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The effects of zinc and melatonin on muscle ischaemi-reperfusion injury in rat

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Abstract: Ischemia-reperfusion leads to damage in cell or tissue due to insufficient blood flow. The aim of present study was to determine the effect of zinc, melatonin and zinc + melatonin supplementations during 3 weeks on muscle tissue and plasma MDA and GSH levels. This study was performed on 38 male Wistar-Albino rats. Experiments groups were designed as sham-control, ischemia-reperfusion (I/R), zinc + I/R, melatonin + I/R and zinc + melatonin + I/R Ischemia-reperfusion was induced by left femoral artery occlusion (1 hour) and reopening (1 hour). At the end of experiments tissue and blood samples were analysed for MDA and GSH. MDA levels were increased, GSH levels decreased in I/R groups. However, zinc and melatonin supplementation inhibited MDA and increased GSH levels in I/R groups. The results of present study show that increased lipid peroxidation in muscle tissue by ischemia-reperfusion may be prevented by zinc and melatonin or zinc plus melatonin supplementation.

Key words: Ischemia-reperfusion; Muscle; Zinc; Melatonin; Free radical.

Introduction

Resumption of blood and oxygen supply to the tissues or organs after ischemia is called reperfusion, and the damage caused in the period when blood supply is restored in the tissues or organs after ischemia is called reperfusion injury. Although there are a number of mechanisms involved in reperfusion injury, free radical derivatives figure prominently among the various factors (1,2). Reperfusion is a prerequisite to recovery the tissue or organ from ischemic damage. However, paradoxically, re-entry of molecular oxygen with reperfusion becomes the source of harmful oxygen radicals associated with lipid peroxidation and impairs the integrity of the cell membrane (3). So, reperfusion of ischemic tissues may increase cellular damage (4). Free radicals classified as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (5). Under normal conditions, free radicals and the antioxidant system remain balanced. However, when free radicals exceed the buffering capacity of antioxidants, oxidative stress occurs. These radicals may harm the organism by altering the structure and functions of molecules such as proteins, DNA, lipids, and carbohydrates. Oxidative stress plays a major role in the pathogenesis of diseases like hypertension and diabetes (6). Of the oxygen used in the oxidative mechanism, only a small portion is converted into highly reactive and toxic reactive oxygen species. However, when the concentration of the reactive oxygen species formed than higher than normal lead to cell organelles, membrane lipids, nuclear and mitochondrial DNA, and macromolecules like proteins (7). Preconditioning has affect oxidative damage and potential effects on tissue damage and functions.

Zinc is an essential element with a multitude of biological roles. An essential mineral for the human body, it serves various biological functions in anti-apoptotic, anti-inflammatory and antioxidant capacities (8,9).

Melatonin is released into the circulation as soon as it is synthesized. It is synthesized differently during the day and night, and its nocturnal synthesis is higher (10). It produces its biological effects through two transmembrane receptors (11).

The present study aims to determine the effects of individual and combined supplementation of zinc and melatonin during 3 weeks on lipid peroxidation in skeletal muscle ischemia-reperfusion injury in rats.

Materials and Methods

Experimental animals

After the approval of the Ethics Board of the Kombassan Experimental Medicine Research and Application Center of Necmettin Erbakan University (Resolution no: 2013-116) was obtained, the study was conducted at the same center. The study included 38 male Wistar-albino rats with a mean weight of 300 g. The rats were divided into five groups. All rats were kept in rooms with controlled temperature and light, and fed *ad libitum*.

Study groups were formed as follows:

1-Sham-control group (n=6): Left leg of rats were opened /closed under general anesthesia.

2-Ischemia-Reperfusion Group (I/R) (n=8): Under general anesthesia the animals was subjected to ischemia (1 hour) and reperfusion (1 hour) by clamping left femoral artery.

3-Zinc + Ischemia-Reperfusion Group (Zn + I/R) (n=8): Firstly supplemented with 3 mg/kg/day intraperitoneally zinc during 3 weeks and ischemia and reperfusion

was induced as described above.
4-Melatonin + Ischemia-Reperfusion Group (Mel + I/R) (n=8): Melatonin was supplemented with 3 mg/kg/day intraperitoneally melatonin during 3 weeks and following ischemia and reperfusion was performed.
5-Zinc + Melatonin + Ischemia-Reperfusion Group (Zn + Mel + I/R) (n=8): Following the supplementation of zinc and melatonin at equal doses during 3 weeks, I/R was induced, and tissue and blood samples were collected after the procedure.

Anesthesia

Rats were anesthetized with intramuscular (i.m.) ketamine hydrochloride (60 mg/kg) and Xylazine (rompun) (5 mg/kg),

Collection of Blood and Tissue Samples

Upon completion of experiments, the animals were sacrificed by rompun anesthesia, and 3 to 4 ml of blood samples were transferred to EDTA tubes. The erythrocyte in the sample was bathed in serum physiologic and stored for GSH analysis, while the plasma was stored for MDA analysis. Additionally, skeletal muscle samples (gastrocnemius) were kept at -80°C until the time of analysis to measure MDA and GSH.

