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Melatonin prevents ischemia – reperfusion injury following superior mesenteric artery occlusion in the rat

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ABSTRACT

Background: Free radicals derived from molecular oxygen have been reported to be responsible for changes in motility and mucosal damages observed in intestinal Ischemia-Reperfusion (I/R) injury. Melatonin has been considered as an antioxidant that prevents injuries resulting from Ischemia/Reperfusion in various tissues. This study was designed to determine the effects of melatonin at a dose dependent manner in intestinal I/R damages by contractile responses of Malondialdehyde (MDA), a product of lipid peroxidation in rats.

Material and methods: A total of 36 young male Wistar – Albino rats (80 – 120 g) were divided equally in to 6 groups and subjected to different concentration of melatonin (10, 20, 30 mg/Kg) .Group 1 was control, group 2 was sham that were subjected to surgical process for Superior Mesenteric Artery (SMA) dissection. Groups 3, 4, 5 and 6 were I/R that were given melatonin at 0, 10, 20 and 30 mg/kg respectively. After laparatomy, a microvascular traumatic clip was placed across the SMA under general anesthesia, and following ischemia for 30 minutes it was removed. The first dose of melatonin was administrated before, and the second dose was administrated just after reperfusion, and the third dose was administrated on the second day, all by intramuscular route. On the third day of the experiment all rats were killed, and their bowels were removed.

Results: The levels of tissue malondialdehyde were found to be significantly lower in group 4 compared to group 3 ($P < 0.05$).There was significant differences in histopathological patterns of group 4 compared with group 3 ($P < 0.01$). MDA levels, in groups 5 and 6, showed no significant changes in comparison to I/R group.

Conclusion: These results showed that Melatonin at dose of 10 mg/Kg has antioxidant effects and prevents rat intestinal ischemia – reperfusion damages.

Keyword: Melatonin, Ischemia-Reperfusion, Superior Mesenteric Artery, Malondialdehyde.

INTRODUCTION

Ischemia–reperfusion injury of the intestine is an important factor associated with a high morbidity and mortality in both surgical and trauma patients (1).The temporary interruption of blood flow to an organ followed by reperfusion of the tissue with oxygenated blood is highly destructive for cells which are affected. This process, which is generally referred to as ischemia/reperfusion, can happen in any organ, and might result in the heart attack or stroke which are especially devastating and often lead to death of the individual. Although blood flow continues in tissues, its interruption in intestine for a short period of time results in, severe hypoxia and ischemic lesions can occur at the end of villi (2). Intestinal I/R is accompanied with decreased contractile activity, increased microvascular permeability and dysfunction of mucosal barrier (1). Two mechanisms have been suggested for the development of post ischemic mucosal damages, enhanced generation of oxygen radicals and activation of phospholipase A_2. Oxygen-free radicals cause epithelial damages largely through peroxidation of lipid membranes, and they may also contribute to tissue damages by attraction neutrophils (2-3) and exaggeration in release of eicosanoid (4). Both of these pathways lead to the accumulation and activation of neutrophils in intestinal tissue. These cells are largely responsible for the formation of severe mucosal lesions (5). Free radicals derived from molecular oxygen have been reported to be responsible for changes in motility and mucosal damage observed in intestinal ischemia-reperfusion injury. Melatonin, which is secreted in circadian rhythm from the pineal gland

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as an endogenous hormone, is synthesized and secreted in retina, salivary gland, liver and intestine as well. Receptors for melatonin have been identified in the digestive system; therefore the indolamine might play a leading role in different aspects of the vast digestive physiopathology. The hormone may interact with receptors and subsequently stimulates the synthesis of gastroprotective hormones, exerts a direct defense for the epithelium, enhances submucosal blood flow and prevents the damages induced by ischemia followed by reperfusion. Unlike the classical antioxidants, melatonin is devoid of prooxidative activity and all known intermediates generated by the interaction of melatonin with reactive species are also free radical scavengers. This phenomenon is defined as the free radical scavenging cascade reaction of the melatonin family. Due to this cascade, one melatonin molecule has the potential to scavenge up to 4 or more reactive species. This makes melatonin very effective as an antioxidant. Under in vivo conditions and at equivalent doses, melatonin is often several times more potent than vitamin C and E in protection of tissues from oxidative injury (micromole/kg) (6). This study was designed to determine the effects of melatonin at a dose dependent manner in intestinal I/R damages by contractile responses of Malondialdehyde (MDA), a product of lipid peroxidation in rats.

