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The Effect of Melatonin on Relaxation Responses of Isolated Ileal Smooth Muscle in Ischemia-Reperfusion Injury in Rats

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Abstract: The aim of this study was to determine the effect of melatonin, a hormone that is known as an antioxidant, on functional responses of isolated ileal smooth muscle in rat intestinal ischemia-reperfusion. A total of 48 Sprague-Dawley rats were divided equally into 6 groups: Group 1 was control, Group 2 was sham-operated, Group 3 was ischemia, Group 4 was ischemia-reperfusion (IR), Group 5 was ischemia plus melatonin (IM) and Group 6 was ischemia-reperfusion plus melatonin (IRM). A dose of 10 mg kg^{-1} melatonin was administered intraperitoneally. Thirty minutes of intestinal ischemia was followed by 90 min of reperfusion. Rats were sacrified after removal of ileal segments. Ileal segments were placed in isolated organ baths and relaxation responses for adrenaline were recorded. Additionally, Ileal segments were examined histopathologically and biochemically. Comparisons of relaxation adrenaline responses of sham-operated group with ischemia group and IR group were found significant (respectively $p<0.05$, $p<0.01$). The comparison of IR group and IRM group was found significant ($p<0.05$). The most extensive changes in morphology were detected in I/R group. In sections from this group, there were inflammation and necrosis in areas throughout the thickness of the intestinal wall (Grade 3.5±0.189). The IRM group was histopathologically graded as 1.375±0.324. According to malondialdehyde values, I/R resulted in approximately 3 fold increase in MDA content of intestinal homogenates which is significantly different from that measured in control homogenates ($p<0.01$). Melatonin-treated groups were statistically indistinguishable from the control and sham-operated groups in terms of tissue MDA content ($p>0.05$). These results suggest that the 10 mg kg^{-1} of melatonin not only physiologically but also biochemically and morphologically could be useful to normalize contractility injured by oxidative stress in intestinal ischemia-reperfusion.

Key words: Ischemia-reperfusion, melatonin, isolated ileum, rat

INTRODUCTION

Ischemia/reperfusion (I/R) damage is a phenomenon often confronted is surgical pathologies such as shock, intussusception, incarcerated hernia and fibrous adhesions, which mechanically occlude mesenteric vessels and it is quite important because it causes severe clinical pathologies by causing destruction in close and far tissues[1]. A mean effect of the I/R damage is formed at the reperfusion phases and free oxigen radicals that appeared in the reoxygnized tissue are held responsible for this mechanism[2,3] .

Melatonin, which is secreted in circadien rhythm from the pineal gland as an endogenous hormone, is synthesized and secreted in retina, salivary gland, liver and intestines as well[4,5]. It has been demostrated that melatonin reduces lipid peroxydation, scavenges the hydroxyl radical, which is a potent initiator of lipid peroxidation and the peroxyl radical, which propagates the process of lipid peroxidation[6,7]. Moreover, peroxynitrite has also been shown to be directly scavenged by melatonin[8]. Another effect of melatonin is to stimulate the activity of the endogenous antioxidant enzyme glutathione peroxydase, which may be due to the effect of the hormone in removing hydrogen peroxide[9].

It has also been reported that melatonin treatment has protective effect of melatonin on contractile activity and oxidative injury induced by ischemia and reperfusion of rat ileum, rat corpus cavernosum and against sepsis-induced functional and biochemical changes in rat ileum and urinary bladder[10-12]. Based on these findings, we investigated the effects of melatonin on ischemia-reperfusion induced relaxant changes in a rat model.

MATERIALS AND METHODS

Animals: This study was performed on 48 adult male Sprague-Dawley rats (weighing 250-450 g). They were

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hosed in aroom at a mean constant temperature plus or minus standard error of mean (SEM) of 22±2°C with a 12 h light-dark cycle. The rats were fed with standard rat food and tap water until experimentation. Twelve hours before the experiments the rats were stopped feeding but allowed free access to tap water. Studies on all groups were made during the same hours. The Dicle University Animal Research Committee approved all experimental procedures. The animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals formulated by the National Institute of Health (NIH publication No: 85-23, revised 1985).

