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EFFECT OF THREE-WEEK ZINC AND MELATONIN SUPPLEMENTATION ON THE OXIDANT-ANTIOXIDANT SYSTEM IN EXPERIMENTAL RENAL ISCHEMIA-REPERFUSION IN RATS

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SUMMARY – Renal ischemia-reperfusion directly affects glomerular and tubular epithelium. Oxygen free radicals have a significant part in the pathophysiology of renal ischemia-reperfusion injury. The present study aimed to identify the effects of 3-week zinc, melatonin, and zinc + melatonin supplementation on malondialdehyde (MDA) levels in tissue and plasma and glutathione levels (GSH) in erythrocytes and tissue of rats with experimentally induced renal ischemia-reperfusion injury. The study included Wistar albino rats with a mean weight of 250 g. Study groups were formed as follows: control, sham-control, ischemia + reperfusion, zinc + ischemia-reperfusion, melatonin + ischemia-reperfusion, and zinc + melatonin + ischemia-reperfusion. Animals were supplemented with zinc and melatonin 3 mg/kg/day i.p. for 3 weeks before the induction of ischemia-reperfusion. Renal ischemia-reperfusion was induced in the left kidney under general anesthesia and consisted of ischemia for 45 minutes and reperfusion for 1 hour. After the procedure, animals were sacrificed and blood and kidney samples were collected to analyze MDA and GSH levels. GSH values in kidney tissues and erythrocytes were found to be elevated in the groups supplemented with zinc and melatonin ($p<0.005$). When MDA values in renal tissue and plasma were examined, it was seen that ischemia significantly elevated this parameter, while zinc and melatonin supplementation significantly inhibited MDA values ($p<0.002$). The results of the study indicated that oxidative injury of the blood and renal tissues of rats increased in association with ischemia-reperfusion, but zinc and melatonin supplementation before ischemia-reperfusion markedly reduced this oxidative damage.

Key words: *Acute kidney injury – prevention and control; Reperfusion injury – prevention and control; Zinc – therapeutic use; Melatonin – therapeutic use; Free radicals; Models, animal; Rats*

Introduction

Ischemia initiates a series of biochemical reactions that can lead to cellular dysfunction and eventual cell death by lowering the energy level in the cell and causing accumulation of toxic metabolites in the tissue[1]. Reperfusion, on the other hand, is resumption of blood supply to the tissue. Free oxygen radicals (ROS), which are released by polymorphonuclear leukocytes (PMNL) that are settled down in the ischemic tissue upon resumption of blood supply (reperfusion) further aggravate tissue injury. The severity of the injury varies depending on the length of ischemia, temperature of the tissue, and tissue-specific factors[2]. Ischemia-reperfusion injury can also be seen in kidneys in the course of clinical conditions such as systemic hypotension, hypovolemic shock, cardiac arrest, renovascular surgery, aorta clamping and organ injury[2-4]. The severity of renal injury increases depending on the length

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of ischemia; thus, the resulting clinical picture may vary from prerenal azotemia without tissue damage to acute renal failure associated with tubular or cortical necrosis[3,5-7].

Lipid peroxidation in biological membranes increases ion permeability of these membranes, resulting in damage to transmembrane proteins like the membrane receptor enzyme. Unsaturated fats and cholesterol in the phospholipids found in the membranes rapidly react with free radicals to yield peroxidation products. One of the major products of lipid peroxidation is malondialdehyde (MDA)[8-10]. Malondialdehyde resulting from lipid peroxidation brings about several unfavorable effects such as changing the ion permeability and enzyme activity by altering the ion exchange and causing cross-linking of membrane components. Glutathione (GSH) is the primary intracellular antioxidant component that protects cells against oxidative stress. It is synthesized from glutamate, cysteine and glycine in a number of tissues, including, most notably, liver tissue[11]. Zinc has been proven to play an important part in the synthesis of proteins, carbohydrates, energy, nucleic acid, and lipids, as well as in homeostasis, oxidative stress, apoptosis, prevention of oxidative stress, and aging[12]. Melatonin, which is both fat- and water-soluble, on the other hand, can easily access all cell organelles, including the nucleus. This characteristic offers melatonin an advantage in protecting DNA against oxidative stress[13-15].

