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ORIGINAL ARTICLE

**Engin Sahna · Hakan Parlakpinar · Feral Ozturk
Yılmaz Cigremis · Ahmet Acet**

The protective effects of physiological and pharmacological concentrations of melatonin on renal ischemia-reperfusion injury in rats

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Abstract Reactive oxygen species have been implicated in the pathophysiology of renal ischemia reperfusion (I/R) injury. The pineal secretory product melatonin is known to be a potent free radical scavenger and antioxidant. This study was designed to investigate the effects of physiological and pharmacological concentrations of melatonin on I/R injury. Rats were pinealectomized (Px) or sham-operated (control) 2 months before the I/R studies. There were eight groups of eight rats each. After a right nephrectomy to produce damage, left renal vessels were occluded for 60 min, followed by 24 h reperfusion, in rats. Malondialdehyde (MDA) levels resulting from I/R were significantly higher in the pinealectomized rats than in the control group. Melatonin administration (4 mg kg^{-1} i.p. either before ischemia or reperfusion) to Px and sham-operated rats significantly reduced the MDA values and returned them to the control values. Morphological changes in the groups were similar to the MDA levels. Serum levels of blood urea nitrogen and creatine were unchanged. These results suggest that physiological and pharmacological melatonin concentrations are important for the reduction of I/R-induced damage. We also demonstrated that melatonin, even when administrated just before reperfusion, had a protective effect on I/R injury. It would seem valuable to test melatonin in clinical trials for the prevention of possible I/R injury.

Keywords Melatonin · Pinealectomy · Renal ischemia-reperfusion

E. Sahna (✉)
Department of Pharmacology, Faculty of Medicine,
University of Firat, 23100, Elazıg, Turkey
E-mail: esahna@hotmail.com
Tel.: +90-424-2370000
Fax: +90-424-2379138

H. Parlakpinar · A. Acet
Department of Pharmacology, Faculty of Medicine,
University of Inonu, Turkey

F. Ozturk
Department of Histology, Faculty of Medicine,
University of Inonu, Turkey

Y. Cigremis
Department of Biochemistry, Faculty of Medicine,
University of Inonu, Turkey

Introduction

Acute renal failure resulting from renal ischemia reperfusion (I/R) injury is of great clinical importance because of its frequent occurrence and high mortality [15]. The temporary discontinuation of renal blood supply is a consequence of diverse clinical conditions such as renal transplantation, aortic aneurysm surgery or hypotension due to resuscitation. Clinical and experimental studies have provided evidence that I/R-injury is mediated by reactive oxygen species (ROS) [16]. These free radicals can attack a wide variety of cellular components, including DNA, proteins, and membrane lipids [17]. Lipid peroxidation yields conjugated dienes and secondary products. Malondialdehyde (MDA), a stable metabolite of the free radical-mediated lipid peroxidation cascade, is widely used as a marker of oxidative stress. Research efforts designed to prevent or ameliorate I/R injury have focused on the pharmacological inhibition of free radical injury. Antioxidants have been found to protect renal cells from cellular injury induced by ischemia and reperfusion [6, 22, 25].

Melatonin is the chief indoleamine produced by the pineal gland and a well-known antioxidant and free radical scavenger [17, 21, 26]. Both in vivo and in vitro, melatonin limits the peroxidative breakdown of lipids and reduces oxidative damage [2, 17]. Melatonin reduced I/R damage to other organs like the heart [11] and brain [8] in rats. In previous studies with pinealectomized rats, we demonstrated that the physiological concentrations of melatonin, which are known to decrease with age, were important in preventing the mortality resulting from irreversible ventricular fibrillation on

reperfusion [23] and cardiac infarct size [24] resulting from I/R. However, the role of physiological or pharmacological levels of melatonin in the prevention of I/R-induced renal injury remains unknown.

This study was designed to investigate the effects of physiological and pharmacological concentrations of melatonin on I/R-induced renal damage in an in vivo rat model. To examine this, we evaluated histopathological changes, MDA levels, serum creatine (Cr) and blood urea nitrogen (BUN) levels, and the effect of exogenous melatonin administration.

Materials and methods

Experimental groups

Male Wistar rats weighing 150–200 g were placed in a quiet room at 21 ± 2°C and60 ± 5% RH with a 12/12 h light/dark cycle. Rats were pinealectomized (Px) or non-Px 2 months before the beginning of the I/R studies. All experiments were performed between 9.00 and 17.00 h.