Preperation of drug: Under steril condition, melatonin melatonin (Sigma Chemical, St Louis, MO) was dissolved freshly in pure ethanol and later was liquidized with isotonic sodium chloride (0.9% NaCl) amounting to a final concentration of 1:10 in a freshly prepared solution form.

Experimental design: The rats were anesthetized intraperitoneally with 100 mg kg^{-1} ketamine. A midline laparatomy was performed after shaving and local cleansing with antiseptic solution. Intestines were exteriorized gently to the left onto a moist gauze; then, the superior mesenteric artery (SMA) was dissected carefully and then occluded by atraumatic microvascular clamp. Afterwards, the intestines were returned to the abdomen, which was then closed temporarily with 3-0 silk suture. Following 30 min of occlusion time, a relaparatomy was performed and the microvascular clamp on the artery was removed for 90 min reperfusion. Thirty minutes before reperfusion, melatonin was applied intraperitoneally in groups 5 and 6. All animals were sacrificed by cervical dislocation. Ileum was immediately removed and intestinal intraluminal contents were rinsed with previously aerated (95% O2 and 5%CO2) Tyrode's solution (124.9 mM NaCl, 2.6 mM KCl, 23.8 mM $NaHCO_3$, 0.5 $MgCl_2$, 0.4 mM NaH2PO4, 1.8 mM CaCl2 and 5.5 mM glucose). A 1 cm long specimen was taken for histopathologic study and for lipid peroxidation measurement.

***In vitro* organ bath experiments:** Ileal tissue was placed in Tyrode's solution. Full thickness of ileal tube strips (15x20 mm) were prepared and mounted in 20 mL organ bath containing Tyrode's solution. The solutions were aerated continuiously with 95%O2 - 5%CO2 at 37°C. The tissues were equilibrated for 60 min under a resting tension of 1 g[13]. In this period, strips were washed out once in every 15 min. Isometric forces were recorded by an external force displacement transducer (FDT-10A, Commat Iletisim Co, Ankara, Turkey) using MP 30 software (MP30 Biopac Systems Inc., Santa Barbara, CA, USA). After a 60-minute period of equilibration, 10^{-7} M adrenaline was added to the organ bath and relaxation responses was obtained. The strips were washed out three times in 5 min intervals. Same procedures were repeated for 10^{-6} M and 10^{-5} M adrenaline. Then, maximum reference relaxation responses were recorded with $BaCl_2$. The recorded results were expressed as gram. These results were expressed as percentage of the maximal relaxation induced by $BaCl_2$.

Forty eight male rats were divided randomly into six groups each consisting of eight rats. In group 1 (control), laparatomy was done, ileum was removed and ileal strips were prepared and mounted in 20 mL organ bath containing Tyrode's solution. In group 2 (sham-operated), surgical process was applied until SMA dissection and waited for 30 min. In group 3 (ischemia group), ischemia process was applied. In group 4 (IR group), I/R process was applied to the rats. In group 5 (MI), 10 mg kg^{-1} melatonin in addition to ischemia process was applied. In group 6 (IRM), 10 mg kg^{-1} melatonin in addition to I/R process was applied.