The present study explored the individual and combined effects of zinc and melatonin, which have protective effects against free radical formation in different tissues, on blood and kidney tissue MDA and GSH levels in experimental renal ischemia-reperfusion.

Materials and Methods

The study included 60 Wistar albino type male rats, each weighing 250 to 260 g, supplied by the Experimental Medicine Research and Application Center of Necmettin Erbakan University. Upon approval from the Selcuk University School of Veterinary Medicine Ethics Committee, the study was carried out at the research center of the same school. The rats were randomly allocated to six groups. All rats were kept in temperature and light-controlled rooms and fed on the same rat feed and water.

Experimental groups were formed as follows:

Control group (n=10): the animals in this group were decapitated without any procedure.

Sham-control group (n=10): the rats in this group were anesthetized with intramuscular (i.m.) ketamine hydrochloride (60 mg/kg) and Xylazine (rompun) (5 mg/kg). Following sterilization of the left kidney area, the kidney was opened as if it would be operated on and then closed before the animals were decapitated.

Renal ischemia-reperfusion group (n=10): the animals were anesthetized and their left kidney was subjected to ischemia for 45 minutes, followed by reperfusion for one hour.

Zinc + renal I/R group (n=10): the experimental animals in this group were supplemented with 3 mg/kg/day intraperitoneal zinc for 3 weeks. After zinc supplementation, ischemia and reperfusion were induced in the animals as described above.

Melatonin + renal I/R group (n=10): following 3 weeks of 3 mg/kg/day intraperitoneal (i.p.) melatonin supplementation, ischemia and reperfusion were induced in the animals.

Zn + melatonin + renal I/R group (n=10): after 3 weeks of equal amounts of zinc and melatonin supplementation, ischemia and reperfusion were induced.

At the end of ischemia-reperfusion procedures, the animals were decapitated and 3- to 4-mL blood samples were taken into EDTA tubes. Erythrocyte and plasma samples obtained from the blood, as well as ischemic-reperfused renal tissues were stored at -80 °C until the GSH and MDA analyses.

Blood and tissue analyses

Protein analysis

Biuret test was used to analyze proteins. The tissue to be analyzed was first weighed and then put into a tube. Then it was broken down in a Misonix Microson ultrasonic cell disruptor at 4°C to obtain a 10% homogenate in 150 mM KCl. The resulting homogenates were centrifuged at 3000 rpm for 15 minutes. On top of 50 µL of the supernatant, 1 mL sodium sulfate and then 1 mL biuret reagent was added. This was followed, 5 minutes later, by the addition of 50 µL of distilled water, 1 mL sodium sulfate and 1 mL biuret reagent, and the resulting sample was tested blindly. For the biuret reagent, 2.5 g copper sulfate and 10 g

sodium potassium tartrate was dissolved in distilled water and 350 mL of 2.5 NaOH added. The volume of the resulting mixture was adjusted to 1 L with distilled water. For 2.5 NaOH, 50 g NaOH was added to distilled water to obtain a 500 mL solution. For sodium sulfate, 20 g of sodium sulfate was diluted in 100 mL distilled water. The values were expressed as g/dL.

Determination of tissue malondialdehyde levels

Tissue MDA levels were determined using Uchiyama and Mihara method. The tissue to be analyzed was weighed, separated into pieces and placed in tubes. It was then processed in a Misonix Microson ultrasonic cell disruptor at 4 °C to obtain a 10% homogenate in 150 mM KCl. Of the homogenized tissue, 2 mL was taken, and after adding 2 mL of 8% $HClO_4$, the sample was centrifuged at 3000 rpm for 15 minutes. Three mL of 1% H_3PO_4 and 1 mL of 0.675% TBA was added to 0.5 mL of the supernatant and incubated in 90 °C water bath for 45 minutes. When the mixture cooled down, 4 mL of n-butanol was added and its absorbance against n-butanol at 532 nm was tested. Its concentration was maintained at c=108.9A. The result was described as mg/g protein[16].

