



## Ukrainian Journal of Nephrology and Dialysis

Scientific and Practical, Medical Journal

### Founders:

- State Institution «Institute of Nephrology NAMS of Ukraine»
- National Kidney Foundation of Ukraine

ISSN 2304-0238;  
eISSN 2616-7352

Journal homepage: <https://ukrjnd.com.ua>

### Research Article

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doi: 10.31450/ukrjnd.4(64).2019.04

### Evaluation of protective effects of melatonin on free radical metabolism in rat kidney during ischemia-reperfusion

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### Citation:

Nisari M, Yay A, Ertekin T, Nisari M, Al Ö, Ceylan D, Önder GÖ, Kavutcu M. Evaluation of protective effects of melatonin on free radical metabolism in rat kidney during ischemia-reperfusion. Ukr J Nephrol Dial. 2019;4(64):20-29. doi: 10.31450/ukrjnd.4(64).2019.04

### Article history:

Received October 16, 2019

Received in revised form

November 08, 2019

Accepted November 14, 2019

**Abstract.** The purpose of this research was to investigate the possible protective effect of melatonin, as a potent antioxidant on I/R-induced renal injury in rats.

**Methods.** We used 28 female Wistar albino rats weight 200–250g. The rats were randomly divided into 4 groups. Control Group (C): They were fed with only standard rat diet and tap water without drug injections or ischemia-reperfusion. Melatonin Group (M): 25 mg/kg melatonin was administered i.p 30 min. Ischemia/Reperfusion Group (I/R): Rats were subjected to 45 min of renal pedicle occlusion followed by 24 hours reperfusion. Melatonin+ischemia/reperfusion Group (M+I/R): Melatonin (25 mg/kg) was administered 30 min prior to ischemia and immediately before the reperfusion period. Rats were subjected to 45 min of renal pedicle occlusion followed by 24 hours reperfusion.

**Results.** While MDA levels increased in the I/R group, SOD and GST activities were seen to be significantly increased. Although the increase of the SOD activity was observed in the M+I/R group, no meaningful difference was found. MDA levels were significantly decreased in M+I/R group compared to the control group, CAT and GST activities were significantly increased.

**Conclusions.** Our results show that the treatment with M may prevent kidney damage due to ischemia result in increasing oxidant stress peroxidation damages further. Melatonin or its metabolites are capable of neutralizing free radicals and non-radical oxygen-based reactants. This study suggests that melatonin may be an effective antioxidant agent.

**Key words:** rat, melatonin, ischemia, reperfusion.

**Conflict of interest statement:** the authors declared no competing interests.

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УДК: 616.61-008.64:616.61-005.4

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## Оцінка протективного впливу мелатоніну на метаболізм вільних радикалів у нирках щурів в умовах ішемічно-реперфузійного пошкодження

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**Резюме.** Метою роботи було дослідити можливий протективний ефект мелатоніну, як потужного антиоксиданту, на ішемічно-реперфузійне пошкодження нирок у щурів.

**Методи.** 28 щурів лінії Wistar, альбіноси, масою 200-250г були залучені до дослідження. Щури були рандомізовані у 4 групи. Контрольна група (С): щурів годували лише стандартною дієтою та водопровідною водою без ін'єкцій лікарських засобів або ішемії-реперфузії. Група мелатоніну (М): мелатонін вводили у дозі 25 мг/кг протягом 30 хв. Ішемічно-реперфузійна (I/R) групу щурів піддавали 45 хв нирковій оклюзії з наступною 24-годинною реперфузією. Група мелатонін + ішемія/реперфузія (М + I/R): Мелатонін (25 мг/кг) вводили за 30 хв до ішемії та безпосередньо перед періодом реперфузії. Щурів піддавали 45 хв нирковій оклюзії з наступною 24-годинною реперфузією.

**Результати.** Незважаючи на підвищення концентрації МДА у крові щурів I/R групи, ми спостерігали підвищення активності СОД та глутатіон S-трансферази у тканині нирок щурів I/R групи. Хоча у групі М + I/R було також визначено збільшення активності СОД, отримані дані статистично не відрізнялись. Рівень МДА був статистично значущо нижчим у групі М + I/R порівняно з контрольною групою, тоді як активність САТ та глутатіону S-трансферази значно підвищеними.

**Висновки.** Наші результати демонструють, що лікування мелатоніном може запобігти ішемічному ураженню нирок. Мелатонін або його метаболіти здатні нейтралізувати вільні радикали та нерадикальні реагенти на основі кисню. Це дослідження свідчить про те, що мелатонін може бути ефективним антиоксидантним засобом.