Histopathologic study: The samples of full-thickness segments of ileum were fixed in 10% formalin solution and embedded in parafin. The tissue samples were stained with H and E for morphological analysis and were evaluated by light microscopy in a blinded fashion. Histopathological examination of reperfused intestinal tissue was performed by employing a staging method described by Hierholzer *et al.*[14] and thus, semiquantitative histological evaluation was graded from 0 to 4. In grade 0, no specific pathological changes are observed: Normal architecture of gut wall, including villi, crypts, lamina propria and muscularis externa. In grade 1, mild mucosal damage is assesed: Denudation of villi epithelium, otherwise normal structure. In grade 2, moderate damage occurs: Loss of villus length and epithelial sloughing with evidence of congestion, hemorrhage and inflammation in the mucosa, but no change in submucosa or muscularis externa. In grade 3, an extensive damage is observed: Loss of a large number of villi including denudation, sloughing and the presence of granulomatous tissue with the damage localized to submucosa and muscularis. In grade 4, there is a severe damage and necrosis: Inflammation and necrosis in areas throughout the thickness of the intestinal wall.

Determinaton of tissue malondialdehyde: The determination of MDA, which was one of the last products of lipid peroxidation in the homogenates prepared, was assessed spectrophotometrically with the method defined by Ohkawa *et al.*[15]. This method is based

on the fact that when the lipid peroxidation product malondialdehyde (MDA) enters reaction with thiobarbituric acid, it forms a pink, maximum absorbent complex at 532 nm wavelenght. The MDA results were specified as nmol mg^{-1} tissue.

Statistical analysis: The results obtained in our study were calculated as an mean±standard deviation (SD). For statistical evaluation, the Kruskal-Wallis was used as variance analysis. As a statistical significance test, Mann Whitney U test was employed. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Isolated ileum responses: The relaxation responses were compared among groups and showed in Table 1 and Fig. 1.

There was no statistical difference between control and sham-operated groups in any concentration ($p>0.05$). The relaxation responses of control group and ischemia group were compared and found statistically significant in any concentration ($p<0.01$). Similar results was found among sham-operated group and ischemia group, but this difference was lower in the concentration of 10^{-7} M adrenalin ($p<0.05$). The relaxation responses of control and sham-operated groups were statistically different from those of ischemia-reperfusion group ($p<0.01$).

Relaxant responses to all doses of adrenalin in the sham-operated group were not different compared with those of the ischemia melatonin and ischemia reperfusion melatonin groups ($p>0.05$). Similarly, the comparison of relaxant responses were not different between ischemia and ischemia reperfusion groups ($p>0.05$). Relaxant responses in the melatonin treated ischemia group weredifferent those of untreated ischemia group ($p<0.05$ at a dose of 10^{-7} M; $p<0.01$ at the other doses).

Relaxant responses to all dose of adrenalin in the melatonin treated ischemia-reperfusion group were different compared with those of the untreated ischemia-reperfusion group ($p<0.05$).

Histopatological findings: Based on the histopathalogical analysis of 8 sections for each group, grading scores were calculated and analyzed statistically as demonstrated on Table 2.

In control and sham-operated groups, no any change was detected as depicted on Fig. 2a, grading as 0. In ischemia group, there was loss of a large number of villi including denudation, sloughing and the presence of granulomatous tissue with the damage localized to submucosa and muscularis. The most extensive changes in morphology were detected in I/R group. In sections from this group, there is a severe damage and necrosis: Inflammation and necrosis in areas throughout the thickness of the intestinal wall (Grade 3.5±0.189) as seen Fig. 2b. In IM group, the lengthening of villi and recovery of epithelial structure were seen. The IRM group was histopathologically graded as 1.375± 0.324 (Fig. 2c).

Table 1: The recorded relaxation responses

	Relaxation responses (%)		
	Adrenalin doses		
Groups	10^{-7} M	10^{-6} M	10^{-5} M
Control*	59±10.227	74±6.766	89±6.051
Sham-operated	33±03.036**	59±3.852	77±7.220
Ischemia***	24±02.795	40±2.831	54±5.227
IR	19±01.869	34±2.411	42±2.996
IM	35±04.273	55±2.025	70±4.256
IRM****	32±03.662	45±3.417	61±7.766

