

Effects of hormone replacement therapy in postmenopausal rats with circadian rhythm disorder and burn injury-induced kidney damage

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This study investigated the effects of burn injury and circadian rhythm disorder on the kidneys of postmenopausal rats and potential protective role of hormone replacement therapy (HRT). Forty-eight female Sprague Dawley rats (20-22 weeks old) were divided into six groups: control, circadian rhythm disorder (PC), burn injury (B), circadian rhythm disorder with burn injury (PCB), circadian rhythm disorder with burn injury treated with estradiol-drospirenone (PCBE), and burn injury treated with estradiol-drospirenone (PBE). Estradiol-drospirenone combination for HRT administration (0.01 mg/kg 17 β -estradiol and 0.02 mg/kg drospirenone) for 14 days. The study lasted 15 days, after which histopathological and immunohistochemical analyses were conducted on kidney tissues. Serum cortisol, creatinine, and blood urea nitrogen (BUN) levels significantly increased in the B, PC, and PCB groups compared to the control ($P < 0.001$). However, these parameters significantly decreased in the PBE and PCBE groups ($P < 0.001$). Histopathological damage and cyclooxygenase-2 (Cox-2), hypoxia inducible factor-2 (Hif-2), and interferon-gamma (IFN- δ) expression were significantly elevated in the B, PC, and PCB groups ($P < 0.001$), whereas estradiol-drospirenone treatment markedly reduced these pathological alterations ($P < 0.001$). These findings suggest that the estradiol-drospirenone combination may have a therapeutic role in mitigating kidney damage caused by circadian rhythm disorders and burn injuries in postmenopausal individuals.

Keywords: Burn injury, Circadian rhythm, Cyclooxygenase-2 (Cox-2), Estradiol-drospirenone, Hypoxia inducible factor-2 (Hif-2), Interferon-gamma (IFN- γ)

Circadian rhythms are essential biological processes that regulate physiological and behavioral functions in alignment with the 24-hour light-dark cycle, primarily coordinated by the suprachiasmatic nucleus of the hypothalamus through photic input from the retina¹. Disruptions in this system due to aging, genetic factors, or hormonal changes such as those occurring during menopause can result in circadian rhythm disorders²⁻⁴. Menopause, characterized by a decline in estrogen levels, is associated with alterations in sleep patterns, locomotor activity, and hormonal rhythms, as demonstrated in postmenopausal rodent models, where changes in clock gene expression, neurotransmitter signaling, and hypothalamic-pituitary axis function have been observed⁵⁻⁷.

Hormone replacement therapy (HRT), including agents such as estradiol and drospirenone, has shown potential in mitigating these disruptions⁸. Estradiol offers renal protection via anti-inflammatory and

antioxidant pathways, while drospirenone, with its anti-mineralocorticoid activity, may influence renal sodium handling and blood pressure regulation⁹. However, the combined effects of estradiol and drospirenone on kidney function, particularly under stress conditions like burn injury and circadian misalignment, remain insufficiently explored and may involve complex interactions with systems such as the renin-angiotensin-aldosterone axis and nitric oxide signaling¹⁰⁻¹².

Burn injuries represent a significant public health concern worldwide, affecting millions of individuals annually and posing significant challenges for healthcare providers¹³. Circadian rhythms regulate various cellular processes involved in wound healing, including inflammation, proliferation, and tissue remodeling¹⁴. Disruptions in circadian rhythms following burn injuries can impede the orderly progression of these healing stages, leading to delayed wound closure, increased risk of infection, and impaired functional outcomes¹⁵.