There were eight groups of eight rats each. Four groups without Px: non-Px + vehicle (Non-Px + veh), non-Px + I/R, non-Px + I/R with melatonin administration before ischemia [Non-Px + I/R + M(bi)] and non-Px + I/R with melatonin administration before reperfusion [Non-Px + I/R + M(br)]. Four groups with Px: Px + veh, Px + I/R, Px + I/R with melatonin administration before ischemia [Px + I/R + M(bi)], and Px + I/R with melatonin administration before reperfusion [Px + I/R + M(br)]. In both Px and non-Px rats, the vehicle or melatonin (4 mg kg^{-1}) were administered by intraperitoneal injection 10 min before ischemia or just prior to reperfusion. Melatonin (Sigma, St. Louis, Mo., USA) was dissolved in ethanol and further diluted in saline (0.09% NaCl w/v) to give a final concentration of 1%.

Pinealectomy

Pinealectomy was performed as described by Kuszak and Rodin [9]. Rats were anesthetized with ketamine hydrochloride (75 mg kg^{-1}) and xylazine (8 mg kg^{-1}) before the operation. The entire procedure was completed within 15 min. Px was confirmed by the histological evaluation of the gland for each animal.

Ischemia-reperfusion procedure

Rats were anesthetized with ketamine hydrochloride and xylazine administered i.p. before the operation. Right nephrectomy was performed through dorso-lateral incisions on all rats and the left renal vessels were occluded for 60 min, followed by 24 h reperfusion.

Evaluation of tissue damage

At the end of each in vivo study, the rats were killed and the kidneys were quickly removed, decapsulated and divided longitudinally into two equal sections. One was placed in formaldehyde solution for routine histopathological examination by light microscopy. The other section was placed into liquid nitrogen and stored at –70°C until assayed for MDA levels. Trunk blood was extracted to determine the serum levels of BUN and Cr.

Biochemical determination

A total of 200 mg of the kidney tissue was homogenized with ice-cold 150 mM KCI for determination of MDA. The MDA content of the homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances [28]. The results were expressed as nmol/g tissue. The serum levels of BUN and Cr were determined using an Olympus Autoanalyser (Olympus Instruments, Tokyo, Japan).

Histological analysis

For light microscopic evaluation, kidneys were fixed in 10% phosphate buffered formalin. Paraffin embedded specimens were cut into 6 μm thick sections and stained with hematoxylin-eosin (H-E) before investigation under a light microscope (Olympus BH-2). The kidneys were examined for tubular dilatation, inflammatory cell infiltration, cast formation in the tubular lumen and tubular epithelial cell detachment by an observer blinded to the animal treatment group.

Statistics

Data are expressed as arithmetic means ± SEM of the number (n) of experiments. Multiple comparisons between the experimental groups were performed by one-way ANOVA with a Tukey post hoc test. When $P < 0.05$, the difference was considered to be statistically significant.

Results

Although there was no difference between the body weights of Px (172 ± 7 g) and non-Px (176 ± 6 g) groups before the Px or non-Px, Px rats had significantly higher body weights (291 ± 6 g) than non-Px rats (258 ± 8 g) 2 months after the operation (i.e. before the I/R experiments). The kidney weights of Px rats (1.48 ± 0.2 g) were also significantly higher than those in the non-Px group (1.07 ± 0.1 g).

Kidney MDA levels

MDA levels were significantly higher in Px rats (25 ± 0.1) than in the sham-operated control group (10 ± 0.1). I/R injury increased MDA levels in both Px and Non-Px rats (52 ± 0.3 and 30 ± 0.1, respectivelly). In both Px and Non-Px rats, melatonin administration (4 mg kg^{-1} i.p. either before ischemia or reperfusion) significantly reduced the MDA values, returning them to control levels. Results are shown in Table 1.

Cr and BUN levels

Serum levels of BUN and creatine were higher in the I/R administration groups but the results were not significant ($P < 0.05$). In melatonin treated groups, these values did not change in Px or non-Px groups (data not shown).