MATERIAL AND METHODS

This study was performed on 36 male Wistar – Albino rats (weighing 80 to 120 g). They were kept in individual cages at rooms of 20 –to 25ºC, humidity of 70- 80 %, and 12-h day and light cycle one week before experimentation. The rats had free access to water and were pair-fed with standard chow. Animals were fasted for 12 h before the experiment but had free access to water.

Studies on all groups were made at the same time. After the experiments animals had free access to both water and food. Animals were divided randomly into 6 groups each of six rats. Group 1 was control; group 2 was sham that was subjected to surgical process for superior mesenteric artery (SMA) dissection. Groups 3, 4, 5 and 6 were I/R that were given 0, 10, 20 and 30 mg/kg of melatonin respectively. After laparatomy, a microvascular traumatic clip was placed across the superior mesenteric artery (SMA) under general anesthesia, and after ischemia for 30 minutes it was removed. The first dose of melatonin was administrated before, the second dose was administrated just after reperfusion, and the third dose was administrated on the second day all by intramuscular route

Preparation of melatonin solution

Under sterile conditions, 48 mg of melatonin was dissolved in 1ml of absolute ethanol and solution diluted by isotonic sodium chloride (0.9 % NaCl) to final concentration of 1: 10.

Mesenteric ischemia and reperfusion

The rats were anesthetized by intraperitoneal injection of 10% ketamine hydrochloride (4 – 5 mg / kg) and 2 % xylazine (1 mg / kg). A midline laparatomy was performed after shaving and local cleansing with antiseptic solution. Intestines were deviated to the left, and the superior mesenteric artery (SMA) was dissected carefully and then occluded by atraumatic micro vascular clip (ischemia). The circulation of the SMA was stopped for 30 minutes and after removal of the clamp, circulation was restarted (reperfusion). Just before reperfusion, the first dose of melatonin was administrated intramuscularly (IM) to groups 4, 5 and 6. Following reperfusion, the second doses of melatonin were administrated to groups 4, 5 and 6.Third dose of melatonin was administrated to groups 4, 5 and 6 in the second day.

Histopathology

Intestines of animals from duodenum up to the middle of the colon were removed and intestinal intraluminal contents were rinsed with 0.9 % NaCl. A sample of 1 cm in length for each part of duodenum, jejunum, ileum and colon were taken for histopathological study. The samples were bathed in 10 % formalin solution and blocked in paraffin. The tissue samples were stained with H&E and evaluated by light microscopy in a blinded fashion. A total of 20 microscopic slides were examined for each parameter.

Histopathological examination of reperfused intestinal tissue was performed by employing a standard staging method described by Hierholzer et al (7), with minor modification: Grade 0: Normal mucosal villi. Grade 1: Development of a subepithelial space at the tip of the villus with capillary congestion. Grade 2: massive development of a subepithelial space at the sides of villus. Grade 3: Denuded villi with disintegration of lamina propria, hemorrhage, and ulceration.

Determination of Malondialdehyde (MDA)

The remaining intestinal tissues were used for lipid peroxidation process. Determination of Malondialdehyde (MDA), which is one of the last products of lipid peroxidation in the homogenates

preparation, was assessed spectrophotometrically according to the method of Esterbauer (8).

Statistical analysis

The results obtained from the groups were calculated as mean ± SD, and the analysis of variance (ANOVA) was used to evaluate the tissue MDA results. The histopathologic results were analyzed by Mann–Whitney U test. The level of statistical significance was accepted as P less than 0.05.

RESULTS

Tissue MDA results

The mean MDA level was higher in I/R than control group (0.8178 ± 0.2818 vs 2.3800 ± 0.3203, respectively, P≤0.05). The levels of tissue MDA were found to be significantly lower in group I/R + Melatonin (10 mg/Kg) compared to group I/R (P≤ 0.05).

In the Control and Sham groups, MDA levels were not significantly different (0.8178 ± 0.2818 and 1.3530 ± 0.3570, respectively, P≤0.05).

MDA level in groups I/R +Melatonin (20 mg/Kg) and I/R + Melatonin (30 mg/Kg) were lower than I/R group, but there was no significant differences between them(Table 1).

