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Original Article: Laboratory Investigation

Protective effects of melatonin on renal failure in pinealectomized rats

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Aim: The purpose of the current study was to investigate the effects melatonin on renal function.
Methods: The histological appearance of the kidney and malondialdehyde, nitric oxide, glutathione and superoxide dismutase contents were determined. Serum creatinine and blood urea nitrogen levels were also assayed. Rats were divided as: Sham, pinealectomized (Px) and pinealectomized and treated with melatonin.
Results: In Px group, malondialdehyde and nitric oxide levels were elevated when compared with the sham group. The Px group exhibited reduced superoxide dismutase activity and glutathione content. All of these harmful changes were restored by melatonin. Melatonin also ameliorated serum creatinine and blood urea nitrogen levels related to renal injury. The score for glomerular, tubular and interstitial changes was significantly higher in the Px group. Melatonin supplementation significantly reduced these parameters.
Conclusions: This protective effect may be associated with both melatonin's lipophilic and hydrophilic effects, thus providing on-site protection against free radical mediated damage.

Key words: aging, kidney, melatonin, pineal gland, rat, reactive oxygen radical.

Introduction

Melatonin plays an important role in various physiological processes, including the regulation of circadian and endocrine rhythms; aging; the stimulation of immune functions; and the prevention of the adverse effects of antibiotics, including renal failure.[1] Melatonin exhibits a diurnal variation and its level declines with advanced age.[2] However, the exact levels required for the prevention of oxidative damage (which also accumulates with advanced age) remain unknown.[3] Despite the analysis of the extra-pineal generation of melatonin, it has been shown that even the amounts of melatonin secreted by the pineal gland contribute to free radical protection. Thus, the removal of this source of melatonin by surgical pinealectomy has repeatedly been shown to exaggerate the amount of molecular destruction resulting from high free radical states.[4] Several conditions are known to disturb the balance between the production of reactive oxygen species (ROS) and cellular defense mechanisms resulting in dysfunction and cellular destruction. An imbalance between pro-oxidant and antioxidant factors plays an important role in many disease processes.[5] In the free radical theory of aging, it is suggested that accumulated free radical damage may be responsible for the degenerative process during aging.[6] It is known that there is a concentration of reduction serum melatonin during aging. This situation may cause damage in the kidney which is one of the more perfusing organs. The mechanism(s) underlying renal failure during aging is thought to be sophisticated, and related to hypoperfusion, loss of pulsatile perfusion, accumulation of free radicals, and a systemic inflammatory response. The pineal secretory product, melatonin, is known to exhibit a free radical scavenging ability and reduces neutrophil accumulation.[7] Melatonin also activates several antioxidative enzymes (including superoxide dismutase [SOD]), modulates gene expression for several protective enzymes and reduces lipid peroxidation.[8]

In elderly people, the pineal gland wrinkles and loses its melatonin synthesis ability. The aim of the present study was to expose rats to an aging process via surgical pinealectomy. For this, the effects of lack of chronic physiological melatonin on kidney tissue were observed in the current study. Changes in oxidant substances were evaluated, especially those in lipid peroxidation, nitric oxide (NO), reduced glutathione (GSH) content and SOD activity levels during this period so that the antioxidant effects of long-term exogenous melatonin could be investigated. Also the mean dimension and the number of glomeruli and the score for histopathological alterations were determined by light microscope.

Methods

Experimental groups

Eighteen male Wistar rats weighing 150–200 g were placed in a constant temperature (21 ± 2°C) and humidity (60 ± 5%) controlled room in which a 12:12 h light dark cycle was maintained. Animals were placed in cages two by two.

The animals were divided into three groups: Group I ($n = 6$) and group II ($n = 6$) were designated as sham-pinealectomized and pinealectomized (Px) rats, respectively. They received 1% ethanol (0.5 mL i.p.) alone. Rats in group III ($n = 6$) were pinealectomized and melatonin (Px + Mel) and were injected with 4 mg/kg/day (i.p.) for 28 days. All injections were administered at 17:00 h. Animals from the sham-operated and Px groups received an equal volume of (0.5 mL) of vehicle solution i.p.