The current study hypothesizes that estradiol-drospirenone treatment could mitigate kidney damage

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induced by the combined stressors of burn injury and circadian rhythm disruption in postmenopausal rats by modulating inflammatory and hypoxic pathways. Specifically, this study investigates the expression of cyclooxygenase-2 (Cox-2), hypoxia inducible factor-2 (Hif-2), and interferon gamma (IFN- γ), which are known to play critical roles in renal inflammation, oxidative stress, and hypoxia-related damage. Cox-2 is an inducible enzyme that mediates prostaglandin synthesis and is upregulated in response to tissue injury, contributing to inflammatory cascades¹⁶. Hif-2 is a hypoxia-inducible factor implicated in the regulation of erythropoietin and vascular factors under low oxygen conditions, which are prominent in burn-related ischemic renal injuries¹⁷. IFN- γ is a pro-inflammatory cytokine that enhances immune responses and may exacerbate tissue damage when dysregulated¹⁸. By evaluating the modulation of these markers, we aim to elucidate the potential protective role of hormone therapy in this complex injury model. This study aims to evaluate potential mechanisms and effects of estradiol-drospirenone combination therapy on the kidney in postmenopausal rats with burn injuries and circadian rhythm disorders.

Material and Method

Animals and ethical approval

The experimental protocol in our study was approved by the Local Ethics Committee for Animal Experiments of Burdur Mehmet Akif Ersoy

University (Approval No: 24.04.2024/1301). Animal Research: Reporting *in vivo* Experiments (ARRIVE) 2.0 were followed for all experimental procedures. Forty-eight (20-22 weeks), female Sprague Dawley rats weighing 250-300 g, obtained from Burdur Mehmet Akif Ersoy University Laboratory Animal Production and Experimental Research Laboratory, were housed in adjustable rooms with a temperature of 21-23°C, humidity of 55-65%, and a 12h light/dark cycle for 10 days before the study for adaptation. The rats were provided with pelleted rat feed containing 24% crude protein, 6.19% crude cellulose, and 3.100 kcal/kg metabolic energy, along with *ad libitum* access to water.

Circadian rhythm disorder model

The circadian rhythm disorder model to be applied was determined according to the model of Tsai *et al.*¹⁹. The study period for experimental rats began with a 10-day adaptation period in which the lights were on from 09:00 to 21:00. This was followed by a 2-week experimental phase characterized by 12-h phase shifts in the light/dark cycle twice a week. Specifically, each light/dark cycle change included a 24-h period in which the lights were on (Fig. 1A). Control rats followed a similar lighting regimen throughout the study; During the phase change period, the lights were on from 09:00 to 21:00, except on Mondays. On these days, the time the lights were on was extended to 24-h to ensure

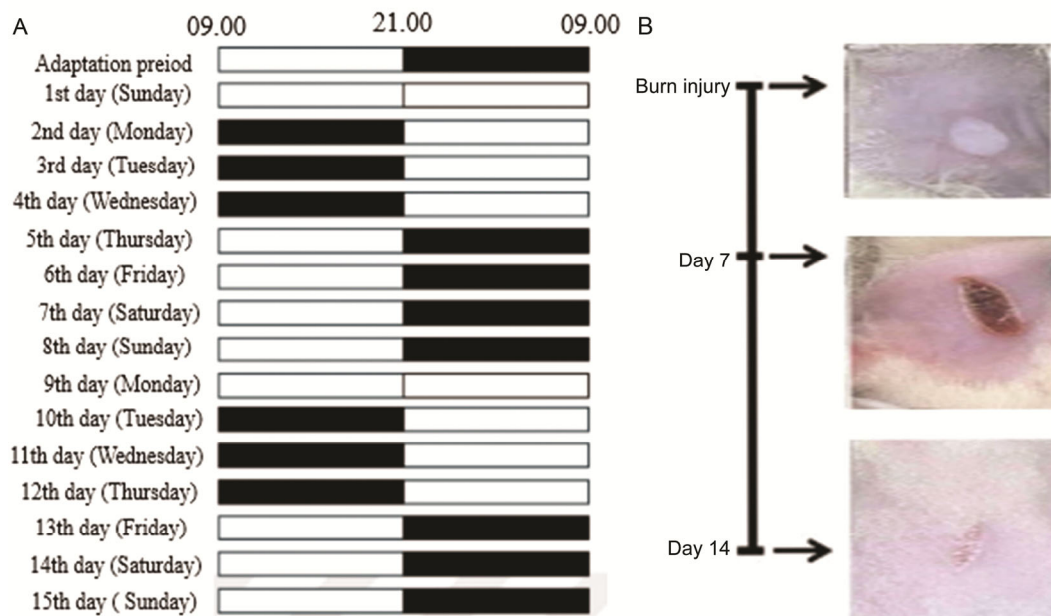


Fig. 1 — (A) The program applied to create circadian rhythm disorder, (B) Burn injury model.

equal light exposure between the control and experimental groups. This experimental design aims to investigate the effects of repeated phase shifts in the light/dark cycle on circadian rhythms and physiological parameters in rats, with control measures implemented to maintain consistency in light exposure between groups.