Histological results

Morphological damage ranged from nil (Non-Px group control Fig. 1) to mild (melatonin administrated Non-Px + I/R group Fig. 2), moderate (Px group Fig. 3, melatonin administrated Px + I/R group Fig. 4) and severe (Non-Px + I/R group Fig. 5 and Px + I/R group Fig. 6). Morphological changes including tubular dilatation, inflammatory cell infiltration, cast formation in the tubular lumen and tubular epithelial cell detachment were clearly observed in the kidneys of the I/R group (Fig. 5) and Px + I/R group (Fig. 6). In the pinealectomy group diffuse inflammatory cell infiltration was detected but tubular alterations were rare (Fig. 3). The glomeruli appeared normal in all groups. Cell detachment and intratubular casts were rare in the I/R + melatonin (br)

Table 1 The effects of pinealectomy (Px) or melatonin (M) administrations on the MDA levels in rats, with or without ischemia-reperfusion (I/R), sham-operated (Non-Px), vehicle (veh), before ischemia (bi), before reperfusion(br). $n = 8$ in each group

Groups	Non-Px + veh	Non-Px + I/R	Non-Px + I/R + M(bi)	Non-Px + I/R + M(br)	Px + veh	Px + I/R	Px + I/R + M(bi)	Px + I/R + M(br)
MDA levels (nmol/g tissue)	10 ± 0.1	30 ± 0.1^{a}	12 ± 0.1^{b}	19 ± 0.1^{b}	25 ± 0.1^{a}	52 ± 0.3^{a}	16 ± 0.1^{b}	21 ± 0.1^{b}

a: significantly different from sham-operated group ($P < 0.05$), b: significant difference resulted from melatonin administration ($P < 0.05$)

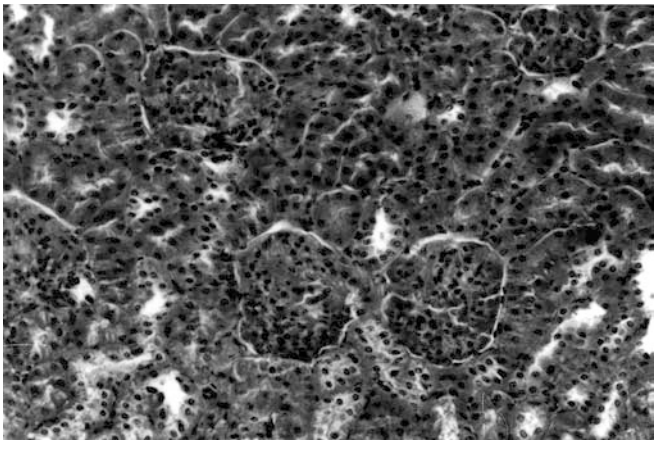

Fig. 1 Light micrograph showing the renal cortex in the control group. Glomeruli and tubules appear normal. H-E, ×66

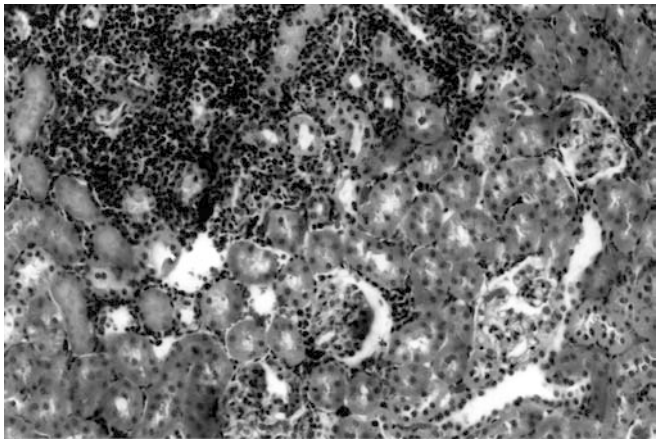

Fig. 3 Light micrograph showing the renal cortex from a Px rat. Notice the diffuse inflammatory cell infiltration. H-E, ×66