Px animals were randomly allocated to vehicle or melatonin treatment groups. Px or sham-operated rats were housed for 5 months

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before the beginning of treatment. Melatonin (Sigma Chemical Co., St Louis, MO, USA) was dissolved in ethanol and further diluted in saline (vehicle) (0.09%NaCl, w/v) to give a final concentration of 1%. After the last injection, all rats were weighed before killing with a high dose of anesthetic agent and the kidneys were quickly removed and weighed, encapsulated and divided equally into two longitudinal sections. One of them was placed in formaldehyde solution for routine histopathological examination by light microscopy. The other half was placed in liquid nitrogen and stored at −85°C until assayed for malondialdehyde (MDA), NO, GSH content and SOD activity. Trunk blood was extracted to determine the serum levels of creatinine (Cr) and blood urea nitrogen (BUN). The experiments were performed in accordance with the guidelines for animal research from the National Institute of Health and were approved by the Committee on animal Research at Inonu University, Malatya.

Pinealectomy

The animals were anesthetized i.p. with 8 mg/kg xylazine (Rompun) in combination with 75 mg/kg ketamine (Ketalar) before the operation. Pinealectomy was performed as described by Hoffman and Reiter.[9] The skin was prepared for the surgery by povidone iodine solution (Betadine, Kim-Pa Corporation, Istanbul, Turkey) and the skin on the top of the head was cut to expose the skull.

The animal was fastened to the dissection table; an incision was made in the skin and the subcutaneous tissue, bringing the lambda into view. The skullcap was opened with the aid of a micromotor, bringing the cerebral hemispheres and the superior sagittal sinus into view. The pineal gland, located under the venous sinus, was removed in one piece using forceps. Next, the bone fragment was returned to its place and the surgical layers were sutured. After surgery, the animals received a single dose of prophylactic antibiotic. The procedure was completed within 15 min. The pinealectomy was confirmed by the histological evaluation of the gland for each animal. Rats in the sham-operated group underwent similar surgical procedures without the removal of the pineal gland.

Biochemical analyses

The MDA content of homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances.[10] Results are expressed as nmol/g tissue.

Since total nitrite was considered as an index of NO production,[11] NO levels were measured as total nitrite. The kidney tissues were homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.5) (containing protease inhibitor, phenylmethylsulfonyl fluoride, PMSF, 1 mM) with a homogenizer (IKA Ultra Turrax T25 basic) at 16.000 r.p.m. for 3 min at +4°C. The homogenate was centrifuged at 10.000 × g for 20 min at + 4°C and the obtained supernatant was used to measure the kidney level of NO. Firstly, all nitrate molecules in supernatant were reduced to nitrite by nitrate reductase, and total nitrite levels were measured by the Greiss reaction described before[12] with minor modifications. Total nitrite levels in the kidney were expressed as nmol/g wet tissue.

GSH, an index of cellular defense mechanisms,[13] was determined by the spectrophotometric method, which was based on the use of Elman's reagent.[14] Results are expressed as nmol/mg tissue.

The SOD enzyme activity determination was based on the production of H_2O_2 from xanthine by xanthine oxidase and the reduction of nitroblue tetrazolium as previously described.[15] The product was evaluated spectrophotometrically. Results are expressed as U/g protein.

Serum levels of Cr and BUN were determined using the Olympus Autoanalyzer (Olympus Instruments, Tokyo, Japan).