Burn injury model

The burn injury model was determined according to the method of Abdullahi *et al.*²⁰. In this burn injury model, the back area of the rats was shaved to ensure direct contact between the skin and the applied heat source. A 100 g iron block was used to cause full-thickness skin burns. This iron block was heated by immersing it in boiling water at 100°C for 15 min. After this heating period, under anesthesia the iron block was contacted with the shaved area of the rat's skin and it was ensured that no additional pressure was applied during contact. Wound contraction was quantified as the percentage reduction in wound area relative to the initial measurement. Lesion diameters were measured from the inner edges with a digital caliper (in mm) on days 1–7 and 14 (Fig. 1B). The epithelialization period was defined as the duration (in days) required for complete resurfacing of the wound bed, with no visible raw area remaining following wound induction. Wound contraction percentage was calculated by dividing the difference between the initial wound area and the final wound area by the initial wound area, and then multiplying the result by one hundred²¹.

Hormone replacement therapy (HRT)

Angeliq® (Schering AG, Berlin, Germany) was used for HRT. The dose of HRT was determined using the method of Topcuoglu *et al.*²². Rats treated daily with oral administration of estradiol-drospirenone combination (0.01 mg/kg 17- β -estradiol and 0.02 mg/kg drospirenone) for 14 days.

Experimental design

In this study, a total of six groups were formed, including control, PC, B, PCB, PCBE, and PBE (8 rats per group). The animals were treated as follows; Control group: Postmenopausal rats were not treated for 14 days; PC group: Postmenopausal rats with circadian rhythm disorder for 14 days; B group: Postmenopausal rats with burn injury for 14 days; PCB group: Postmenopausal rats with circadian rhythm disorder with burn injury for 14 days; PCBE group: Postmenopausal rats with circadian rhythm

disorder with burn healing with estradiol-drospirenone combination (0.01 mg/kg 17 β -estradiol and 0.02 mg/kg drospirenone) daily treatment p.o. for 14 days; PBE group: Postmenopausal rats with burn injury with estradiol-drospirenone combination (0.01 mg/kg 17 β -estradiol and 0.02 mg/kg drospirenone) daily treatment p.o. for 14 days.

The study was concluded on the 15th day, and all animals were euthanized under anesthesia with ketamine (90 mg/kg i.p.) and xylazine (10 mg/kg i.p.) using cervical dislocation. Kidney tissue samples were collected.

Determination of serum cortisol, creatinine and BUN levels

The blood samples were taken directly into tubes and centrifuged at 4000 rpm for 10 min. Cortisol levels of serum was performed according to the procedure of a commercial ELISA kit (BT-LAB E0609Mo) and measured at 450 nm with an ELx800-Biotek instrument. The levels of creatinine and blood urea nitrogen (BUN) in the serums were measured by an autoanalyzer (Gesam chem 200 Gesam Production SRL, Campobello, Italy). The creatinine and BUN levels were detected according to Kinetic UV optimized IFCC methods and Jaffè method, respectively.

Histopathological analysis

Kidney samples were taken out during the necropsy, examined macroscopically for any unusual characteristics, and then preserved in 10% neutral formalin solution²³. Subsequently, the tissue samples were treated using Leica ASP300S automated tissue processing equipment and embedded in paraffin wax. Using a Leica RM2155 rotary microtome, the paraffin blocks were cooled before being sectioned into 5 μ m thick pieces. These slices were stained with hematoxylin-eosin (H&E) and examined under a light microscope²⁴.