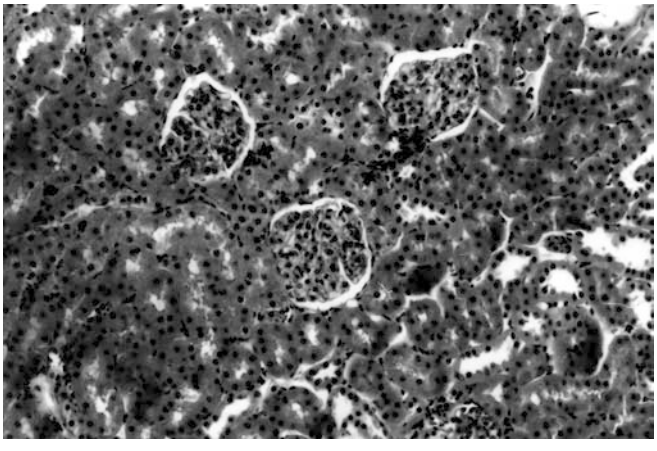

Fig. 2 Light micrograph of the renal cortex after I/R following pretreatment with melatonin before ischemia. The tubules are almost normal. Inflammatory cell infiltration is seen around the glomeruli (*arrow*). H-E, ×66

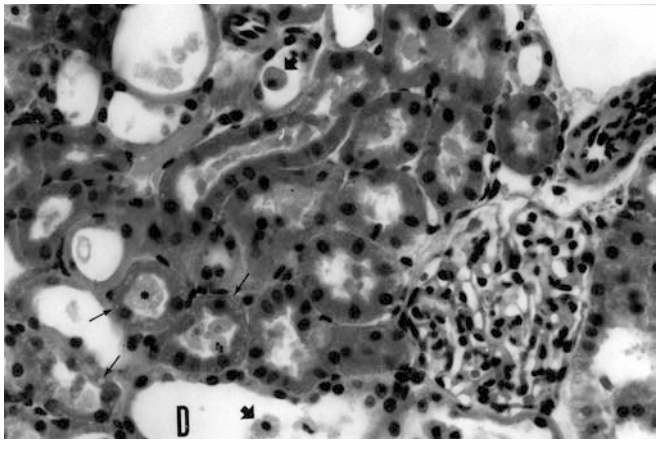

Fig. 4 Light micrograph showing the renal cortex from a Px rat after an I/R period following pretreatment with melatonin before reperfusion. Tubular (*D*), intratubular cast (*asterisk*) and cell detachment (*thick arrows*) are present. Some of the tubular epithelial cells show fine intracytoplasmic vacuoles (*thin arrows*). H-E, ×66

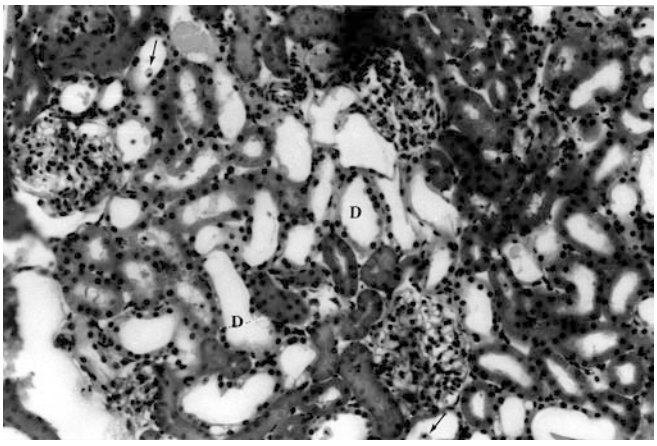

Fig. 5 Light micrograph of renal cortex after I/R period. Tubular dilatation (*D*) and tubular epithelial cell detachment (*arrows*) are visible. H-E, ×66

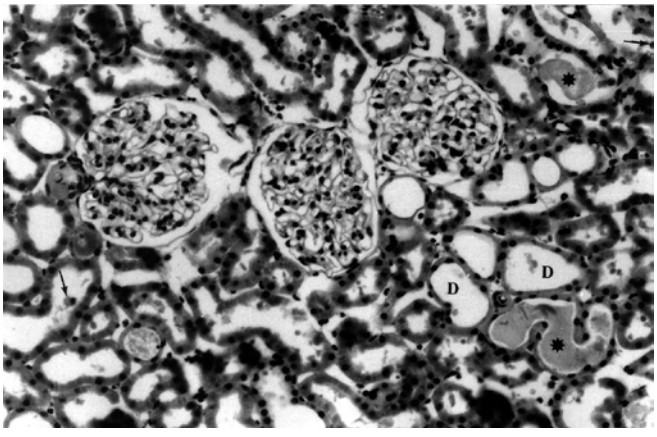

Fig. 6 Light micrograph showing the renal cortex from a Px rat after I/R. Tubular dilatation (*D*) intratubular cast (*asterisk*) and tubular epithelial cell detachment (*arrows*) are visible. H-E, ×66

group and absent in the I/R + melatonin (bi) group (Fig. 2). In the Px + I/R + melatonin (br) group, tubular cells showed intracytoplasmic vacuoles (Fig. 4). Melatonin apparently reduced kidney tissue damage in the Px or non-Px groups. Especially when administrated before ischemia. Px alone also altered kidney morphology.