**Ключові слова:** щури, мелатонін, ішемія, реперфузія.

**Introduction.** Ischemia is the limitation of the blood supply to tissues causing the absence of oxygen and glucose for cellular metabolism. Ischemia can cause functional and structural cell damage depending on the block of the blood flow [1, 2]. Reperfusion is the rearrangement of the absence of the factors causing ischemic tissue blood flow. Reperfusion ensures the energy needs in ischemic tissue and allows the removal of toxic metabolites. Renal ischemia/reperfusion (I/R) injury occurs in many clinical events, including renal transplantation, aortic aneurysm surgery, partial nephrectomy, cardiopulmonary bypass, renal artery angioplasty, and elective urological operations [3-5]. Ischemia/reperfu-

sion (I/R) injury is a complex event frequently seen in vascular surgery which can cause functional and structural cell damage. The incident happening during I/R have admitted considerable caution over the last twenty years, and different mechanisms have been suggested to clarify the source of tissue injury. Although hypoxia and acidosis are most often included in the damage that occurs during the ischemic period, oxygen-free radicals, endothelial factors and neutrophils are caused to reperfusion damage and occurring the formation of reactive oxygen species (ROS) [6].

Oxidative stress occurs following ischemia-reperfusion. Against endogenous and exogenous oxidative stress in tissues has been antioxidants [7]. Melatonin secretion by the pineal gland and is endogenous antioxidants were discovered in 1958 by Lerner et al. [8].

Melatonin (5-methoxy-N-acetyl-tryptamine) is, mainly secreted from the pineal gland, also from the lens and bone marrow cells, besides this hormone is synthesized and secreted in the gastrointestinal system [7, 9, 10].

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Melatonin is synthesized from tryptophan in the pineal gland, is secreted the dark and is bound to plasma proteins. This hormone is metabolized in the liver [10]. Melatonin, anti-aging is a molecule against chemical carcinogens DNA protection, regulation of endocrine rhythms, to expose some antigonadotropic effect, creating a protective effect on the nervous system, is involved in the regulation of many physiological functions, such as the elimination of the stimulation and free radicals on the immune system. In different organs and tissues, melatonin has been indicated by a direct free radical scavenger and has the antioxidant effect [11, 12]. It is important that melatonin has high lipophilicity to protect oxidative stress [13]. Additionally, melatonin plays an important role in protecting cells against free radical damage and neutrophil-induced toxicity by limiting increased myeloperoxidase activity [14]. It is known that free radicals are protected against kidney I/R damage [15-18].

**The objective** of this study was to confirm the putative protective effect of melatonin against oxidative stress during I/R injury of the kidney. Also in this study, we apply the dose rate is close to the amount of melatonin secreted melatonin in humans (25 mg/day). The results of the literature data demonstrated that this study is one of the rare experiments on female rats.

**Materials and methods.** Animals and study design. In this study, [28] female Wistar albino aged 8-10 weeks and weighing 250-300 g were obtained from Erciyes University experimental and Clinical Research Center (DEKAM). The study was registered at DEKAM with the permission of Erciyes University Experimental Animals Local Ethics Committee, Approval No. 13/133, and dated 13/11/2013.

The rats were randomly divided into four groups; control (C), ischemia/reperfusion (I/R), melatonin (M) and melatonin+ischemia/reperfusion (M+I/R) and each one containing 7 rats. 24 hours later, the rats were killed and the left kidneys were quickly removed, decapsulated and divided longitudinally into two equal sections. One was placed in a 10% formaldehyde solution. For light microscopic evaluation, kidneys were embedded in paraffin wax. About 5  $\mu$ m sections were stained with hematoxylin-eosin (H-E) before investigation under light microscopy and stained with TUNEL. The other section of tissues were stored at -80°C until assayed.

**Surgery and experimental protocol.** Rats weighed between 200-250 g were used in this study, animals in all groups were anesthetized with an intraperitoneal injection of 80 mg/kg ketamine hydrochloride (Ketalar, Pfizer, Groton, CT) and 10 mg/kg xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany). Rats were placed on a thermal blanket to avoid hypothermia, and under the rib of left abdominal skin was shaved and washed with antiseptic. The dorsolateral incision (2-3 cm) was opened by using sterile scalpel and forceps. Then adipose tissue and kidney were taken out together using a sterile scalpel. The left renal hilus was

dissected and the left renal pedicle (artery and vein) was occluded for 45 min to induce ischemia. 10 ml of warm normal saline was instilled into the peritoneal cavity to help maintain fluid balance during ischemia. When the animals were shown signs of emergence from anesthesia during surgery the rats were anesthetized with an intraperitoneal injection of 40 mg/kg ketamine hydrochloride so anesthesia was allowed to continue. At the end of the ischemic period, reperfusion was established by the removal of the clamp. After the start of the reperfusion, the kidney was placed in the abdominal cavity with the adipose tissue. Atraumatic needle (6-0 number Vicryl) was used to sew of the subcutaneous connective tissue and muscle layers. Then the suture line was washed with antiseptic.