The histological lesions in the tissues were graded using an ordinal approach that evaluated hemorrhage, necrotic and degenerative changes, inflammatory cell infiltrations, and hyperemia. Scores for normal tissues ranged from 0 to 3, with values in between for mild, moderate, and severe pathologies²⁵. The grading criteria for the histopathological changes were explained in Table 1.

Immunohistochemical examination

Furthermore, three sets of sections from each block were obtained on slides coated with poly-L-lysine and subjected to immunohistochemical staining for the following antibodies: anti-interferon gamma (IFN- γ)

Table 1 — Histopathological grading score description

0	No lesion
1	Mild hyperemia, slight hemorrhage, no inflammation and vacuolar degeneration
2	Severe hyperemia, slight hemorrhage, slight inflammation and slight necrosis
3	Severe hyperemia, severe hemorrhage, marked inflammation and marked necrosis

(ab7740), anti-cyclooxygenase-2 (Cox-2) antibody [EPR12012] (ab179800), and anti-hypoxia inducible factor-2 (Hif-2)-alpha [BL-95-1A2] - BSA free. The antibody expression method employed was streptavidin-biotin. Immunohistochemistry was performed using streptavidin-alkaline phosphatase conjugate and biotinylated secondary antibodies after the sections were incubated for 60 min with a 1/100 dilution of the primary antibodies. The chromogen was diaminobenzidine (DAB), and the secondary antibody was the EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) from Abcam, Cambridge, UK. Instead of receiving primary antibodies, antigen dilution solution was administered to the negative controls. A qualified pathologist from another university conducted each test who was blinded to the samples.

Each section was examined separately so that each antibody could be examined using immunohistochemistry²⁶. Using a grading system from (0) to (3) and the following markers: (0) negative staining, (1) focal weak staining, (2) diffuse weak staining, and (3) diffuse strong staining, the degree of the cells' immunohistochemical reactivity was assessed semiquantitatively. In each slice, ten distinct regions were assessed using an objective magnification of 200 \times . Olympus Co., Tokyo, Japan supplied the Database Manual Cell Sens Life Science Imaging Software System for the morphometric analysis and microphotography. Immunopositive cell scores were evaluated using the Image J 1.46r program (National Institutes of Health, Bethesda, MD).

Statistical analysis

The results were expressed as arithmetic mean \pm standard error (SE). The data obtained were first analyzed for parametric-nonparametric with the Shapiro Wilk test. The comparison of the results obtained from all groups was performed by One-Way ANOVA and the differences between the groups were determined by Tukey test values below $P < 0.001$ were considered significant. To compare the groups'

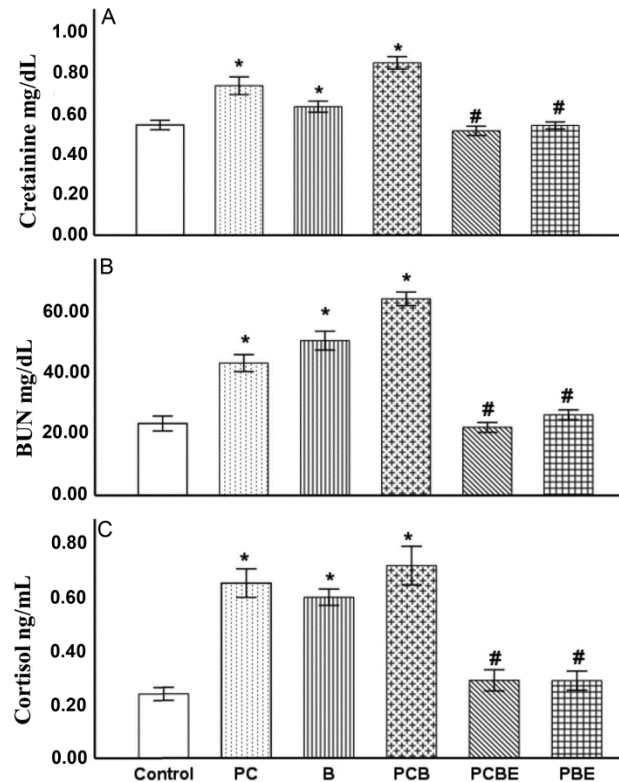


Fig. 2 — Cortisol, creatinine and BUN levels in serum control, B, PC, PCB, PBE and PCBE groups. [All values are expressed as mean \pm SEM (n = 8). * $P < 0.001$ when compared to the control group. # $P < 0.001$ when compared to the B, PC and PCB groups]

immunohistochemistry scores statistically, the Oneway ANOVA Duncan test from the SPSS-22.00 package program was used. $P < 0.05$ served as the threshold for significance.