Discussion

In this study, we demonstrated that the renal injury resulting from I/R was significantly increased after pinealectomy. The I/R mediated renal damage in both Px and non-Px rats was prevented by melatonin administration. These findings strongly suggest that physiological or pharmacological concentrations of melatonin, a pineal secretory product, are important in protecting the kidney from I/R-induced damage in rats in vivo.

The protective effect of melatonin on I/R injury (such as, arrhythmias and cell death) has been shown for other organs in the rat, such as the heart [11, 23, 24]. Kilic et al. [8] suggest that pinealectomy aggravates and melatonin administration attenuates brain damage in focal I/R.

To investigate the role of melatonin in I/R-induced renal damage, either Px (confirmed by histologically) or non-Px rats were used. Although the retina also has a high capacity to synthesize melatonin, it does not seem to contribute significantly to the plasma melatonin concentrations, probably because of rapid catabolism in the retina itself [7]. The amplitude of the melatonin rhythm is reduced by 60–100% after pinealectomy [29]. Pinealectomy may cause hypertension after 60 days [23, 30], therefore we used rats that were pinealectomized 2 months before I/R experiments to eliminate possible tissue damage due to pinealectomy-induced hypertension.

The antioxidant action of melatonin is probably responsible for its protective activity during reperfusion. In addition to the damage caused by the lack of blood flow and oxygen delivery, the restoration of blood flow has also been reported to contribute to cell damage due to the generation of free radicals [16]. The production of oxygen free radicals during ischemia and reperfusion of the kidney has been implicated as a major pathophysiological component of acute renal failure [6, 16]. Therefore, experimental approaches to the amelioration of renal I/R injury have mainly focused on the prevention of free radical induced cellular damage. The beneficial effects of many free radical scavengers and antioxidants on I/R-induced damage have been demonstrated [6, 22, 25].

In the renal vascular bed, I/R rapidly results in cellular injury associated with lipid peroxidation. As lipid peroxidation is the main pathway for tissue radical damage, irrespective of the source of free radicals, blocking this pathway appears to be an attractive strategy to protect the kidney from ROS-mediated damage. We found that I/R caused renal damage associated with increased MDA levels, a marker for lipid peroxidation. Decrease of the endogenous melatonin plasma levels by pinealectomy significantly further increased both the apparent histological renal damage and the MDA level. This indicates a protective role for melatonin against oxidative-stress related damage, but leaves open the possibility that other factors were changed after pinealectomy which were relevant in this respect. That melatonin plays a role was finally confirmed when the plasma melatonin level was increased by i.p. administration of melatonin. This selective increase of melatonin decreased the histologically visible damage and returned the MDA levels to control levels. This opens the clinical application of melatonin administration for the prevention of oxidative stress related damage. The finding that even late administration of melatonin, just before reperfusion, acted protectively, suggests that it may act very rapid.

By what exact mechanism does melatonin exert this protective function? Melatonin is known to scavenge the ROS such as hydroxyl radicals, superoxide anion radicals, singlet oxygen and the nitrogen-based reactant peroxynitrite anions, as well as to stimulate antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase [17, 21, 26]. Many studies have shown that, in vivo and in vitro, melatonin limits the peroxidative breakdown of lipids and reduces oxidative damage [2, 4, 17, 20]. Nava et al. [13] showed that melatonin attenuates acute renal failure and oxidative stress induced by mercuric chloride in rats. Previous studies have demonstrated that melatonin protects the kidney against gentamicin [14], adriamycin [12], daunorubicin [4] and doxorubicin [4] toxicity.