Histological determination

For the light microscopic examination, kidney tissue was cut into the small pieces. The samples were fixed in 10% buffered formalin and prepared for routine paraffin embedding. Sections of tissues were cut at 5 μm, mounted on slides, and stained with hematoxylin-eosin (H-E), Masson's trichromic and periodic acid Schiff (PAS) and examined by a Lyca DFC280 light microscope and Leica Q Win and Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, U.K). The mean dimension of the glomeruli was semi quantitatively determined by measuring the dimension of a minimum of 100 glomeruli per section. A minimum of 20 fields at 20× magnification were assessed for calculating the mean number of glomeruli. Kidney damage was scored by grading glomerular, tubular and interstitial changes. Glomerular damage (sclerotic changes such as matrix expansion, the narrowing or disappearance of the Bowman's space, the adhesion of the capillary tuft to the Bowman's capsule, the capillary collapse and the thickening of the glomerular basement membrane) was evaluated as: 0, absent; 1, <25% of glomeruli affected; 2, 25–50% glomeruli affected; 3, >50% of glomeruli affected. The grading for tubular changes (intracellular vacuolization) was scaled as: 0, absent; 1, <25% of tubules injured; 2, 25–50% of tubules injured; 3, >50% of tubules injured. The presence of interstitial inflammation was judged as: 0, absent; 1, mild; 2, moderate; and 3, severe.

Statistical analysis

The results are given as mean ± standard error (S.E) in the text. Normality of the distribution was confirmed using the Kolmogorov-Smirnov *Z*-test. The homogeneity of variance assumption was examined using Levene's test. Kidney MDA, NO, GSH, SOD, BUN, and Cr levels were analysed by the one-way analysis of variance (ANOVA) test. Post-hoc comparisons were performed using Tukey's test. The Mann–Whitney *U*-test was used to compare the histopathological data between the groups. The differences were considered significant when $P < 0.05$. Statistical analyses were performed using The Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) (Ver.10.0).

Results

Biochemical results

Table 1 presents the tissue levels of MDA, NO, GSH, SOD, BUN and Cr levels. Briefly, pinealectomy caused a significant increase in MDA, the end product of lipid peroxidation (516.28 ± 18.02 vs 359.42 ± 8.88) and NO (361.71 ± 14.17 vs 245.71 ± 8.13) productions, and a decrease in GSH contents (77 ± 29.93 vs 543.85 ± 71.76) and SOD (249.57 ± 30.59 vs 631.85 ± 101.93, respectively) activities (when compared with the sham-operated group) in the rats kidneys. Melatonin given to Px rats significantly decreased MDA (376 ± 7.98), NO (253.71 ± 13.11) production and elevated GSH (344.28 ± 42.28) content and SOD (519.71 ± 34.11) activity in the kidney tissue.

Serum levels of Cr and BUN were significantly higher in the Px-operated animals, when compared to the sham group. Pre-treatment of the animals with melatonin significantly reduced the high level of serum Cr and BUN levels (Table 1).

There were no statistical changes either in the rats' weight or the kidneys' weight when compared with each other (data were not shown).

Table 1 The effects of long-term pinealectomy (Px) with or without melatonin (Mel) treatment in rats

Parameters	Sham-operated	Px	Px + Mel
MDA (nmol/g tissue)	359.42 ± 8.88	516.28 ± 18.02*	376.0 ± 7.98**
NO (nmol/g tissue)	245.71 ± 8.13	361.71 ± 14.17*	253.71 ± 13.11**
SOD (mU/mg protein)	631.85 ± 101.93	249.57 ± 30.59*	519.71 ± 34.1**
GSH (nmol/g tissue)	543.85 ± 71.76	77.0 ± 29.93*	344.28 ± 42.28**
BUN (mg/dL)	14.12 ± 0.58	35.91 ± 1*	18.14 ± 0.45**
Cr (mg/dL)	0.44 ± 0.01	0.98 ± 0.02*	0.51 ± 0.02**

*$P < 0.05$ *vs* Sham-operated group; **$P < 0.05$ *vs* Px group. Values are expressed as mean ± Standard Error.
MDA, malondialdehyde; NO, Nitric Oxide; SOD, superoxide dismutase; GSH, glutathione; BUN, blood urea nitrogen; Cr, creatinine.