Results

Biochemical findings

Cortisol levels were expressed in ng/mL, while creatinine and BUN levels were reported in mg/dL. The levels of cortisol, creatinine and BUN in the blood serum significantly increased in B, PC and PCB groups compared to the control group ($P < 0.001$) (Fig. 2). A significant decrease in blood serum cortisol, creatinine and BUN levels were observed in PBE and PCBE groups compared to B, PC and PCB groups ($P < 0.001$) (Fig. 2).

Histopathological findings

The PC and PCB groups showed a statistically significant decrease in histopathological scoring compared to the control group ($P < 0.001$ for both) (Fig. 3). The PCBE and PBE groups showed a significant decrease in this scoring compared to the PC, PCB and B groups ($P < 0.001$ for both) (Fig. 3).

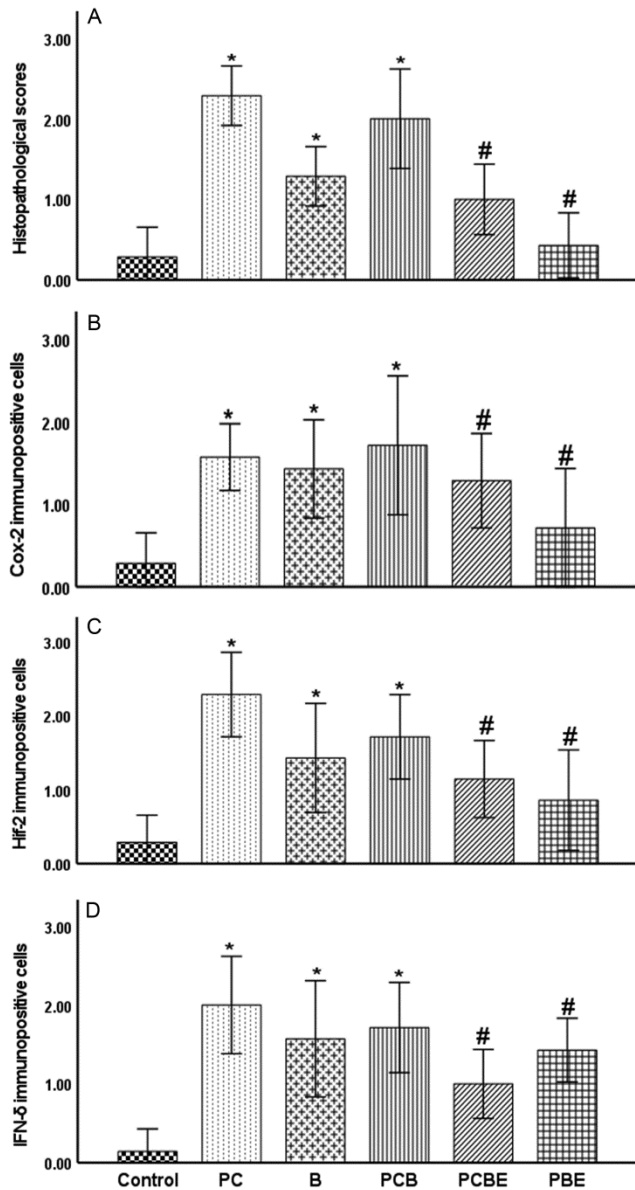


Fig. 3 — Histopathological scores and Cox-2, Hif-2, and IFN- γ immunopositive cells between the control, B, PC, PCB, PBE and PCBE groups. [All values are expressed as mean \pm SEM (n = 8). * P <0.001 when compared to the control group. # P <0.001 when compared to the B, PC and PCB groups]

The renal histology in the control group was shown to be normal after a histological study (Fig. 4A). Conversely, the PC group exhibited hyperemia, mild to severe inflammatory cell infiltration, and microhemorrhages (Fig. 4B). B group had mild to severe hyperemia (Fig. 4C). Significant hemorrhages and hyperemia in the PCB group (Fig. 4D). Significant hyperemia in the PCBE group without any hemorrhage (Fig. 4E). The PBE group showed no abnormal signs Fig. 4F.