IR: ischemia-reperfusion; IM: ischemia+melatonin; IRM: ischemia-reperfusion+melatonin; * Between Control ve Sham-operated groups there was no statistical significance ($p>0,05$). Control group was statistically different compared those of both ischemia and IR groups in any dose of adrenaline ($p<0,01$). Sham-operated group was different compared with ischemia group. This difference was lower in the concentration of 10^{-7} M adrenalin ($p<0.05$). Sham-operated group was significantly different compared with IR ($p<0.01$). Ischemia group had lower difference in lower doses of adrenaline (at a dose of 10^{-7} M), and higher difference at other doses of adrenaline compared with IM group ($p<0.05$; $p<0.01$). The comparison of IR and IRM was found significant ($p<0.05$). Each group consists of 8 rats. Values were expressed as percentage of the maximal contraction induced by $BaCl_2$ and shown as mean±SD

Table 2: Semiquantitative histological grading of cross-sections of the rat ileum (8 sections/group)

Groups	Mean±SD
Control	$0.0±0.0^{a}$
Sham-operated	$0.375±0.183^{b}$
I	$2.375±0.324^{c}$
IR	$35.0±0.189^{d}$
IM	$0.75±0.25^{e}$
IRM	$1.375±0.324^{f}$

(a,b) p=0.063, (a,e) p=0.01, (b,e) p=0.263, (b,f) p=0.021, (c,d) p=0.015, (c,e) p=0.003 (a,c), ((a,d) p<0.000; (a,f), (b,c), (b,d), (d,f) p<0.001

Table 3: Values of MDA measured in each experimental group as mean±SD

groups	MDA nmol g^{-1} tissue
Control	$19.26±6.163^{a}$
Sham-operated	$18.33±4.866^{b}$
Ischemia	$39.06±3.720^{c}$
Ischemia-reperfusion (IR)	$55.73±3.100^{d}$
Ischemia + melatonin (IM)	$19.19±2.384^{e}$
IR + melatonin (IRM)	$24.50±2.486^{f}$

(a,d), (b,d), (c,d), (c,e), (e,f) p<0.01; (a,c), (b,c) p<0.05; (a,e), (b,e), (a,f), (b,f) p>0.05

MDA levels: MDA contents of homogenates obtained from intestinal samples of control animals averaged 19.26±6.163 nmol g^{-1} tissue, while those obtained from animals subjected only I/R was found to be 55.73±3.100 nmol g^{-1} tissue Table 3. I/R resulted in approximately 3 fold increase in MDA content of

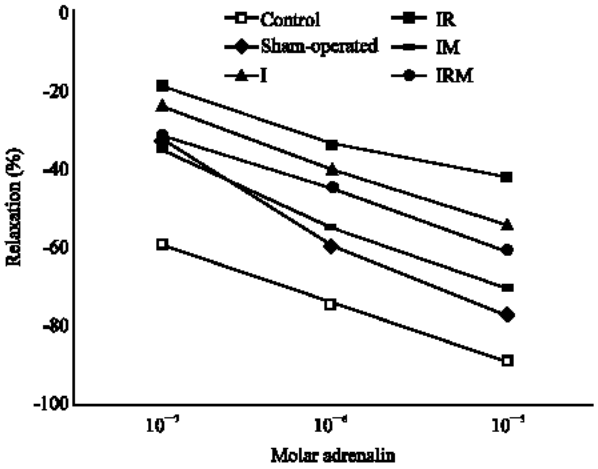

Fig. 1: Relaxant responses of groups: Best relaxant responses are seen in control and sham-operated groups although relaxant responses in the IR group are worse than those in the ischemia group. Relaxant responses to all dose of adrenalin in the melatonin treated ischemia and ischemia-reperfusion groups were different compared with those of the untreated ischemia and ischemia-reperfusion groups ($p<0.05$)