Histopathologic Results

The histopathological results were assessed in duodenum, jejunum, ileum and colon of the rats. There were significant differences in the histopathologic results of duodenum between I/R, I/R + Melatonin (10 mg/kg) and I/R + melatonin (20 mg/Kg), (P ≤ 0.01) and also between I/R and control groups (P ≤ 0.01). The differences in histopathological scores in groups of control and sham (P ≥ 0.05) and between I/R + melatonin (30 mg/Kg) and I/R groups (P ≥ 0.05) were not significant statistically.

Histopathological results of jejunum and ileum between I/R and I/R + Melatonin groups showed a significant difference (P ≤ 0.01), whereas, the same relationship was not observed in groups I/R + melatonin (20 mg/Kg), I/R + melatonin (30 mg/Kg) and I/R (P ≥ 0.05; Tables 3 and 4, Fig 1-4).

Histopathological scores of colon between treatment group (melatonin) and I/R, were not significantly different (P ≥ 0.05; Table 5, Fig 1-4).

DISCUSSION

The mechanism of hypoxia-induced intestinal damage is not clear, but ischemic mucosal damage takes place as the results of many factors (2). The damages formed on the surface of mucosa are followed by transmucosal and transmural damages (2).Damages occurring after reperfusion are more sever than those induced by ischemia (2, 4) even in cat's small intestine permeability increasing (4).

This study showed a significant protection of melatonin against I/R - induced intestinal injury, small bowel I/R increased intestinal MDA levels, indicating that OFRs are produced as a result of I/R, induced lipid peroxidation in rat ileum. Melatonin (10 mg/kg) treatment appeared to be protective against damages observed in I/R via inhibition of lipid peroxidation, as confirmed by a reduction of MDA. The higher dose of melatonin (20, 30 mg/kg) was ineffective in terms of reduction in lipid peroxidation. Some recent studies have shown that melatonin treatment abolished the increase in MDA levels probably by scavenging the reactive oxygen metabolites and inhibition of lipid peroxidation. These finding are consistent with other reports which show that melatonin readily protects the gastric and intestinal mucosa from the damages caused by a variety of agents such as absolute ethanol (9) stress (10) or ischemia – reperfusion (10-14). A number of reports have shown that melatonin is as effective as an antioxidant in the various models of oxidative stress at the dose of 10 mg/kg dose (10, 15) and reduces MDA content toward control levels (16). In these studies, however, melatonin was administered more than one dose with prolonged reperfusion period during the experimental procedure. On the other hand, we have observed that a single dose of 10 mg/kg was sufficient to ameliorate the I/R-induced damages in the present model of intestinal I/R.

This study demonstrated that pre-treatment with melatonin lowered mucosal damages at histopathological level. Melatonin can pass through the plasmatic membrane; may activate nuclear receptors. Moreover, the same antioxidant and anti-inflammatory role of melatonin in chronic vascular disease, atherosclerosis, renal, skeletal muscle, liver, spinal cord, skin and pancreas ischemia have been reported (17-23). Also it has been found that melatonin at the dose of 50 mg/kg improved smooth muscle response, which was reduced as a result of injury during the intestinal I/R (24).

The antioxidative mechanisms of melatonin seems to be different from classical antioxidants such as vitamin C, vitamin E and glutathione. As electron donors, classical antioxidants undergo redox cycling; thus, they have the potential to promote oxidation as well as prevent it. Melatonin, as an electron-rich molecule, may interacts with free radicals via an additive reaction to form several stable end-products which are excreted in the urine. Melatonin does not undergo redox cycling

Table 1. Malondialdehyde levels of the intestinal tissues of the study groups

Group	No	MDA (µg/µl)
1	5	0.8178 ± 0.2818
2	4	1.3530 ± 0.3570**
3	6	2.3800 ± 0.3203
4	5	1.3140 ± 0.2639*
5	6	1.4190 ± 0.07060
6	5	1.6860 ± 0.1447

Values are expressed as mean ± SD.
Group 1- Control, Group 2- Sham, Group 3- I/R Group, 4- I/R + Melatonin (10 mg/Kg)
Group 5- I/R+ Melatonin (20 mg/Kg), Group 6- I/R + Melatonin (30 mg/Kg)
* There were significant differences in comparison with I/R group (P< 0.05).
**There were no significant differences in comparison with Control group (P< 0.01).