Immunohistochemical findings

Under a microscope, Cox-2, Hif-2, and IFN- γ immune-stained slides showed no immunopositive cells in the control group (Fig. 5A-7A), whereas the PC group showed a significant increase in expression (P <0.001) (Fig. 5B-7B). The B group had mild to moderate emotions as well (Fig. 5C-7C). There were noticeable expressions in the PCB group (Fig. 5D-7D). The PCBE group showed decreased expressions (Fig. 5E-7E). Nothing or very little expressions in the PBE group (Fig.5F-7F). Positive expressions were seen in tubular epithelial cells, endothelial cells, and interstitial cells. The immunohistochemical expression scores' statistical analysis results are displayed in Fig. 3. The study's findings indicate that PC and B caused pathological findings but estradiol-drosperinone combination therapy decreased the renal lesions.

Burn wound-healing efficacy

On the 15th day of the study, wound closure rates were evaluated in the experimental groups. The highest level of burn wound healing was observed in the PBE group, with a wound closure rate of approximately 92%. This was followed by the PCBE group, which exhibited a 90% closure rate. In contrast, the burn injury group without treatment (B group) showed a wound healing rate of around 68%, while the PCB group had a significantly lower wound closure rate of approximately 62%. The PC group displayed a 70%

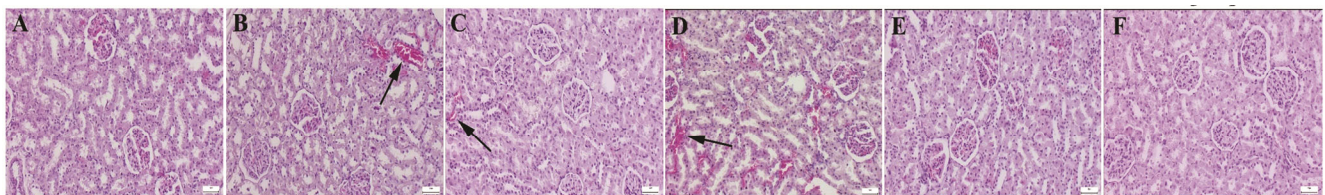


Fig. 4 — Histopathological appearance of the kidney between the groups. (A) Normal tissue histoarchitecture in control group. (B) Marked hyperemia and hemorrhage (arrow) in PC group. (C) Moderate hyperemia and slight hemorrhage in B group. (D) Marked hyperemia and hemorrhages in PCB group, (E) Decreased pathological findings in PCBE group, (F) No pathological findings in PBE group, [Scale Bars= 50 μ m]

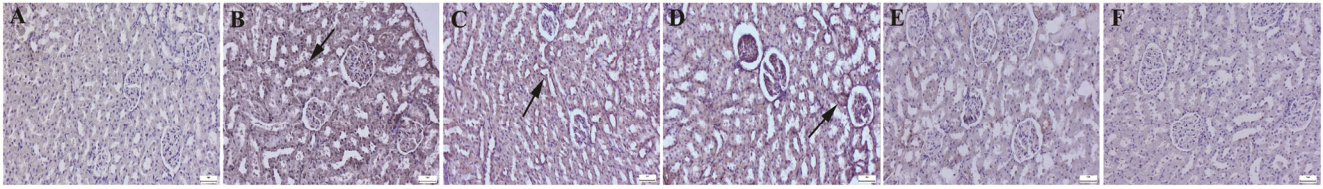


Fig. 5 — Representative Cox-2, immunohistochemical findings among the groups. (A) Negative expressions in control group. (B) Marked increased in expressions (arrow) in PC group. (C) Slight expressions (arrow) in B group, (D) Marked expression (arrow) in PCB group, (E) Decreased expression (arrow) in PCBE, (F) Almost negative expression in PBE group, Streptavidin Biotin Peroxidase Method [Scale Bars= 50 μ m]