intestinal homogenates which is significantly different from that measured in control homogenates ($p<0.01$), while iskemi resulted in approximately 2 fold increase in MDA content of intestinal homogenates which is different from that measured in control homogenates ($p<0.05$). Similar results were found in sham-operated group. It was very interesting to observe that melatonin administration significantly reduced the intestinal MDA content to the control levels. It was very interesting to observe that melatonin administration in IM and IRM groups in 30 min before the reperfusion significantly reduced the intestinal MDA content to the control levels. Melatonin-treated groups were statistically indistinguishable from the control and sham-operated groups in terms of tissue MDA content ($p>0.05$). The comparison of ischemia group with ischemia-reperfusion and ischemia + melatonin groups were statistically significant ($p<0.01$). Similarly, the comparison of I/R and IRM was found to be statistically significant ($p<0.01$).

DISCUSSION

Our results showed that intestinal I/R resulted in a decreased ileal relaxation in response to adrenalin induction. Administration of 10 mg kg^{-1} melatonin appeared to be improving the reduced relaxant responses and returned them both to control and sham-operated

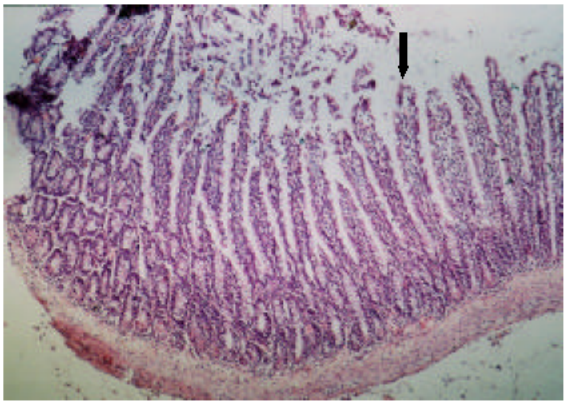

Fig. 2a: Control group (normal villus) (H-E X 100) Grade 0

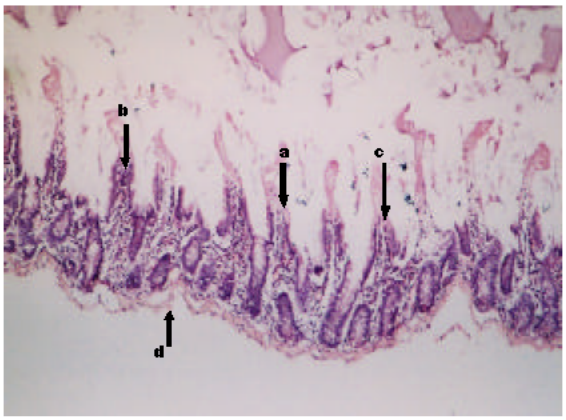

Fig. 2b: IR Group, a: shortening, sloughing and necrosis of villi, b: Infiltration of inflammatory cells, c: damage to epithelial mucosa, d: damage to muscularis and serosal layers (H-E X 100) Grade IV

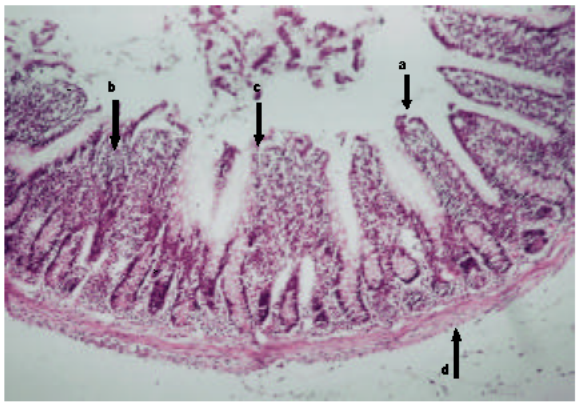

Fig. 2c: IRM Group, a: lengthening of villi b: mild infiltration of inflammatory cells lamina propria, c: recovery of epithelial surface d: intact serosal and muscular layers (H-E X 100) Grade II

levels. The relaxant responses were in accordance with the results of MDA levels and histopathologiacal grading. Our results also showed that surgical manuplation of gastrointestinal tract by itsefl resulted in reduced intestinal relaxation, enhanced MDA levels and damaged ileal structure.