**Experimental groups.** The control group (C: n=7): Animals were not administered melatonin by i.p. injection or ischemia/reperfusion. Ischemia/reperfusion group (I/R: n=7): Animals were not administered melatonin by i.p. injection, but left renal vessels were occluded for 45 min, followed by 24 h reperfusion.

**Melatonin group (M: n=7):** Melatonin (25 mg/kg) were administered by i.p. injection and do, not ischemia/reperfusion. **Melatonin+I/R group (M+I/R: n=7):** in all of the animals, melatonin (25 mg/kg) was administered by i.p. injection 1 h before ischemia then left renal vessels were occluded for 45 min, followed by 24 h reperfusion.

Rats were put at an average ambient temperature of 27°C under a 12 h/12 h light/dark cycle and were fed on a standard rodent diet and filtered tap water.

**Biochemical analysis.** First, the kidney tissues were washed with cold NaCl solution (0.154M) to remove blood contamination and then homogenized in the homogenizer. The preparation procedure was ground in the tissue glass mixer using a rotor formed by a simple electric motor. The homogenization as a tissue blender similar to the typical kitchen blender is used to emulsify and pulverize the tissue (DiAx 900; Heidolph Instruments GmbH&Co KG, Schwabach, Germany) at 1000 U for about 3 min. After centrifugation at 10,000g for about 60 min, the upper clear layer was taken.

**Measurements of superoxide dismutase (SOD).** Superoxide dismutase activity was measured according to the method described by Durak et al [19]. A portion of the homogenate was extracted in ethanol/chloroform mixture (5/3 v/v) to discard the lipid fraction in the activity measurements of total superoxide dismutase. After centrifugation at 10,000g for 60 min, the upper clear layer was removed and used for the analyses. The SOD activity method is based on the measurement of absorbance increase at 560 nm due to the reduction of NBT to NBTH2. One unit of SOD activity was defined as the enzyme protein amount causing 50% inhibition in the NBTH2 reduction rate and the results were recorded in U/mg protein.

**Measurements of catalase activities (CAT).** In the upper clear layer, CAT enzyme activity was measured using the methods described by Aebi et al [20]. The

CAT enzyme activity assay method is based on the measurement of absorbance decrease due to  $H_2O_2$  consumption at 240 nm. The activities of CAT were recorded in IU/mg protein.

Measurements of glutathione S-transferase activities (GST). GST enzyme activity was measured using the method described by Habig et al [21]. The GST activity method is based on the measurement of absorbance increase at 340 nm due to the reduction of the DNPG. The results were expressed in IU/mg protein. The measurements of activity were made using the value of  $\epsilon$  of the DNPG complex ( $\epsilon:10\text{ M}\cdot\text{l}\cdot\text{cm}^{-1}$ ).

Measurements of malondialdehyde levels (MDA). According to the method defined by Van Ye et al [22], MDA levels are based on the reaction of MDA with thiobarbituric acid (TBA). In the TBA test reaction, MDA and TBA react in acid pH to form a pink pigment with an absorption maximum at 532 nm. Arbitrary values obtained were compared with a series of standard solutions (1,1,3,3-tetra ethoxy propane). Results were expressed as nmol/L. During the experimental period, all procedures were performed at 4 + C.

Histopathologic evaluation. For histological examination, routine paraffin wax embedding procedures were used. The kidney tissues were removed, fixed in 10% formalin and processed by routine histological methods and embedded in paraffin blocks. 5  $\mu\text{m}$  thick paraffin sections were cut from each specimen and were put on poly-L-lysine slides. All sections were stained with hematoxylin-eosin (H&E) and PAS staining to evaluate a morphological overview of the tissue and its structure. The images were captured using an Olympus BX51 microscope and analyzed.

TUNEL immunofluorescence staining. To detect apoptosis within the cells of the kidneys, in situ TdT mediated X-dUTP nicked labeling (TUNEL) reaction to the paraffin sections were applied by using

ApopTag® Fluorescein In Situ Apoptosis Detection Kit (EMD Millipore, Darmstadt, Germany) in accordance with the manufacturer's recommendations. Briefly, serial 5  $\mu\text{m}$  thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol and incubated 5 min in phosphate-buffered saline (PBS) at room temperature. Slides were incubated 15 min with proteinase K and washed with distilled water. After the wash, several times in PBS pre-treated with 3% hydrogen peroxide for 10 min. The specimens were incubated with fluorescein-labeled deoxy-UTP at 37°C for 1 hour at a humidity ambient. The nucleus was visualized with 4, 6-diamino-2-phenylindole (DAPI). The images were taken randomly for evaluating the TUNEL-positive cells by using an immunofluorescence microscope (Olympus BX51, Tokyo, Japan).