Determination of tissue malondialdehyde (MDA) levels

Tissue MDA levels were determined using Uchiyama and Mihara (12) 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. The homogenized tissue was placed into 2 ml of 8% $HClO_4$ and centrifuged at 3000 rpm for 15 minutes. After 0.5 ml supernatant was added 3 ml of 1% H_3PO_4 and 1 ml of 0,675% TBA, it was incubated in a 90°C water bath for 45 minutes. When the mixture cooled down, it was added 4 ml of n-butanol 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.

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 according to Ellman's method. After 200 µl of supernatant was added 8 ml of phosphate buffer (pH 6,8), 78 µl of 1 N NaOH and 100 µl of Ellman's solution, the sample was left to wait for 5 minutes and its absorbance was tested against distilled water at 412 nm in the spectrophotometer. Activity was measured according to the following formula: $a = (A_{standard} / A_{sample}) \times C_{standard}$. The standard was taken to be $c_{standard}$ = 15,34 g/100 ml. The tissue protein was obtained using the biuret method and the values were presented as nmol/g protein (13).

Determination of plasma malondialdehyde levels

Blood samples put into EDTA tubes were centrifuged at 3000 rpm for 5 minutes and their plasma was separated. After 2.5 ml of 10% TCA (Merck catalogue no: 818 K02907810) was transferred into a tube, 0.5 ml of plasma sample was added. After being vortexes, the tubes were capped and left to incubate in a 90°C water bath for 1.5 minutes. Then they were cooled in cold water, and their absorbance against blank was read at 532 nm in the spectrophotometer. The results were presented as nmol/ml.

Erythrocyte glutathione analysis

Of the bathed erythrocyte 45 µl was taken and diluted with distilled water at a rate of 10%. After being added 10% sulfosalicylic acid, the mixture was kept in ice for one hour and then centrifuged at 4000 rpm for 3 minutes. Of the supernatant, 200 µl was taken, and added 8 ml of phosphate buffer with a pH value of 6,8, 78 µl of 1 N NaOH, and 100 µl of Ellman reagent. The mixture was left to wait for 5 minutes and then tested against the blind sample tube at 412nm.To prepare the Ellman 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. results were given as mg/dl.

Statistical analysis

SPSS software was used in the statistical evaluation. Data are presented as mean ± SD. Kruskal Wallis variance analysis was used in comparisons between groups, and Mann Whitney U test was employed for $p<0.05$ value. Values for which $p<0.05$ was accepted as significant.

Results

Tissue GSH values were presented at table 1. A comparison between groups showed that I/R groups supplemented with zinc and melatonin had the highest tissue GSH values ($p<0.05$). I/R group, on the other hand, had the lowest tissue GSH level. When tissue MDA is considered, the highest levels were found in the ischemia-reperfusion group ($p<0.001$). Other groups were not different in terms of their MDA levels.

When the erythrocyte GSH levels were compared, Group 5, Zn + Mel + I/R group, was seen to have the highest erythrocyte GSH level ($p<0.001$), I/R group, on the other hand, had the lowest erythrocyte GSH level ($p<0.001$, table 2). An examination of plasma MDA levels showed that this parameter had the highest plasma MDA levels ischemia-reperfusion group MDA level ($p<0.05$), while there was not any statistically signifi-

Table 1. Tissue GSH and MDA Levels in Experimental Groups.

Groups	GSH (nmol/g protein)	MDA (mg/g protein)
Sham-Control	0.15±0.06 *	0.12±0.06 *
Ischemia-Reperfusion Group (I/R)	0.12±0.04 *	0.49±0.31 **
Zinc+ I/R	0.26±0.04 **	0.09±0.03 *
Melatonin + IR	0.24±0.15 **	0.13±0.07 *
Zn+Melatonin + I/R	0.23±0.01 **	0.12±0.07 *

Zinc, melatonin or zinc + melatonin supplementtaion increased GSH levels while reduce MDA levels in mussle tissue was compared to sham-control (for GHS $P<0.05$; for MDA $P<0.001$).

Table 2. Erythrocyte GSH and Plasma MDA Levels in Experimental Groups.

Groups	Erythrocyte GSH (mg/dl)	Plasma MDA (nmol/ml)
Sham-Control	0.24±0.06 **	0.09±0.01 *
Ischemia-Reperfusion Group (I/R)	0.12±0.06 *	0.29±0.12 **
Zinc+ I/R	0.23±0.04 **	0.06±0.02 *
Melatonin + IR	0.19±0.05 **	0.09±0.03 *
Zn+Melatonin + I/R	0.27±0.04 ***	0.11±0.06 *

I/R caused significantly increase plasma MDA levels anc reduces erythrocyte GSH leves, However, Zinc, melatonin or zinc + melatonin supplementation inhibited MDA levels and increased erythrocyte GSH levels (GSH; $P<0.05$; MDA; $P<0.001$).

cant difference between the plasma MDA levels of the other groups.