Tissue glutathione analysis

To determine GSH levels, the tissue was homogenized to obtain a 10% homogenate in 150 mm KCl at 4 °C, as described for MDA, and centrifuged at 3000 rpm for 15 minutes. The GSH quantities in the samples were measured in accordance with Ellman's method. Eight mL of phosphate buffer (pH 6.8), 78 mL of 1 N NaOH and 100 µL of Ellman's solution was added to 200 µL of the supernatant, then the sample was left to rest for 5 minutes and its absorbance was tested against distilled water at 412 nm in a spectrophotometer. Activity was measured according to the following formula: $a = (A_{standard}/A_{sample}) \times C_{standard}$. The standard was taken to be $c_{standard}$ = 15.36 g/dL. The values obtained by tissue protein biuret method were calculated as nmol/g protein[17].

Quantification of plasma malondialdehyde levels

Blood samples put into EDTA tubes were centrifuged at 3000 rpm for 5 minutes to separate plasma. Then, 0.5 mL of plasma sample was added to 2.5 mL of 10% TCA (trichloroacetic acid crystals, Merck catalogue no. 818 K02907810) in an experimental tube; the tubes were capped and vortexed. The samples were incubated in a 90 °C water bath for 15 minutes, cooled down in cold water, and tested against the blind sample at 532 nm on a spectrophotometer. The blind sample was prepared by putting the same amount of distilled water instead of plasma in the blind tube and carrying out the same series of procedures. The results were presented as nmol/mL.

Erythrocyte glutathione analysis

Blood samples put into EDTA tubes were centrifuged at 3000 rpm for 5 minutes to quantify erythrocyte GSH levels. Erythrocyte samples were bathed in 0.9% serum physiological saline 3 times. Of the bathed erythrocyte samples, 50 µL was taken and 450 µL of distilled water and 500 µL of 10% sulfosalicylic acid was added. After cooling down on ice for 1 hour, the mixture was centrifuged at 4000 rpm for 3 minutes. Then, 8 mL of phosphate buffer (pH 6.8), 78 mL of 1 N NaOH and 100 µL of Ellman's solution was added to 200 µL of the supernatant; the sample was left to rest for 5 minutes and its absorbance was tested against distilled water in the reagent tube at 412 nm on a spectrophotometer. To prepare Ellman's solution, 100 mg of 5'-5'-dithiobis-2-nitrobenzoic acid (DTNB; Sigma, catalogue no. D-8130) was dissolved in 100 mL of phosphate buffer with a pH value of 7.8. GSH standard was prepared as 15.34 mg/100 mL by dissolving 15.34 mg of reduced glutathione (Sigma, catalogue no. G-4251) in 100 mL of 1 nm sodium EDTA. The results were expressed as mg/dL.

Statistical analysis

The SPSS statistical software was used on statistical analyses. The results were described as mean ± standard deviation. Kruskal-Wallis variance analysis was used on between-group comparisons and Mann-Whitney U test was employed for $p<0.05$ level. The level of statistical significance was set at $p<0.05$.

Results

Table 1 shows tissue GSH and MDA values in the experimental groups. Comparison of the values

Table 1. Renal tissue glutathione (GSH) and malondialdehyde (MDA) levels

Group	GSH (nmol/g protein)	MDA (mg/g protein)
Control	0.37±0.15[b]	0.12±0.08[b]
Sham-control	0.36±0.18[b]	0.07±0.03[b]
Renal ischemia-reperfusion (R-I/R)	0.44±0.12[b]	0.28±0.20[a]
Zinc + renal I/R	0.60±0.21[a]	0.11±0.06[b]
Melatonin + renal I/R	0.57±0.18[a]	0.08±0.05[b]
Zn + melatonin + renal I/R	0.52±0.09[a]	0.06±0.04[b]

Different letters in the same column (a, b) indicate significance (p<0.005 for GSH; p<0.002 for MDA)

among groups revealed that groups 4, 5 and 6 had the highest tissue GSH values (p<0.005). GSH value in group 3 was higher than that in groups 1 and 2, but the difference was not statistically significant. As for tissue MDA levels, the highest MDA levels were found in the ischemia-reperfusion group (p<0.002). Erythrocyte GSH and plasma MDA levels in the experimental groups are presented in Table 2. When erythrocyte GSH levels were examined, it was seen that renal ischemia-reperfusion group had the lowest (p<0.005) and melatonin + I/R group the highest erythrocyte GSH levels, which were significantly elevated as compared with the levels in other groups (p<0.005). There was no statistically significant difference between the values in other groups. Examination of plasma MDA levels showed that the ischemia-reperfusion group had the highest plasma MDA levels (p<0.001), while there was no significant difference among other groups.