For quantitative analysis, ten visual fields were randomly photographed for each TUNEL stained section from each experimental group under a microscope at x400 magnification. The number of TUNEL-positive cells nuclei (apoptotic nuclei) was counted with Image J software.

Statistical analyses. The Statistical Package for the Social Sciences (SPSS, IBM) 21.0 program was used for statistical analysis. Biochemical parameters between study groups were assessed by using a one-way analysis of variance test (ANOVA) followed by Student-Newman-Keuls multiple comparison tests. The normal distribution of histopathologic parameters was assessed using the Shapiro-Wilk test. Variable of apoptosis parameters were assessed using Kruskal-Wallis analysis followed by Dunn multiple comparison tests.

The results were expressed as mean standard deviation, and statistical significance was set at  $p < 0.05$  for all analyses.

**Results.** The results of these studies are shown in Table 1 and Figure 1.

Table 1

**SOD, CAT and GST activities and MDA levels (Mean  $\pm$  SD) in rat the kidney tissue**

Groups	SOD (U/mg pr)	CAT (IU/mg pr)	GST (IU/mg pr)	MDA ( $\mu\text{mol/mg pr}$ )
Control (C)	11.2 $\pm$ 0.36	20.4 $\pm$ 6.33	2.16 $\pm$ 0.41	3.1 $\pm$ 0.12
Ischemia/reperfusion (I/R)	14.3 $\pm$ 0.99	23.8 $\pm$ 10.57	4.23 $\pm$ 0.98	4.5 $\pm$ 0.23
Melatonin (M)	9.89 $\pm$ 0.29	19.9 $\pm$ 3.61	1.48 $\pm$ 0.43	2.1 $\pm$ 0.03
Melatonin+I/R (M+I/R)	13.6 $\pm$ 0.36	24.3 $\pm$ 11.31	3.37 $\pm$ 0.36	1.9 $\pm$ 0.25
Statistical evaluation (ANOVA and Student-Newman-Keuls test)				
Groups	SOD	CAT	GST	MDA
C-I/R	$P < 0.05 \uparrow$	$p > 0.05$	$P < 0.05 \uparrow$	$p > 0.05$
C-M	$P < 0.05 \downarrow$	$p > 0.05$	$P < 0.05 \downarrow$	$P < 0.05 \downarrow$
C-(M+I/R)	$p > 0.05$	$P < 0.05 \uparrow$	$P < 0.05 \uparrow$	$P < 0.05 \downarrow$
I/R-M	$P < 0.05 \downarrow$	$P < 0.05 \downarrow$	$P < 0.05 \downarrow$	$P < 0.05 \downarrow$
I/R-(M+I/R)	$p > 0.05$	$p > 0.05$	$p > 0.05$	$P < 0.05 \downarrow$
M-(M+I/R)	$P < 0.05 \uparrow$	$P < 0.05 \uparrow$	$P < 0.05 \uparrow$	$p > 0.05$

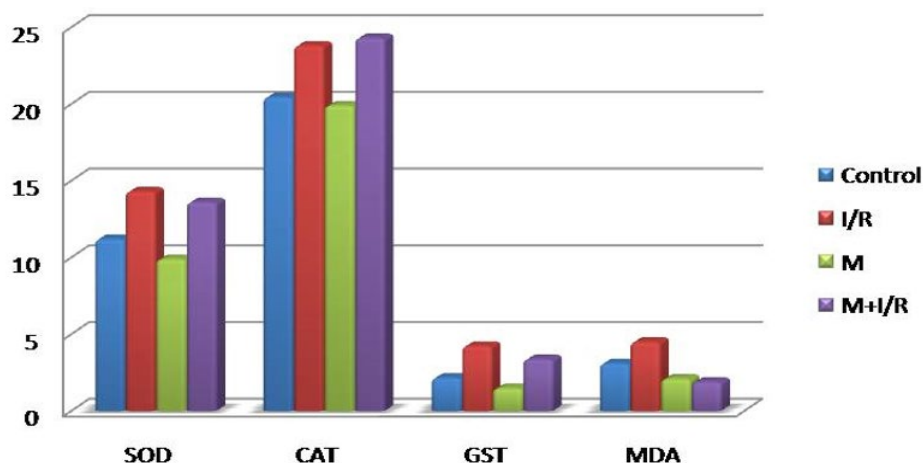


Fig. 1. Control, I/R, M and M+I/R of enzyme activities in rat kidney tissues.

In M group SOD activity was significantly decreased by control while in I/R group it was significantly increased by control ( $p < 0.05$ ). When the groups were compared in terms of CAT enzyme activity, a significant difference was observed.

CAT enzyme activity was significantly higher in the M+I/R group ( $p < 0.05$ ), no statistically significant change was observed in the other groups ( $p > 0.05$ ).

GST enzyme activity was significantly higher in I/R and M+I/R groups compared to group C, the GST activity in group M was significantly lower compared to group C ( $p < 0.05$ ). In addition, the GST in group M was significantly decreased compared to groups I/R and I/R-M.