Table 2. Results of the duodenum histopathological analysis of the study groups

Grade	Group 1 (n=5)	Group 2 † (n=4)	Group 3 †† (n=6)	Group 4 ** (n= 5)	Group 5 * (n = 6)	Group 6 (n = 5)
0	3	3	-	3	3	-
1	2	1	-	2	2	2
2	-	-	2	-	-	-
3	-	-	4	-	-	3

† There were no significant differences in comparison with control group (group 1) (P > 0.05).
†† There were significant differences in comparison with control group (Group 1) (P< 0.01).
* There were significant differences in comparison with I/R group (Group 3) (P < 0.05).
** There were significant differences in comparison with I/R group (Group 3) (P < 0.01).

Table 3. Results of the Jejunum histopathological analysis of the study groups.

Grade	Group 1 (n=5)	Group 2 † (n=4)	Group 3 †† (n=6)	Group 4 ** (n= 5)	Group 5 (n = 6)	Group 6 (n = 5)
0	5	4	-	3	2	1
1	1	1	-	1	1	-
2	-	-	1	-	-	-
3	-	-	5	1	3	5

† There were no significant differences in comparison with control group (Group 1) (P > 0.05).
†† There were significant differences in comparison with control group (Group 1) (P < 0.01).
** There were significant differences in comparison with I/R group (Group 3) (P < 0.01).

Table 4. Results of the ileum histopathological analysis of the study groups

Grade	Group 1 (n=5)	Group 2 † (n=4)	Group 3 †† (n=6)	Group 4 ** (n= 5)	Group 5 (n = 6)	Group 6 (n = 5)
0	5	4	-	4	3	1
1	1	1	3	1	2	2
2	-	-	1	-	-	1
3	-	-	-	-	1	1

† There were no significant differences in comparison with control group (Group 1) (P < 0.05).
†† There were significant differences in comparison with control group (Group 1) (P < 0.01).
** There were significant differences in comparison with I/R group (Group 3) (P < 0.01).

Table 5. Results of the colon histopathological analysis of the study groups.

Grade	Group 1 (n=5)	Group 2 † (n=4)	Group 3 (n=6)	Group 4 (n= 5)	Group 5 (n = 6)	Group 6 (n = 5)
0	5	4	1	3	3	2
1	1	-	5	2	2	3
2	-	-	-	-	-	-
3	-	-	-	-	-	-

† There were no significant differences in comparison with control group (Group 1) (P > 0.05).

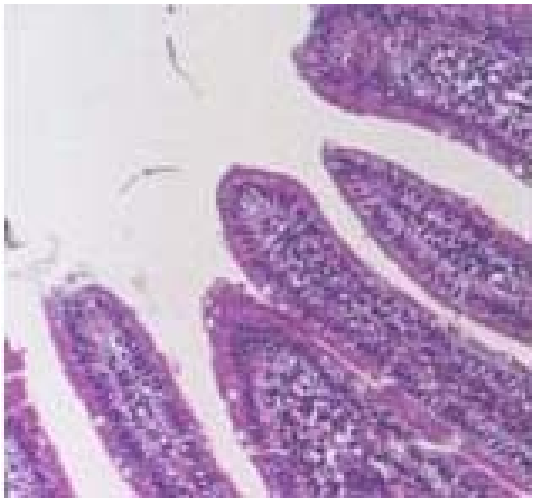

Figure 1. Light microscopic visualization of the jejunum in rat. Normal (grade 0). Haematoxylin and Eosin, magnification 200 X.

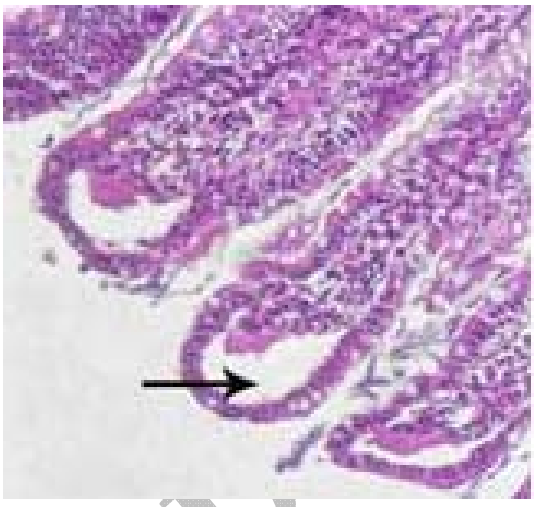

Figure 2. Light microscopic visualization of the intestine of rat. Black arrow show subepithelial space at the tip of villus (Grade 1). Haemato-xylin and eosin, magnification 200 X.