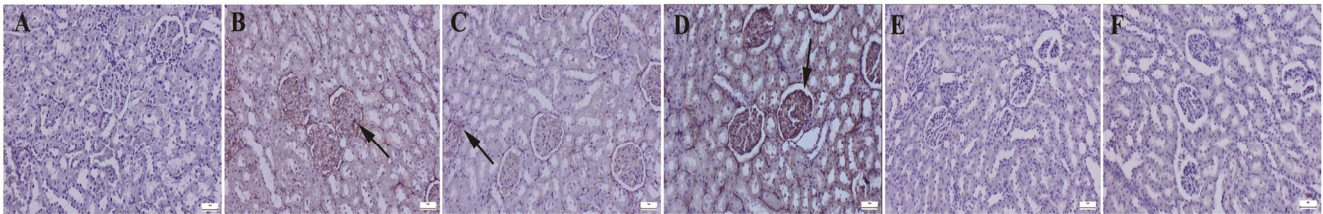


Fig. 6 — Hif-2 expressions between the groups. (A) Negative expressions in control group. (B) Marked increased in expressions (arrow) in PC group. (C) Slight expressions (arrow) in B group, (D) Marked expression (arrow) in PCB group, (E) Decreased expression (arrow) in PCBE, (F) Almost negative expression in PC group, Streptavidin Biotin Peroxidase Method [Scale Bars= 50 μ m]

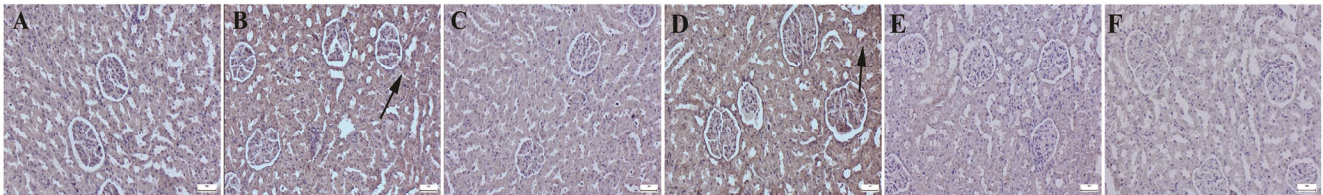


Fig. 7 — IFN- γ expressions between the groups. (A) Negative expressions in control group. (B) Marked increased in expressions (arrow) in PC group. (C) Slight expressions (arrow) in B group, (D) Marked expression (arrow) in PCB group, (E) Decreased expression (arrow) in PCBE, (F) Almost negative expression in PBE group, Streptavidin Biotin Peroxidase Method [Scale Bars= 50 μ m]

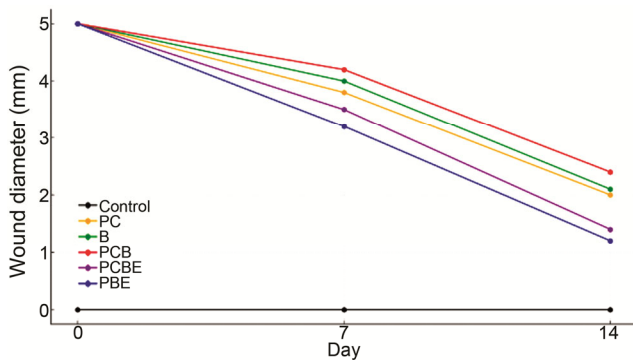


Fig. 8 — Burn wound closure percentage of wounds in experimental groups according to days.

healing rate, and the control group maintained normal skin integrity without burn injury (Fig. 8).