MDA level was higher in the I/R group compared to the C group ( $p < 0.05$ ). Besides, its level was lower in M+I/R group compared to the group M ( $p > 0.05$ ).

**Histologic findings.** We examined the role of melatonin in IR-induced renal and tubular damages. In order to investigate the overall structure of kidney tissues, we assessed the histologic sections stained with hematoxylin-eosin. The control and melatonin groups had normal architecture. There was no considerable pathologic alteration in these groups. The histological examination of kidneys obtained from the I/R group had severe renal damage, which included tubular epithelial necrosis of the proximal convoluted tubule, congestion surrounding the tubules and vacuolation involving approximately all tissue fields. The renal histology stained with PAS especially was a significant loss of brush border in epithelial cells of proximal tubules. Tubular injury in M+I/R had a little histological change (Figures 2 and 3).

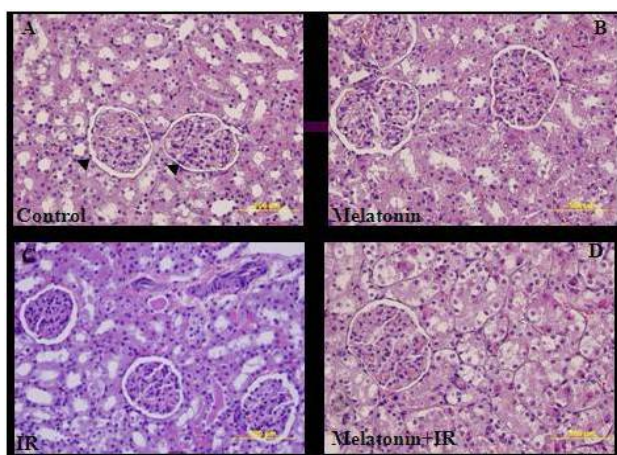


Fig. 2. Light microscopic features of the rat's kidney sections from each group (H&E). (A) The control kidneys had normal architecture. (B) The kidneys in melatonin-treated and non-ischemic groups did not show histological abnormalities (C) The kidney after IR damage showed epithelial necrosis, vacuolization, congestion both interstitial connective tissue and surrounding the tubules (arrows). (D) Effects of melatonin on rat renal IR injury. The melatonin-treated IR injury had less histological abnormalities compared with that observed in kidneys from IR rats (H&E; magnification, x400).

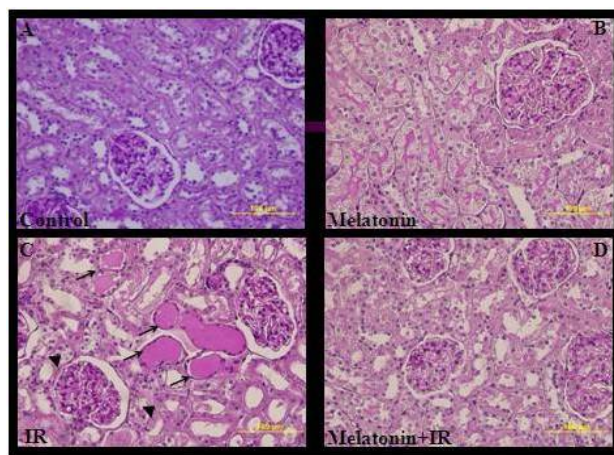


Fig. 3. The Photomicrograph shows PAS-stained sections (A) The proximal tubules have a brush border, whereas the distal tubules have a cleaner, sharper luminal surface. Glomerular basement membrane and brush border of proximal tubule epithelial cells very clear with PAS staining. (B) The kidney of non-ischemic melatonin-treated had nearly the same architecture compared with the control group. (C) Kidney sections obtained from the IR group. Severe damage can be seen as a loss of brush border and cell damage. (D) Tubular injury in melatonin-treated IR injury rats shows decreased/moderate damage. (PAS; magnification, x400).

Melatonin downregulates apoptotic cells. We investigated the influence of melatonin on the I/R of the kidney in rats. The TUNEL method was used to evaluate apoptotic cell count (Figures 4 and 5).