In addition to being an effective free radical scavenger, melatonin may also decrease intracellular calcium concentrations [29] and inhibit human platelet aggregation and tromboxane-B2 production [3]. Furthermore, it has been shown that melatonin can suppress sympathetic nerve function and decrease catecholamine release [10]. These factors may also contribute to the protective effects of melatonin on I/R injury. Eckert et al. [5] suggest that α_1-adrenoceptor and Ca^{2+} overload in renal artery myocytes during renal surgery plays a major role in post-surgical organ dysfunction.

Melatonin is an amphiphilic molecule [18], allowing it to pass all biological barriers easily, thus quickly penetrating into cells of the ischemic region and all subcellular compartments where it scavenges damaging reactants [20]. This is a clear advantage of melatonin over other antioxidants which penetrate cells more slowly. The property of melatonin to rapidly enter cells may explain why melatonin administration just before reperfusion in rats still had a protective effect on reperfusion-induced cellular damage in the present study.

In this study, we demonstrated that Px alone altered kidney morphology and lipid peroxidation and the renal injury resulting from I/R was significantly increased after pinealectomy. These findings strongly suggest that physiological concentrations of melatonin are important in protecting the kidney from I/R-induced damage in rats. Px or melatonin administration also generally tended to increase and decrease renal injury, respectively. The results of this study are consistent with previous results which demonstrated that the physiological levels of melatonin are important in protecting against I/R-induced injury [8, 20, 23, 24]. Akcetin et al. [1] demonstrated a diminished cytosolic antioxidative capacity in the kidneys of aged rats. The use of antioxidative treatment was therefore suggested, especially after renal transplantation from aged donors. Free radical damage has frequently been implicated in aging, and the total antioxidative capacity of serum is related to melatonin levels [17, 19], thus the reduction in melatonin with age may be a factor in increased oxidative damage in the elderly. These findings, together with our current results, indicate that melatonin replacement therapy or melatonin supplementation may attenuate I/R injury.

In addition, we demonstrated that melatonin administration immediately before reperfusion still had a protective effect on I/R-induced tissue damage in Px rats. Since most of the drugs used to prevent I/R injury are only known to be effective when they are given before ischemia, it would seem valuable to test melatonin in clinical trials for the prevention of possible I/R-induced damage associated with transplantation, partial nephrectomy and enucleation of renal cell carcinoma.

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References

1. Akcetin Z, Erdemli G, Bromme HJ (2000) Experimental study showing a diminished cytosolic antioxidative capacity in kidneys of aged rats. Urol Int 64: 70
2. Carneiro RCG, Reiter RJ (1998) δ-Aminolevulinic acid-induced lipid peroxidation in rat kidney and liver is attenuated by melatonin: an in-vitro and in-vivo study. J Pineal Res 13: 287
3. Del Zar MM, Martınuzzo C, Falcon C, Cardınalı DP, Carreras LO, Vacas MI (1990) Inhibition of human platelet aggregation and tromboxane-B2 production by melatonin: evidence for a diurnal variation. J Clin Endocrinol 70: 246
4. Dziegiel P, Suder E, Surowiak P, Jethon Z, Rabczynski J, Januszewska L, Sopel M, Zabel M (2002) Role of exogenous melatonin in reducing the nephrotoxic effect of daunorubicin and doxorubicin in the rat. J Pineal Res 33: 95
5. Eckert RE, Karsten AJ, Utz J, Ziegler M (2000) Regulation of renal artery smooth muscle tone by α_1-adrenoceptors: role of voltage-gated calcium channels and intracellular calcium stores. Urol Res 28: 122
6. Giovannini L, Migliori M, Longoni BM, Das DK, Bertelli AA, Panichi V, Filippi C, Bertelli A (2001) Resveratrol, a polyphenol found in wine, reduces ischemia reperfusion injury in rat kidneys. J Cardiovasc Pharmacol 37: 262
7. Grace MS, Cahill GM, Besharse JC (1991) Melatonin deacetylation: retinal vertebrate class distribution and *Xenopus laevis* tissue distribution. Brain Res 559: 56
8. Kilic E, Ozdemir YG, Bolay H, Kelestimur H, Dalkara T (1999) Pinealectomy aggravates and melatonin administration attenuates brain damage in focal ischemia. J Cereb Blood Flow Metab 19: 511
9. Kuszak J, Rodin M (1977) A new technique of pinealectomy for adult rats. Experientia 33: 283
10. K-Laflamme A, Wu L, Foucart S, de Champlain J (1998) Impaired basal sympathetic tone and alpha1-adrenergic responsiveness in association with the hypotensive effect of melatonin in spontaneously hypertensive rats. Am J Hypertens 11: 219
11. Lagneux C, Joyeux M, Demenge P, Ribuot C, Godin-Ribuot D (2000) Protective effects of melatonin against ischemia-reperfusion injury in the isolated rat heart. Life Sci 66: 503
12. Montilla P, Tunez I, Munoz MC, Lopez A, Soria JV (1997) Hyperlipidemic nephropathy induced by adriamycin: effect of melatonin administration. Nephron 76: 345
13. Nava M, Romero F, Quiroz Y, Parra G, Bonet L, Rodriguez-Iturbe B (2000) Melatonin attenuates acute renal failure and oxidative stress induced by mercuric chloride in rats. Am J Physiol Renal Physiol 279: 910
14. Ozbek E, Turkoz Y, Sahna E, Ozugurlu F, Mizrak B, Ozbek M (2000) Melatonin administration prevents the nephrotoxicity induced by gentamicin. BJU Int 85: 742
15. Paller MS (1998) Acute renal failure: controversies, clinical trials, and future. Semin Nephrol 18: 482
16. Paller MS, Hoidal J, Ferris TF (1984) Oxygen free radicals in ischemic acute renal failure in the rat. J Clin Invest 74: 1156