Discussion

In this study, ischemia and then reperfusion was induced in the skeletal muscle of male rats that increased MDA level showed that ischaemia-reperfusion were done.

Ischemia-reperfusion can cause severe oxidative damage in the tissues of animals (14,15). Skeletal muscle injury can cause edema, and thus lead to acute compartment syndrome in the extremities (16). MDA is one of the most commonly used parameters as a marker of oxidative stress and tissue injury. Therefore, MDA levels were determined in both the muscle tissue and plasma in our study. In a study by Gurji et al. (17), it was established that ischemia induced by keeping the femoral artery in the hind leg clamped for 90 minutes and 4 hours of reperfusion exacerbated the damage in goats. Similarly, Kirişçi et al. (18) found that ischemia (120 min)-reperfusion (120 min) in the skeletal muscle of rats have elevated tissue MDA levels. Tong et al. (15) also established elevated MDA levels in muscle ischemia-reperfusion. In our study, too, significant increases were found in the levels of MDA as a marker of tissue damage in the gastrocnemius muscle tissue after 1 hour of ischemia and 1 hour of reperfusion, and this is consistent with the reports cited above. However, supplementation of zinc and melatonin (individually and in combination) for 3 weeks before ischemia-reperfusion significantly suppressed the elevated MDA levels both in the tissue and plasma and reduced them to control levels, with zinc administration reducing those levels even below those in the control group. Our results are supported by previous studies, which demonstrated that both zinc and melatonin prevented MDA increases associated with ischemia-reperfusion (19,20,21,22). Actually, the importance of zinc supplementation is further illustrated by the results of previous studies, which reported that zinc supplementation suppressed the increase in lipid peroxidation resulting from ischemia-reperfusion (23,24). Studies investigating the effect of melatonin therapy in ischemia-reperfusion showed that melatonin supplementation reduced organ injury. In their study, Erdem et al. (26) demonstrated that in skeletal muscle ischemia-reperfusion injury induced by clamping the femoral artery, 2 hours of ischemia followed by 1,5 hour of reperfusion significantly elevated MDA levels, which were then lowered by melatonin treatment. It was also shown by Wang et al. (27) that intravenous administration of melatonin 10 minutes before ischemia and 10 minutes after reperfusion significantly restored mitochondrial dysfunction associated with ischemia-reperfusion. In our study, zinc and melatonin was administered for 3 weeks, in a way that can described as chronic, through the intraperitoneal route and then ischemia-reperfusion was induced in the skeletal muscle to examine the changes in tissue and plasma levels of MDA as a marker of organ and system injury. Similar to the reports of the previous studies, initially, MDA values in both tissue and plasma were elevated significantly in association with ischemia-reperfusion injury. However, this increase was curtailed by 3-week zinc and melatonin supplementation in both tissue and plasma, indicating the capability of these two substances to prevent damage in the muscle tissue and plasma. These results are consistent with the results of previous studies (28,29).

GSH reduced levels were reduced in I/R groups both muscle tissue and erythrocyte, however zinc and melatonin supplementation increased GSH levels. Previous studies used different zinc preparations in muscle ischemia-reperfusion. Atahan et al. determined (30), the effect of zinc aspartate administration on long-term ischemia-reperfusion (3 hours of ischemia and 24 hours of reperfusion) and reported that the zinc supplementation elevated GSH levels. In another study, it was again found that zinc aspartate supplementation exercised a protective effect on the skeletal muscle by elevating glutathione levels (31).Bülbüloğlu et al. (32) demonstrated that 50 mg/kg zinc provided protection against oxidative stress in the small intestine tissue by increasing glutathione peroxidase. In our study, zinc sulfate and melatonin (3 mg/kg/day) supplemented for 3 weeks before ischemia-reperfusion in our experimental model was found to protection against oxidant damage by elevating the GSH levels in both the muscle tissue and erythrocytes. In another study, Yılmaz et al (33) reported that long-term zinc sulfate supplementation inhibited lipid peroxidation by increasing GSH amounts in the renal tissue.

Yılmaz et al. (33), for instance, established in their study that melatonin protected renal tissue against oxidative stress caused by ischemia-reperfusion. Melatonin produces this effect by elevating GSH levels was shown in the heart tissue by Liu et al. (34), liver by Deng et al. (35), and spinal cord injury by Aydemir et al. (36). However, it should be noted that erythrocyte GSH levels were the highest in group 5 (zinc + melatonin). This suggests that combined supplementation is more effective than individual supplementation in elevating erythrocyte GSH values.

The results of present study show that muscle I/R leads to oxidant damage in rats. However, zinc, melatonin or zinc + melatonin supplementation prevent oxidant damage by increasing GSH levels.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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