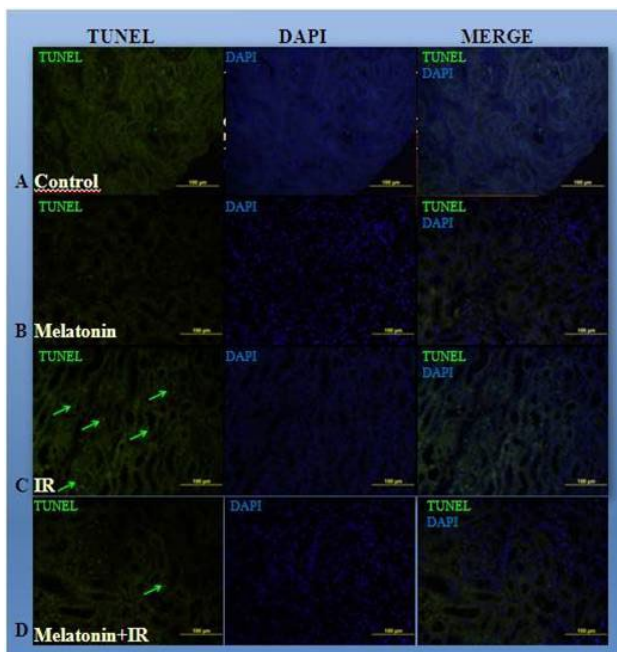


Fig. 4. UNEL+ cells reflective green immunofluorescence. (A) There were only a few TUNEL+ cells in the kidney of the control group. (B) The melatonin-treated group had also less TUNEL+ cells like the control group. (C) It was determined that many apoptotic cells in the tubules both cortex and medulla of IR group. (D) Effects of melatonin on TUNEL+ cells in renal ischemia-reperfusion injury in rats. There was TUNEL+apoptotic cells decreased on section profiles of this group compared with I/R group.

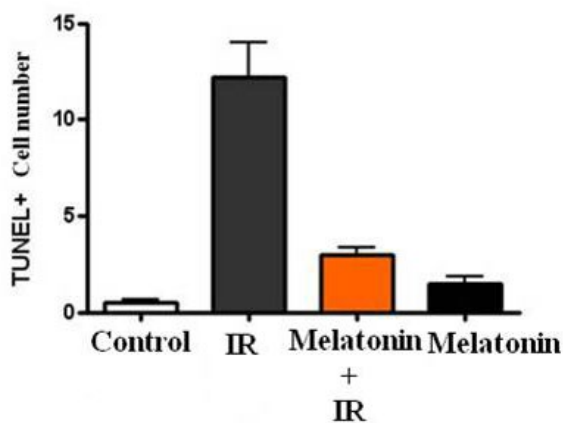


Fig. 5. Graph showing quantification of experimental groups. TUNEL+cell number in kidney tissue of the I/R group was higher compared to the control and other groups.

The number of TUNEL positive cells increased in the I/R compared to the control group. TUNEL positive cell number decreased in M+I/R. But these apoptotic cell number downregulated in the group I/R. Melatonin significantly reduced the renal apoptosis in I/R rats. These groups were indicated that melatonin may protect kidney tissue from apoptosis.

**Discussion.** The most important reason for hypoxia has been ischemia because of arterial or venous blood flow disorders in organ and tissue leading to insufficient perfusion. It is the restoration of blood flow to an organ or tissue. It leads to damage as well as ischemia. caused by the return of blood to the tissue after an ischemia or oxygen deficiency period. The absence of oxygen and nutrients from blood in the course of the ischemic period forms a condition in which the renewal of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than a renewal of normal function. Occurring rapidly with the introduction of free oxygen radicals and derivatives of molecular oxygen into the cell is one of the reasons why reperfusion injury [23].

The most common cause of acute renal failure is renal ischemia, which causes renal functional degradation through a combination of renal vasoconstriction, renal tubular obstruction, tubular back leakage of glomerular filtrate, and decreased glomerular permeability. It's reported that reactive oxygen species (ROS: O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and OH•) play a crucial role in the pathophysiology of various disease states such as ischemia-reperfusion injury in the last decade.

When a tissue/organ is damaged it produces excessive amounts of superoxide (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). During ischemia and reperfusion overproduction of ROS cause oxidative stress that results in changes in mitochondrial oxidative phosphorylation, depletion of adenosine triphosphate (ATP), an increase in intracellular calcium, and activation of proteases and phosphatases leading to the breakdown of membrane phospholipids and cellular cytoskeleton and loss of cellular integrity [24, 25].

The decrease in renal blood flow and following reperfusion have occurred forms of tissue damage at diverse degrees. SOR plays an important role in I/R injury after renal ischemia [25]. Moreover, it is described that lipid peroxidation levels are increased due to the increase in SOR after reperfusion [26].

Cells have mechanisms to reduce in part or prevent oxidative damage. It is announced that to have become more sensitive with the formation of the tissue damage of ischemic tissue, depending on the oxidant decreases the amount of glutathione during ischemia and accelerated inactivation of enzymes such as CAT, SOD, and GPx and the cells are impacted of rapidly occurring oxygen radicals during reperfusion [27].

Melatonin is a methoxyindole derivative produced by different tissues and organs in mammals such as the retina, bone marrow, gastrointestinal system, kidneys, and skin but predominantly by the pineal gland. Melatonin, which is secreted at the highest level in mammals at night, is considered as a strong antioxidant and the main source of melatonin in the blood is pinealocytes in the central nervous system [28]. Many of its metabolites, as well as melatonin, have proven to be effective antioxidants because their antioxidative properties are synergized with metabolites of free radical scavengers and also act as an effective radical scavenger [29].