Discussion

Circadian rhythms play a crucial role in regulating physiological processes, including sleep-wake cycles, hormone secretion, and metabolism²⁷. Disruptions in these rhythms have been associated with numerous health conditions, including obesity, diabetes and

cardiovascular diseases²⁸. Postmenopausal women frequently exhibit alterations in their circadian rhythms, which may contribute to the increased risk of developing these disorders²⁹. Animal models, such as rats, offer valuable insights into the complex interactions between hormonal changes and circadian rhythm disturbances in postmenopausal individuals³⁰. Circadian rhythm disorders can lead to various physiological dysfunctions, including disruptions in wound healing and changes in immune responses. Our study supports previous research indicating that circadian rhythm disorders may exacerbate inflammatory processes and tissue damage³¹, as evidenced by the observed hyperemia, inflammatory cell infiltration and microhemorrhages in the kidneys of rats exposed only to circadian rhythm disruption group. These findings underscore the importance of maintaining appropriate circadian rhythm synchronization for overall health and tissue homeostasis¹⁵.

Burn injuries are a significant concern in clinical practice, often resulting in severe tissue damage and

impaired wound healing³². Burn injuries and disruptions to the circadian rhythm further complicate this scenario, potentially leading to dysregulation of cortisol, a critical stress hormone¹⁵. The findings suggest that the estradiol-drospirenone combination effectively modulates cortisol levels in postmenopausal rats with burn injuries and circadian rhythm disorders. The initial spike in cortisol levels in response to stress and injury is typical, but the hormonal treatment appeared to facilitate a quicker return to baseline levels, indicating a potential regulatory effect on the hypothalamic pituitary adrenal (HPA) axis³³. This regulation may be attributed to estradiol's known effects on reducing stress-induced HPA axis activation and drospirenone's antimineralocorticoid activity, which can counteract cortisol's catabolic effects³⁴. Furthermore, estradiol's role in stabilizing circadian rhythms could contribute to the observed normalization of cortisol levels.

Circadian rhythms are essential for maintaining physiological homeostasis, and disruptions in these rhythms have been implicated in various pathological conditions, including renal dysfunction³⁵. The study by Sun *et al.*³⁶ investigated the interplay between circadian rhythm disorders, inflammatory markers such as Cox-2, Hif-2, and IFN- γ , and renal health, particularly in the context of burn injuries and postmenopausal status. A study by Chen *et al.*³⁷ demonstrated that CLOCK-deficient mice exhibited increased renal fibrosis and oxidative stress following ureteral obstruction. This was associated with heightened expression of Cox-2 and transforming growth factor-beta (TGF- β), suggesting that circadian disruption can exacerbate renal injury through upregulation of inflammatory pathways. In the context of burn injuries, research has shown that such trauma can disrupt circadian rhythms, leading to altered expression of clock-controlled genes in the liver. This disruption may impair metabolic and immune responses, potentially affecting multiple organs, including the kidneys^{38,39}. While direct studies focusing on the combined effects of circadian rhythm disorders, burn injuries, postmenopausal status, and their impact on renal function via Cox-2, Hif-2, and IFN- γ are limited, the existing literature underscores the significance of circadian regulation in inflammatory and oxidative stress responses that are critical to renal health^{40,41}. Further research is warranted to elucidate these complex interactions and their implications for therapeutic interventions.

Overall, our findings suggest that estradiol-drospirenone combination may have potential

therapeutic benefits in mitigating renal damage associated with circadian rhythm disorders and burn injuries in postmenopausal individuals. Further research is warranted to elucidate the underlying of renal complications.

Conclusion

The present study demonstrated that estradiol-drospirenone treatment partially alleviated renal damage induced by burn injury and circadian rhythm disruption in ovariectomized rats. Histopathological evaluations showed mild improvement in tissue integrity, while biochemical and immune histochemical analyses revealed a reduction in oxidative stress and inflammation markers, including Cox-2, Hif-2 α , and IFN- γ . These findings suggest that the observed protective effects may be mediated through modulation of inflammatory and hypoxia-related pathways. However, the treatment did not fully restore normal renal function or completely eliminate tissue damage. Therefore, while estradiol-drospirenone appears to be a promising therapeutic agent for mitigating kidney injury under these complex pathological conditions, further studies involving varying doses and treatment durations are warranted to better understand its full therapeutic potential.

Conflict of interest

The authors have no conflict of interest to declare.

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