Table 2 The demonstration of the mean number of the glomeruli in 20 fields per section and the mean dimension of a minimum of 100 glomeruli per section and the score for histopathological alterations

Groups	Number of the glomeruli	Glomerular dimension	Histopathological score
Sham	10.82 ± 0.36	91.65 ± 0.33	0.00 ± 0.00
Px	13.41 ± 1.39	90.27 ± 1.97	5.62 ± 0.56*
Px + Mel	14.77 ± 0.82	98.11 ± 6.14**	1.37 ± 0.32***

*$P < 0.05$ *vs* Sham group; **$P < 0.05$ *vs* Px; ***$P < 0.001$ *vs* Px. Values are expressed as mean ± Standart Error.
Px, pinealectomized; Px + Mel, Pinealectomized and treated with melatonin.

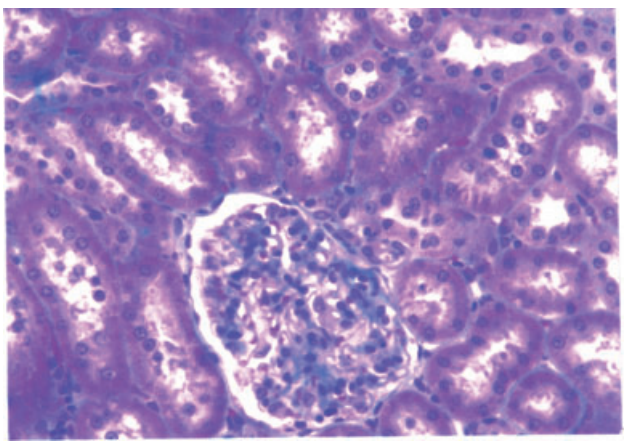

Fig. 1 Control; The glomerulus and tubules are normal in histological appearance. Masson's trichrome; X 40.

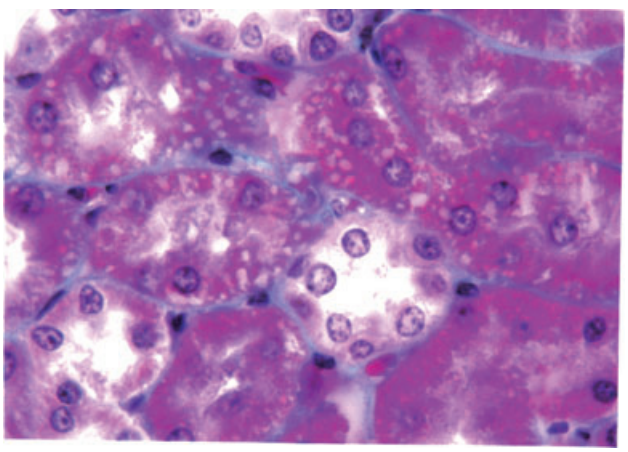

Fig. 2 Pinealectomized (Px) group; Severe proximal tubular vacuolization is seen. Masson's trichrome; X 100.

Histological results

The mean dimension, the number of the glomeruli and the score for histopathological alterations are expressed in Table 2.

The score for glomerular, tubular and interstitial changes was significantly higher in the Px group than the Px + Mel group ($P < 0.05$). Melatonin supplementation significantly reduced these parameters ($P < 0.001$). The sham group revealed normal kidney histology (Fig. 1). The most prominent alterations observed in the Px group were tubular changes such as proximal tubular vacuolization (Fig. 2); rare tubular dilatation; sclerotic changes such as matrix expansion; shrinkage or disappearance of the Bowman's space; the adhesion of the capillary tuft to the Bowman's capsule; capillary collapse and thickening of the glomerular basement membrane (Fig. 3); and interstitial inflammation (Fig. 4). Many of the tubular cells contained large lipofuscin granules (Fig. 5). Melatonin treatment significantly improved these histological changes. The histological appearance of the kidneys of this group was similar to that of the sham group (Fig. 6). However, rarely vacuolization and mild sclerotic changes were observed.

Discussion

In the current study, we have evaluated the following endpoints of renal damage: (i) renal hemodynamics (ii) detailed oxidant-antioxidant status (iii) kidney histopathology.