17. Reiter RJ, Guerrero JM, Garcia JJ, Acuna-Castroviejo D (1998) Reactive oxygen intermediates, molecular damage and aging. Relation to melatonin. Ann N Y Acad Sci 854: 410
18. Reiter RJ (1991) Melatonin: that ubiquitously acting pineal hormone. News Physiol Sci 6: 223
19. Reiter RJ (1992) The aging pineal gland and its physiological consequences. BioEssays 14: 169
20. Reiter RJ, Tan DX, Qi W, Manchester LC, Karbownik M, Calvo JR (2000) Pharmacology and physiology of melatonin in the reduction of oxidative stress in vivo. Biol Signals Recept 9: 160
21. Reiter RJ, Tan DX, Manchester LC, Qi W (2001) Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. Cell Biochem Biophysics 34: 237
22. Rhoden EL, Pereira-Lima L, Teloken C, Lucas ML, Bello-Klein A, Rhoden CR (2001) Beneficial effect of alpha-tocopherol in renal ischemia-reperfusion in rats. Jpn J Pharmacol 87: 164
23. Sahna E, Olmez E, Acet A (2002) Effects of physiological and pharmacological concentrations of melatonin on ischemia-reperfusion arrhythmias in rats: can the incidence of sudden cardiac death be reduced? J Pineal Res 32: 194
24. Sahna E, Acet A, M.K Ozer, Olmez E (2002) Myocardial ischemia-reperfusion in rats: reduction of infarct size by either supplemental physiological or pharmacological doses of melatonin. J Pin Res 30: 234
25. Seth P, Kumari R, Madhavan S, Singh AK, Mani H, Banaudha KK, Sharma SC, Kulshreshtha DK, Mahshwari RK (2000) Prevention of renal ischemia/reperfusion-induced injury in rats by picroliv. Biochem Pharmacol 59: 1315
26. Tan DX, Reiter RJ, Manchester LC, Yan MT, El-Sawi M, Sainz RM, Mayo JC, Kohen R, Allegra M, Hardeland R (2002) Chemical and physical properties and potential mechanisms: melatonin: melatonin as a broad spectrum antioxidant and free radical scavenger. Curr Top Med Chem 2: 181
27. Vanecek J (1995) Melatonin inhibits increase of intracellular calcium and cyclic AMP in neonatal rat pituitary via independent pathways. Mol Cell Endocrinol 107:1497
28. Vanecek J (1998) Cellular mechanisms of melatonin action. Physiol Rev 78: 68
29. Vermeulen NPE, Baldew GS (1992) The role of lipid peroxidation in the nephrotoxicity of cisplatin. Biochem Pharmacol 44: 1193
30. Zanoboni A, Forni A, Zanoboni-Muciaccia W, Zanussi CT (1978) Effect of pinealectomy on arterial blood pressure and food and water intake in the rat. J Endocrinol Invest 1: 125