Table 2. Erythrocyte glutathione (GSH) and plasma malondialdehyde (MDA) levels

Group	Erythrocyte GSH (mg/dL)	Plasma MDA (nmol/mL)
Control	0.32±0.04[b]	0.10±0.03[b]
Sham-control	0.32±0.06[b]	0.12±0.02[b]
Renal ischemia-reperfusion (R-I/R)	0.22±0.06[c]	0.22±0.06[a]
Zinc + renal I/R	0.27±0.07[b]	0.08±0.05[b]
Melatonin + renal I/R	0.36±0.11[a]	0.10±0.07[b]
Zn + melatonin + renal I/R	0.29±0.06[b]	0.08±0.03[b]

Different letters in the same column (a, b, c) indicate significance (p<0.005 for GSH; p<0.001 for MDA)

Discussion

In the present study, the values of MDA as an indicator of lipid peroxidation in renal tissue in rats increased significantly in both tissue and plasma in association with 45 minutes of ischemia and 1 hour of reperfusion. However, 3-week intraperitoneal supplementation of zinc and melatonin both individually and in combination before ischemia-reperfusion was found to markedly inhibit oxidative stress in both renal tissue and blood, as evidenced by elevated levels of glutathione as an element of the antioxidant defense system.

A variety of methods have been employed to show lipid peroxidation in tissue. One of the most commonly used methods is MDA analysis. In our study, MDA values were examined as a marker of lipid peroxidation both in renal tissue and plasma. The significant increase in this parameter, particularly in the ischemia-reperfusion group, is consistent with the studies reporting increased MDA values in renal ischemia-reperfusion injury[18-21]. Renal tissue and plasma MDA values were also used to determine the effects of 3-week supplementation of zinc and melatonin both individually and in combination before the induction of ischemia reperfusion on renal tissue and plasma MDA levels. This parameter was found to be elevated almost 2.5-fold in the ischemia-reperfusion group in comparison to control values. However, there was no significant difference in MDA values between the zinc- and melatonin-supplemented groups and control group, demonstrating that the oxidative stress caused by 45 minutes of ischemia followed by 1 hour of reperfusion was significantly inhibited by prior zinc and melatonin supplementation. Previous studies have reported that zinc and melatonin reduce oxidative damage asso-

ciated with ischemia-reperfusion in different organs and these reports lend support to our findings[22-24]. In their experimental study in rats, Boran and Ozkan[12] evaluated the effects of zinc aspartate administration to injured testes and demonstrated that zinc aspartate pre-treatment reduced ischemia-reperfusion injury through its antioxidant effect. The protective effect of i.p. zinc sulfate supplementation on ischemia reperfusion injury in renal tissue found in our study is consistent with the results of the aforementioned study.

In the same vein, melatonin, a strong antioxidant, was administered for 3 weeks at a dose of 3 mg/kg/day and reduced the levels of MDA, an indicator of oxidative damage caused by ischemia-reperfusion, both in the tissue and plasma examined. These results are consistent with those reported from similar studies which demonstrated the strong antioxidant effects of melatonin in a renal ischemia-reperfusion model[25-28].