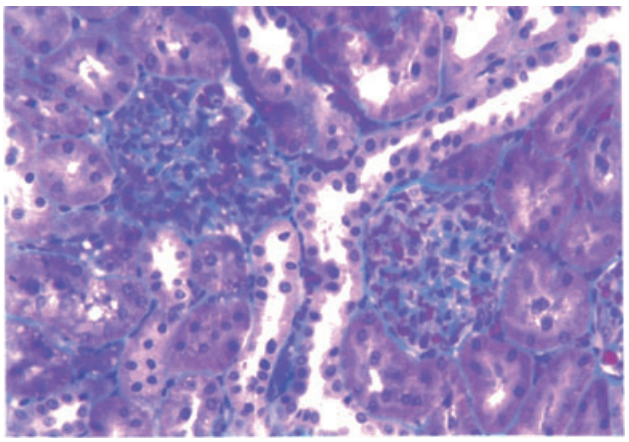

Fig. 3 Px group; Matrix expansion, the disappearance of the Bowman's space, the adhesion of the capillary tuft to the Bowman's capsule, the capillary collapse and the thickening of the glomerular basement membrane are visible. Masson's trichrome; X 40.

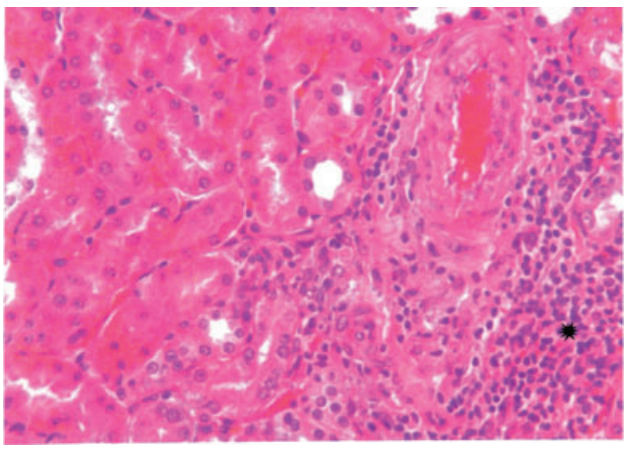

Fig. 4 Px group; Prominent interstitial inflammation is seen. H-E; X 40.

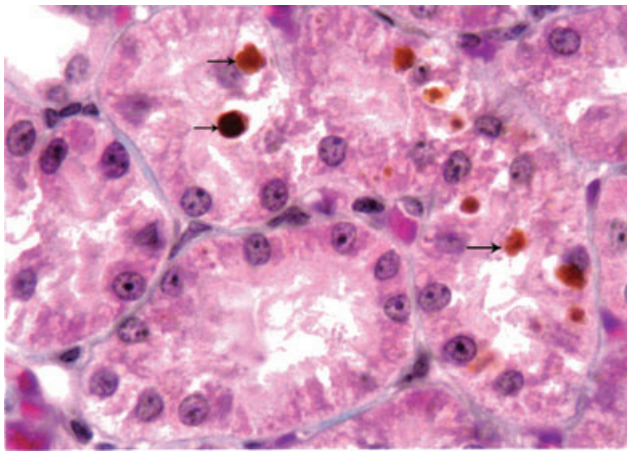

Fig. 5 Px group; Tubules containing many large lipofuscin granules are seen. Masson's trichrome; X 100.

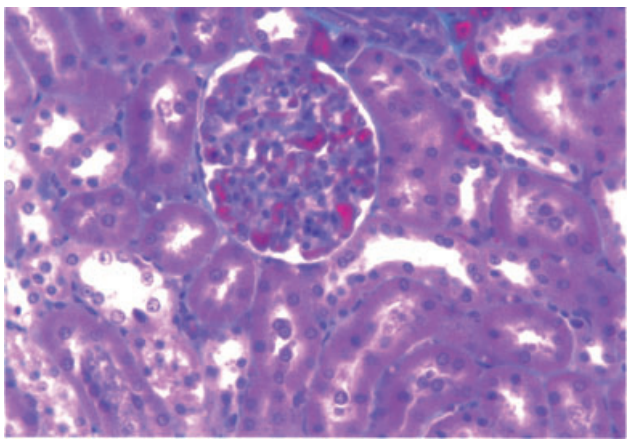

Fig. 6 Pinealectomized and treated with melatonin (Px + Mel) group; The histological appearance of the glomerulus and tubules are nearly the same as those of the control group. Masson's trichrome; X 40.

