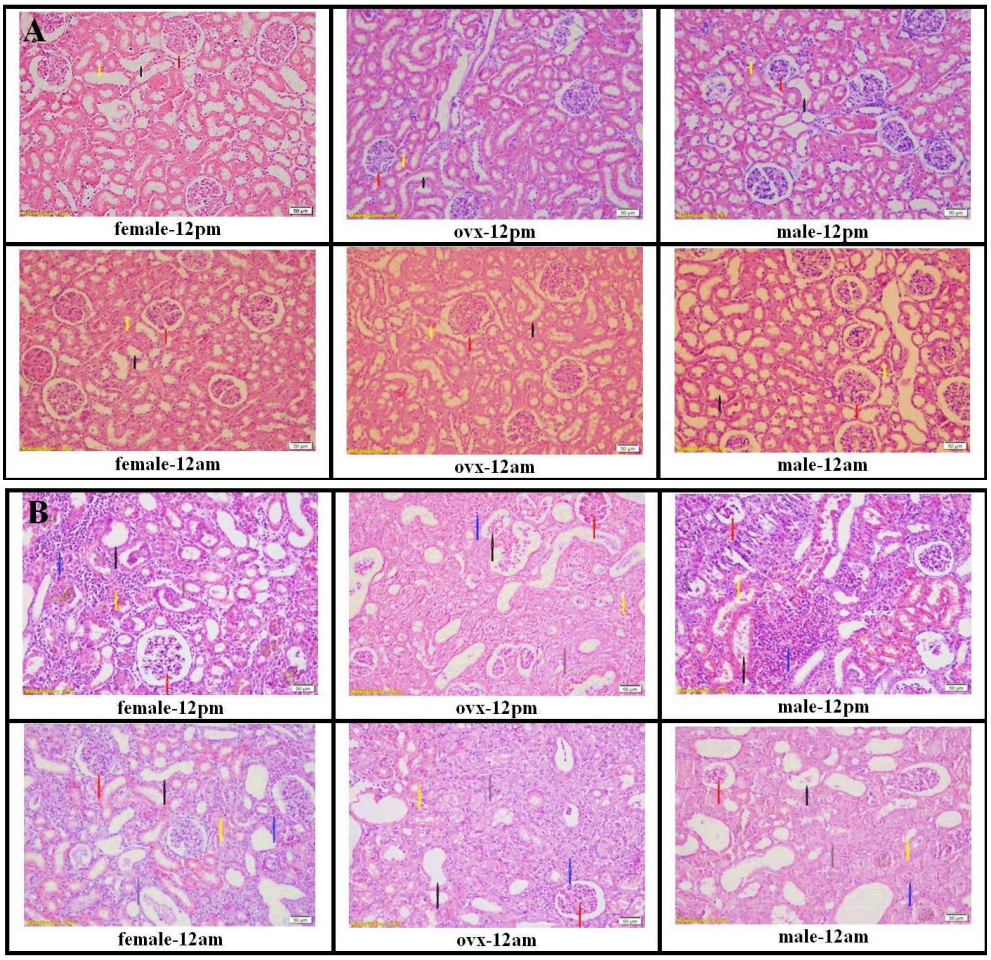

Figure 10. Histopathology of kidney in control groups (A) (red arrow: normal glomerulus; yellow and black arrows: normal tubules) and CKD groups (B) (red arrow: glomerulosclerosis, yellow arrow: tubular necrosis, blue arrow: interstitial inflammation, gray arrow: interstitial fibrosis, black arrow: tubular dilatation). OVX: ovariectomized

attributed to lower levels of the water channel aquaporin-2 in the kidneys at night (36). Furthermore, sex hormones in intact females may reduce urine volume by affecting plasma melatonin levels (37-39). Studies have demonstrated that melatonin administration has a protective effect in kidney injury models (40, 41). In CKD, urine volume notably increased during both day and night, which is consistent with previous findings in rodents (10, 42) and humans (11). CKD can lead to changes in urine concentration and volume due to resistance to antidiuretic hormone (ADH) (43). Several studies have shown that various factors, such as ADH, atrial natriuretic peptide (ANP), sympathetic activity, and the renin-angiotensin-aldosterone system, can influence urine volume (35, 44, 45). In addition, the increase in urine volume was lower in females with CKD than in males. Studies on gender differences in CKD response and urine volume changes have shown conflicting findings, probably due to V2 receptor expression, altered plasma arginine vasopressin (AVP) rhythm, and neural responses in females compared to males during both day and night (36, 46). However, the inconsistent results in studies may be attributed to factors such as disease severity, kidney injury type, and genetic and sex differences.