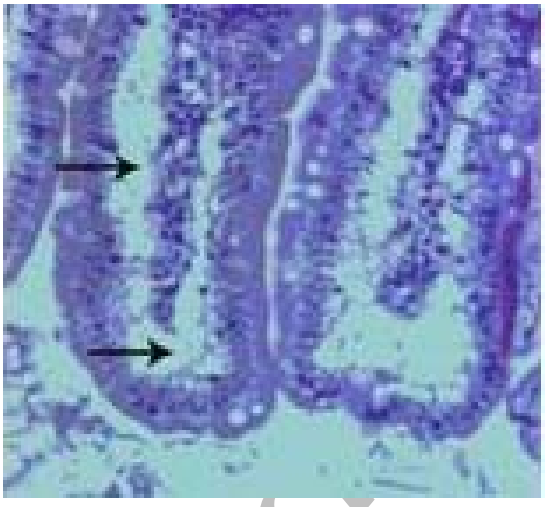

Figure 3. Light microscopic visualization of the intestine of rat. Black arrow show massive development of a subepithelial space at the sides of villus (Grade 2). Haematoxylin and eosin, magnification 200 X.

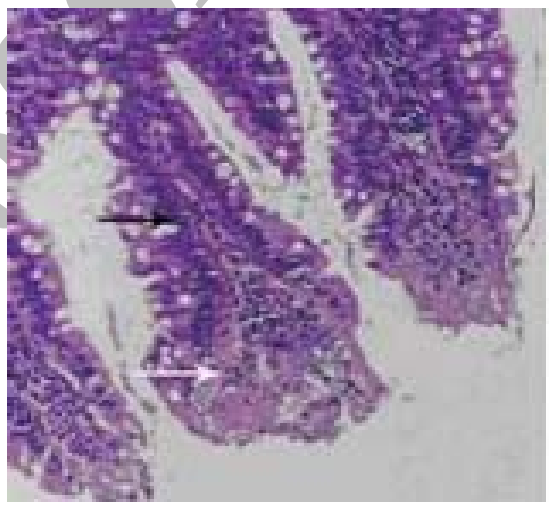

Figure 4. Light microscopic visualization of the intestine of rat. White arrow shows disintegration of lamina propria and black arrow shows ulceration and hemorrhage (Grade 3). Haematoxylin and eosin, magnification 200 X.

and, thus, does not promote oxidation as shown under a variety of experimental conditions. From this point of view, melatonin can be considered a suicidal or terminal antioxidant which is distinguished from the opportunistic antioxidants. Interestingly, the ability of melatonin to scavenge free radicals is not by a mole to mole ratio and indeed, one melatonin molecule scavenges two HO (25). Experimental evidences suggest that the generation of oxygen–derived free radicals is significantly responsible for I/R injury in gastrointestinal tissues. Biochemical mechanisms which allow these radicals to be produced include the XO system, which is modified during ischemia. On reperfusion XO catalyzes conversion of hypoxanthine to uric acid and releases superoxide radicals and H_2O_2. These oxygen radicals may be converted to the highly cytotoxic hydroxyl radical. This initiates the process of lipid peroxidation and release of substrates that recruit and activates polymorph nuclear leukocytes. Evidence supporting the involvement of XO as source of reactive oxygen metabolites is provided by studies in which tissue is depleted by administration of tungsten supplemented, molybdenum – deficient – diet (1, 26). In addition, this assumption is supported by studies which showed that XO inhibitor such as

allopurinol or oxypurinol, attenuate I/R injury. The results of this study clearly show that the protective effects of the hormone against I/R is linked to its ability to activate the XO system consequently to reduce superoxide anion generation. Protective effects of melatonin against ischemia/reperfusion induced by oxidative organ injury in the rat has been reported. (17, 25, 27).

CONCLUSION

From the result of this study it may concluded that treatment with melatonin at the dose of 10 mg/kg beforehand may ameliorates structural and functional damages observed in an experimental I/R, by reduction in inflammation and inhibition of lipid peroxidation. Further studies are required for a better understanding of the exact physio-pathological mechanism responsible for the protective effects of melatonin in I/R damages.

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