Our first notable finding is the protective effect of melatonin on renal hemodynamics. In the present study, the increased serum Cr and BUN levels induced by pinealectomy were significantly blocked by exogenous melatonin. The protective effect of melatonin on Cr and BUN levels could be attributed to its antioxidant properties because it has been found that ROS may be involved in the impairment of glomerular filtrate rate (GFR).[16] Recently, it has been reported that for humans, serum Cr in association with certain other clinical characteristics may be a more accurate measure of the GFR than Cr clearance.[17] The impairment in glomerular function was accompanied by an increase in BUN. Serum Cr concentration is more significant than the BUN level in the earlier phases of kidney disease. On the other hand, BUN begins to rise only after a marked renal parenchymal injury occurs.[18] In this study proteinuria was not seen. In the pinealectomized rats (parallel to histological findings such as the glomerular tuft collapsed with a narrowing of the Bowman's space) the ratio of BUN/Cr increased more than 20 (35.91 $\pm$ 1/0.98 $\pm$ 0.02, respectively). These findings are compatible with the ischemic lesion related to renal damage.

Another major finding of our study is the observation that pinealectomy caused significant kidney damage when compared with sham-operated rats. Tissue damage in the Px rats is prevented by the administration of exogenous melatonin. This protection was manifested by the reduced production of MDA, NO and the increased content of GSH, SOD activity and less tissue damage in the kidneys. Healthy kidneys consume relatively large amounts of oxygen, accounting for approximately 10% of the total oxygen consumed by the body; thus, the aberrant generation of ROS occurs prominently in the kidney. In health, ROS generated by the kidneys are metabolized by adaptive scavenger mechanisms. However, in endogenous or exogenous renal injury, excessively produced ROS may cause acute or progressive renal damage.[19] Merely removing one source of melatonin, which is accomplished by the surgical removal of the pineal gland, has been shown to augment oxidative stress in the kidney under conditions of induced free radical generation and under conditions of aging.[20] Due to this knowledge, we suppose that if excessive ROS production, which occurs when the antioxidative defense mechanisms are reduced, is inhibited in the kidney, the formation of renal damage is prevented. In the current study we focused on the role of endogenous melatonin and the effects of the long-term administration of exogenous melatonin in kidney tissue after pinealectomy.

In the free radical theory of aging, it is suggested that accumulated free radical damage may be responsible for the degenerative process

during aging.[6] The total antioxidant capacity of human serum positively correlates with its melatonin concentration.[21] Thus, the reduction of melatonin production with increasing age may be a factor is the increased oxidative damage in the elderly.[22] Studies derived mainly from observations on Px rats suggest that diminished melatonin secretion may be associated with the acceleration of the aging process.[23] We previously reported that the generation of oxygen-derived free radicals is significantly responsible for Px-induced tissue injury in various organs including the heart,[4] kidney,[1,24] testis[25] and skin.[26] However these studies were focused on the short-term effects of pinealectomy and melatonin was applied daily for 3 days. To date, there has been no evidence concerning the long-term influence of pinealectomy or long-term melatonin administration on kidney tissue injury.

Herein, MDA levels in the Px group were found to be significantly higher than in the sham group. Melatonin also significantly reduced MDA production. MDA was used to evaluate the severity of lipid peroxidation, releasing the toxic effects of active oxygen radicals, which destroy unsaturated fatty acids in the cell membrane.[27] Although tissue MDA levels were clearly decreased by melatonin, its mechanism is not clearly defined. Melatonin may directly eliminate ROS or directly increase the antioxidant enzyme activities and prevent the inhibition of these enzymes. A likely explanation of this result is that melatonin enhances the antioxidant defense system and stabilizes cell membrane fluidity against oxidative stress, thus helping renal cells to resist oxidative damage. Our recent studies[1,24] have shown that melatonin is able to prevent renal damage induced by pinealectomy. As stated before, the favorable effect of melatonin is related to the decreased lipid peroxidation. Many researchers such as Abdel-Wahhab *et al*.[28] and Sahna *et al*.[29] have reported that the concentration of circadian melatonin was significantly reduced in Px rats, and that melatonin decreased the MDA levels as a result of the excessive production of free radicals.