In the present study, GSH levels were determined in renal tissue and erythrocytes as a marker of the antioxidant system. The erythrocyte level of this parameter was highest in the melatonin-supplemented group. In the ischemia-reperfusion group, however, tissue GSH level increased, while erythrocyte GSH level decreased. Elevated tissue GSH levels found after ischemia-reperfusion are believed to have resulted from the increased antioxidant system activity of the respective tissue in response to oxidative stress. As a matter of fact, tissue GSH levels have been reported to increase due to renal injury in a previous study[29]. However, it was established in another experimental study that tissue GSH levels decreased due to renal ischemia in rats[30]. In our study, tissue GSH levels increased, while erythrocyte GSH levels significantly decreased in relation to ischemia-reperfusion. In another experimental study, it was found that tissue GSH levels did not change significantly in renal ischemia-reperfusion injury in rats[31]. It is obvious that the results of studies of tissue GSH levels vary. This variation in the results may be attributed to differences in the experimental models used. In the present research, supplementation of melatonin and zinc, individually and in combination, for 3 weeks prior to ischemia-reperfusion brought about significant increases in tissue and erythrocyte GSH levels. However, the increase in erythrocyte GSH levels caused by melatonin alone was greater than the increase induced by zinc. Actually, it was demonstrated in previous studies that zinc supplementation directly restored renal ischemia-reperfusion injury[32], or zinc brought about this effect through the antioxidant enzymes in the structures of which it is involved[33,34]. The increased GSH levels recorded after 3-week zinc supplementation in our study are in harmony with the results of the studies cited above. One group in our study was supplemented with melatonin for 3 weeks prior to the induction of ischemia-reperfusion to examine the effects of the former on the latter. Melatonin supplementation in the respective group was found to significantly elevate GSH levels, particularly in erythrocytes. Melatonin, which is an extremely strong antioxidant and a molecule with free radical scavenging characteristics, has receptors in a variety of tissues, including those of the kidney[26,35]. A number of previous studies of renal ischemia-reperfusion have reported the protective effects of melatonin on tissues[19,20,36]. The present study explored the combined effects of zinc and melatonin in preventing ischemia-reperfusion injury. Although antioxidant GSH levels increased after the combined supplementation of the two molecules, this increase was not different from the increase brought about by individual supplementation of these molecules.

The results of the study indicated that supplementation of zinc and melatonin in advance, either individually or in combination, to cases at risk of renal ischemia-reperfusion injury may produce a protective effect by inhibiting the oxidative systems and activating the antioxidant systems in renal tissue and blood.

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Sažetak

UČINAK TROTJEDNOG DAVANJA SUPLEMENATA CINKA I MELATONINA NA OKSIDACIJSKI-ANTIOKSIDACIJSKI SUSTAV U EKSPERIMENTALNOJ BUBREŽNOJ ISHEMIJI-REPERFUZIJI KOD ŠTAKORA

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Bubrežna ishemija-reperfuzija izravno djeluje na glomerularni i tubularni epitel. Slobodni radikali kisika imaju značajnu ulogu u patofiziologiji bubrežne ishemijsko-reperfuzijske ozljede. Cilj ovoga ispitivanja bio je utvrditi učinke trotjednog davanja suplemenata cinka, melatonina i cinka + melatonina na tkivne i plazmatske razine malondialdehida (MDA) te na razine razine glutationa (GSH) u eritrocitima i tkivu štakora s eksperimentalno izazvanom bubrežnom ishemijsko-reperfuzijskom ozljedom. Ispitivanje je provedeno na Wistar albino štakorima srednje težine 250 g podijeljenim u sljedeće skupine: kontrolna, lažno kontrolna, ishemija + reperfuzija, cink + ishemija-reperfuzija, melatonin + ishemija-reperfuzija i cink + melatonin + ishemija-reperfuzija. Životinje su dobivale suplemente cinka i melatonina, 3 mg/kg/dan i.p. kroz 3 tjedna prije negoli je izazvana ishemija-reperfuzija. Bubrežna ishemija-reperfuzija izazvana je u lijevom bubregu u općoj anesteziji, a sastojala se od ishemije u trajanju od 45 minuta i reperfuzije u trajanju od 1 sata. Nakon zahvata životinje su žrtvovane, a uzorci krvi i bubrega uzeti su za analizu razina MDA i GSH. Vrijednosti GSH u bubrežnom tkivu i eritrocitima bile su povišene u skupinama koje su dobivale suplemente cinka i melatonina ($p<0,005$). Ispitivanje vrijednosti MDA u bubrežnom tkivu i plazmi pokazalo je da je ishemija značajno povisila ovaj parametar, dok je davanje suplemenata cinka i melatonina značajno inhibiralo vrijednosti MDA ($p<0,002$). Rezultati ove studije pokazali su da se oksidativno oštećenje u krvi i bubrežnom tkivu štakora povećalo uz ishemiju-reperfuziju, ali je davanje suplemenata cinka i melatonina prije ishemije-reperfuzije znatno smanjilo oksidativno oštećenje.

Ključne riječi: *Akutna ozljeda bubrega – prevencija i kontrola; Reperfuzijska ozljeda – prevencija i kontrola; Cink- terapijska primjena; Melatonin – terapijska primjena; Slobodni radikali; Modeli, životinjski; Štakori*