This study revealed that urinary protein levels were higher during the day compared to night under normal conditions. However, urinary protein levels increased in CKD, which aligns with other research indicating that a diet high in adenine can elevate urinary protein levels (3). The increased protein excretion in CKD may be attributed to increased glomerular capillary pressure and albumin filtration (36), tubulointerstitial damage (47), glomerular injury (48), and decreased renal NO production (29). In ovariectomized females and males, urinary protein levels were more elevated during the day, consistent with previous studies demonstrating decreased nocturnal urinary protein (29, 49). Previous studies have suggested sex differences in renal protein excretion (34, 50, 51). This may be due to the protective effects of sex hormones in intact females, which reduce CKD damage, and the detrimental effects of testosterone in males on urinary protein levels (52). For example, it has been reported that estrogen administration in postmenopausal women decreases urinary protein (23). In addition, gonadectomy in male nephrectomized rats and estrogen administration in these animals reduced urinary protein (21). Estrogen appears to stimulate renal nitric oxide synthase activity, increasing NO production and consequently reducing proteinuria (23). Overall, these findings highlight the protective role of female sex hormones in reducing proteinuria.

Our study showed that CKD significantly increased

interstitial inflammation, tubular dilatation, tubular atrophy, and fibrosis in the kidney. Furthermore, kidney injury was more severe during the day compared to the night. Since sleep disorders are common in CKD patients (53), some of these daily variations are likely related to the circadian rhythm of melatonin. For example, a study has shown that melatonin administration can reduce interstitial fibrosis in CKD rats (54). Notably, histopathological markers were lower in female groups compared to male and ovariectomized groups, suggesting a protective role of female sex hormones in chronic kidney injury. For example, estrogen administration has been shown to reduce glomerular fibrosis in kidney injury models (50) and in rats with type 1 diabetes (29). In addition, estradiol has been found to decrease glomerulosclerosis and tubulointerstitial fibrosis by reducing extracellular matrix synthesis, inhibiting endothelin synthesis, and decreasing its inflammatory effects (55). Studies on gonadectomy in kidney injury animal models have shown reduced glomerular, tubular, and renal fibrosis (21, 23). Conversely, testosterone administration in CKD animal models has been associated with apoptosis and fibrosis (52). Overall, female sex hormones, particularly estrogen, may reduce kidney injury by stimulating NOS activity, decreasing extracellular matrix synthesis, increasing its degradation, and inhibiting endothelin synthesis (20, 23).

Conclusion

The present study demonstrates that CKD induces significant changes in histopathological indices. These alternations are more pronounced in males and OVX rats than in intact females, especially at 12:00 p.m. CKD also decreased urea, creatinine, and GFR levels. However, CKD increases urinary volume and protein levels in urine. These changes were more significant in male and OVX animals, respectively. In addition, urinary protein levels showed a significant difference at 12:00 p.m. compared to 12:00 a.m. The effects of sex and sex hormones on CKD severity may be reasonable based on our interpretations. However, the disruption of circadian rhythms may not be justifiable based on the current limited data. Additionally, the time factor might have contributed to the severity of observed damage. To effectively assess and compare circadian rhythms across different groups, it would be necessary to collect samples at multiple time points and measure circadian rhythm-related genes at these specific times.

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Authors' Contribution

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Supervision: Shahriar Dabiri.
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Writing – Original Draft: Shahrzad Sadat Eftekhar Vaghefi, Rana Eftekhar Vaghefi.
Writing – Review & Editing: Shahrzad Sadat Eftekhar Vaghefi, Fatemeh Shahsavari.

Data Availability Statement

All necessary data will be available from the corresponding author upon reasonable request.

Ethical Approval

All experimental protocols were performed following the guidelines for animal experimentation at Kerman University of Medical Sciences and were approved by the Animal Care and Use Committee of Kerman University of Medical Sciences, Kerman, Iran (ethical code: IR.KMU.REC.1399.093).

Founding

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