In this study, we found that NO production was elevated in the Px group when compared with the sham group. Besides the physiological roles of NO, several pathophysiological roles have been found,[30] and in several studies, NO has been shown to play a pivotal role in aging machinery.[31] In accordance with our current results, we previously reported that renal ischemia-reperfusion (I/R), elevated NO levels.[32] When the production of NO by the nitric oxide synthase (NOS) is exacerbated by an oxidative stress or by a pathological condition, its production must be controlled. The reaction of nitrogen monoxide and superoxide anion generates peroxynitrite ($ONOO^-$), which is a highly reactive molecule. $ONOO^-$, reacts with cellular components such as membrane lipids and proteins, thereby disturbing their function and, consequently, their cellular homeostasis.[33] Melatonin, at least at some sites inhibits NOS, a pro-oxidative enzyme.[34] Related to this notion, Topal *et al*.[35] and Teixeira *et al*.[36] reported that melatonin decreases the NO levels.

In the current study, our other important finding is the elevation of the GSH level in the melatonin group when compared with the Px group. GSH is an important constituent of intracellular protective mechanisms against a number of noxious stimuli including oxidative stress.[37] As an antioxidant, it can catch free radicals, reduce H_2O_2 and stabilize sulfhydryl groups. H_2O_2 is transformed into oxygen and water with the aid of Glutathione peroxidase enzyme (GSH-Px), whereby GSH itself is oxidized to Oxidized glutathione (GSSG).[38]

The other result of the present study is that reduced SOD activity in Px rats was significantly ameliorated by melatonin. Melatonin's antioxidant actions probably derive from its stimulatory effects on GSH-Px, SOD, Glutathione reductase enzyme (GSH-Rd), glucose-6-phosphate dehydrogenase and its inhibitory action on NOS.[8] Urata *et al.* [39] have found that the rate-limiting enzyme in GSH synthesis, gamma-glutamylcysteine synthetase, is also increased after the administration of melatonin to rats. Tunez *et al.* [40] reported that melatonin caused an increase in GSH levels. The results of this study, suggest that melatonin may be a very potent agent in replenishing the GSH tissue level, which plays a major role in the antioxidant defense mechanism. As noted above, experimental evidence has shown that melatonin also promotes the activity of GSH-Rd thereby helping to maintain high levels of reduced GSH.[41]

Our biochemical data were correlated with morphometrical changes. The score for glomerular, tubular and interstitial changes was significantly higher in the Px group than the melatonin-treated group. Melatonin supplementation significantly reduced these parameters. Quantitative morphological changes including mean dimension and the number of the glomeruli in the kidneys were also examined by using light microscopy. As shown in the figures, mainly tubular and interstitial changes attracted attention. In Table 2, the glomerular matrix expansion, the narrowing of the Bowman's space, the presence of adhesion and GBM thickening, and the collapse of the glomerular tuft are grouped and expressed as a histological score. The results clearly reveal the protective effect of exogenous melatonin on renal tissue at both a biochemical and histopathological level, although the mechanism has not been unambiguously elucidated.

Herein, we confirm the results of our previous studies[1,24] showing that physiological concentrations of melatonin are important in preventing the nephrotoxicity induced by chemotherapeutic drugs (which often have toxic effects in kidney tissue[24]) and in reducing I/R-induced renal damage.[26] This is also the first finding that shows the relationship between melatonin and Px-induced long-term renal damage. In conclusion, we suggest that a lack of physiological melatonin causes renal failure, and melatonin replacement therapy may reduce Px-induced renal injury, especially in older patients with low plasma melatonin concentrations due to the calcification of the pineal gland.

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