Melatonin has been used as an antioxidant for many years and several of its metabolites are also able to protect cells from oxidative damage caused by reactive species and it amplifies the antioxidant capacity of melatonin [30].

In a study in the blood samples and kidney tissues of the rats, Aktöz et al. [31] showed that I/R significantly increased urea, creatinine, and MDA levels, and decreased SOD and CAT activities. It may be due to the response of the kidney tissue to the antioxidant defenses system. In our study histopathological findings of the I/R group confirmed that there was renal impairment by cast formation and tubular necrosis in the tubular epithelium. In the IR+Melatonin group, while MDA levels significantly decreased, SOD activities increased. In the IR+Melatonin group, the level of tubular necrosis and cast formation are significantly decreased than those seen in the I/R group. These results may indicate that melatonin pretreatment protects against biochemical, and morphological damage in renal I/R injury. Our results show that in melatonin-treated groups SOD activity, which was significantly decreased by control while I/R groups were significantly increased by control. While activity was significantly higher in the M+I/R group compared with the C group was not statistically significant. In addition, the I/R group compared to the decrease in M group was statistically significant while the I/R group compared to the decrease in M+I/R group was found to be not statistically significant. When the groups were compared in terms of CAT enzyme activity, a significant difference was observed. While CAT enzyme activity was significantly higher in the M+I/R group compared with the C group no statistically significant change was observed. When groups were compared in terms of kidney tissue MDA levels, while the MDA level was significantly higher in the I/R group compared with the C was not found to be statistically significant. Whereas the MDA level was significantly lower in the M and M+I/R group compared with the C group was found to be statistically significant ( $p < 0.05$ ). In addition, while the M+I/R group was higher compared with the M was not found to be statistically significant. The differences between the biochemical parameters may depend on the duration of the effect of melatonin, dosage of melatonin and I/R time.

Hagar et al. [32] induced renal ischemia for 45 min during which time they performed a right nephrectomy, and allowed animals to reperfuse for 24 h. Induction of renal ischemia-reperfusion resulted in renal dysfunction, as indicated by elevated levels of blood urea nitrogen and serum creatinine. In addition, the renal MDA level was increased. In our study, when groups were compared in terms of MDA levels, a significant difference was observed; the MDA level was higher in the I/R group than in the C group. Lipid peroxidation, as a free radical generating system, has been proposed to be closely related to IR induced tissue injury and MDA is a good indicator of the degree of lipid peroxidation. The levels of MDA are significantly increased by IR, which

reflects increased lipid peroxidation due to increased oxidative stress.

Korkmaz and Kolankaya [33] induced renal ischemia for 45 min during which time they performed a right nephrectomy, and allowed animals to reperfuse for 3 h. In the present experiment, the levels of MDA are significantly increased and GSH activities are significantly decreased. In our study, when groups were compared in terms of kidney tissue MDA levels, the MDA level was significantly lower in the M and M+I/R group compared with the I/R group ( $p < 0.05$ ). GSH enzyme activity was not evaluated in our research.

Şener et al. [34] reported that local treatment with melatonin inhibited the increase in malondialdehyde levels after 2 and 0.5 hr I/R respectively, followed by an additional 12 hr of ischemia. This protocol was been repeated for 3 days by Şener et al. Then in treatment groups, twice a day during reperfusion periods, melatonin was applied 5 mg per rat. Consistent with our findings, the antioxidant effect of the application of melatonin may protect against oxidation of the kidney.

Sinanoglu O et al. [35] were evaluated the preventive role of M and 1,25-dihydroxyvitamin D3 (VD3) in biochemical and apoptotic events leading to tissue injury and renal dysfunction after I/R. According to the studies, kidneys and blood were obtained for histopathological and biochemical evaluation after reperfusion. M and VD3 had an ameliorative effect on biochemical parameters such as blood urea nitrogen, serum creatinine, alanine aminotransferase, aspartate aminotransferase, and apoptosis in the kidneys against renal I/R injury in rats. Additionally, VD3 combined with M significantly reduced apoptotic and histological alterations when compared with M or VD3 alone. This preventive effect on renal tubular apoptosis was remarkable when M was combined with VD3. In our study, the number of TUNEL-positive cells more increased in the I/R group compared to the C group. In the M group, TUNEL positive cell number was nearly the same with the C group. When groups were compared as statistical analyses, were not differences between C and M groups. In addition, we were observed that there was a significant difference between I/R and C, between I/R and M+I/R and between I/R and M groups ( $p < 0.05$ ).

Ahmadiasl et al. [36] described 45 min of renal pedicle occlusion followed by 24 hr reperfusion. M (10 mg/kg, IP) and erythropoietin (EP) (5000 U/kg, IP) were administered prior to ischemia. They have found that I/R decreased the tissue levels of SOD and CAT. Consistent with this finding, I/R and similar oxidative stress state, ROS accumulation, decrease in antioxidant enzyme activities, and expression, or a combination of both, cause deep injury to cellular components such as proteins, lipids, and DNA. Also, they have found that the levels of CAT and SOD increased by EPO + M which indicates that EPO and M combination treatment stimulates antioxidative enzymes, however, this stimulation was not significant when compared with other treatment groups. We have found that in I/R

groups both SOD and CAT activities, which were significantly increased compared to control. While SOD activity was statistically significant CAT activity was not statistically significant. This difference is that melatonin (25 mg/kg, i.p) was administered 30 min before subjecting rats to I/R and was high dose melatonin. The animals are exposed to short-term and a high dose of melatonin is made acute administration.

Sehajpal et al. [37] described to 40 min of renal pedicle occlusion followed by 24 hr reperfusion. M (4 mg/kg, i.p) was administered 30 min before subjecting rats to I/R. They reported that a significant reduction in renal GST level was observed in I/R group compared with the control group (without M).

Yip et al tested whether combined melatonin (M) and exendin-4 (Ex4) treatment can better preserve glomerular structural integrity after IR injury compared with either alone [38]. They used adult male Sprague Dawley rats (n = 50) were equally divided into sham control, IR, IR-Ex4 (10 µg/kg s.c 30 min after reperfusion and daily for 5 days), I/R+Mel (20 mg/kg i.p. at 30 min postreperfusion) and IR-Ex4-M were euthanized at day 14. Changes in podocyte injury score (PIS) and kidney injury score were highest in I/R group and lowest in sham operated control SC, significantly higher in I/R-Ex4 and IR-Mel groups than in I/R-Ex4-M, and significantly higher in I/R+M group than in I/R-Ex4 group. Immunohistochemical microscopic findings of FSP-1 and WT-1 (two glomerular damage markers), and KIM-1, and snail (two renal tubular damage markers) showed identical patterns, while ZO-1, p-cadherin expressions showed podocin, dystroglycan, fibronectin, and synaptopodin (six glomerular integrity index) showed an inverse pattern in comparison to podocyte injury score PIS among the five groups. They found that combined melatonin–exendin-4 therapy further protected glomerulus from IR injury [38].

Ma et al. first clarified the mechanisms underlying mitochondrial dysfunction during IR and melatonin's protection of mitochondria under this condition. Thereafter, a special focus is placed on the protective actions of melatonin against IR injury in the brain, heart, liver, and others. They reported that increase the potential of melatonin as a therapeutic agent in the future [39].

Panah et. al. concentrated on determining the anti-inflammatory and anti-oxidative effects of melatonin on the complications of I/R. This study was demonstrated that reduction in serum levels of renal function and oxidative stress/inflammatory markers in the melatonin group indicates that melatonin can inhibit I/R outcomes in RTPs through its anti-oxidant and anti-inflammatory properties [40].

This case is the result of oxidative stress after ischemia which is used melatonin the short-term against damaged kidney tissue is thought that is depended on acute injury.

Histopathological changes are confirmed that melatonin has protective effects on IR-induced renal injury. These differences may depend on using a female animal, ischemia/reperfusion period, melatonin doses and the time of administration of melatonin before ischemia.

**In conclusion**, it was shown that the protective effect of melatonin on I/R induced renal injury, mechanism of apoptosis effects and the importance of oxidative stress in apoptosis is related to its antioxidant properties. In the present day, showing the roles of free radicals effects on aging and on various pathologies, especially cancer, cardiovascular and neurodegenerative diseases that brought up the use of antioxidants has accelerated steadily working on this issue. Melatonin is still keeping primacy during comparison with other antioxidants, both it has free radical scavenging ability and wherein increasing antioxidant enzyme activities.

**Conflict of interest statement:** the authors declare no conflict of interest.

**Ethical approval:** all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Author contributions.**

**M. Nisari:** management of the research, collected, analyzed, interpreted the biochemical data and wrote the manuscript.

**A. Yay:** applied the hematoxylin-eosin and tunnel staining procedure, interpreted histological data.

**T. Ertekin:** Ischemia-reperfusion application and interpreted data.

**M. Nisari:** Ischemia-reperfusion application and interpreted data.

**Ö. Al:** Ischemia-reperfusion application and surgery procedure.

**D. Ceylan:** applied the biochemical analysis procedure.

**G. Ö. Önder:** applied the hematoxylin-eosin and tunnel staining procedure

**M. Kavutcu:** idea of the research, applied the biochemical analysis procedure and interpreted biochemical data.

All authors read and approved the final manuscript.

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