In various organs, including the small istestine, tissue damage and impaired function develop after temporary ischemia, not only during the hypoxic period, but also after reoxygenetion. The primary etiological agents of intestinal I/R are considered to be oxygen free radicals (OFRs), mainly derived from xanthine oxidase[17-20]. Secondary pathological events are because of the recruitment and activation of neutrophils in the intestine[21]. Reperfusion of ischemic tissue is the major reason for the formation of toxic OFRs, such as superoxide anion, hydroxyl radical, hydrogen peroxide and peroxynitrite[19]. OFRs can damage cellular membrane and subcellular structures, which contain large amounts of phospholipids and protein, resulting in lipid peroxidation and sequentially structural and metabolic alterations and leading to cell death and necrosis[22]. Increasing amount of evidence has indicated that intestinal I/R causes an acute inflammatory response that is particularly enhanced by reperfusion. Activated neutrophils augments ischemic injury by releasing cytotoxic OFRs and proteolytic enzymes[20]. Muscle and nerve cells in intestinal tissue are very vulnerable to ischemia. In intestinal tissue exposed to ischemia, energy depletion occurs in the cells[20]. Furthermore, during reperfusion period, generation of oxygen free radicals interfere with the function of cells by disruption the ionic homeostasis[23,20]. The damage formed on the surface of mucosa is followed by transmucosal and transmural damage[24,25]. It has been reported that ischemia-induced tissue damage is milder than the damage occuring after reperfusion[2,26].

There are several free radical scavenger to reduce tissue damage and remove oxygen radicals generated during IR tissue injury. Melatonin is one of these important free radical scavengers[1,10,11]. Melatonin is a very potent and efficient endogenous radical scavenger. The pineal indolamine reacts with the highly toxic hydroxyl radical and provides on site protection against oxydative damage to biomolecules within every cellular compartment. Because of melatonin's high diffusion ability, melatonin can enter subcellular compartments of the cells without any trouble. Melatonin can show its effects through its receptors and also without receptors by entering nucleus which makes it one of the most powerful antioxidants. In addition to pineal gland, liver, retina, salivary glands, thyroid and gastrointestinal system are also reported to be sites of melatonin synthesis[27]. Researches suggest that chronically inflamed bowel and ischemic small intestines diseases are sources of significant oxidative stres[28].

A number of reports show that melatonin is as effective as an antioxidant in the various models of oxidative stress at its 10 mg kg^{-1} dose[29-33]. In these studies, however, melatonin was administered more than one dose with prolonged reperfusion period during the experimental procedure. Ozacmak *et al.* showed that 10 mg kg^{-1} melatonin was insufficient as a single shot[11]. On the other hand, we have observed that a single shot of that amount was sufficient to ameliorate the I/R-induced damage in the present model of intestinal I/R. Ballabeni *et al.* demonstrated that I/R caused adaptive alterations in motor changes, which were reversible, since I/R-related enteric dismotility disappeared when duration of the reperfusion prolonged to 72 h[34].

Bubenik GA showed that melatonin reduces the tone of spontaneous contractions of isolated rat ileum but not the amplitude or frequency of contractions, whereas serotonin (5-HT) increased the tone and reduced the amplitude of contractions. Pretreatment with melatonin significantly reduced the 5-HT effect[35]. It is demonstrated that melatonin reduced the force of spontaneous contraction of ileal segments of rat by 25%. They concluded that the action of melatonin on smooth muscle contraction might be through inhibiting the contractile response of 5-HT[11]. In another study, it was shown that melatonin might interact with an apamin sensitive, possibly Ca^{2+}-activated, K^+ channel; thereby, causing an inhibition of ileal smooth muscle contractions in response to carbacol or KCl[36]. It was also demonstrated that intraperitoneal administration of melatonin at 1 or 10 μg kg^{-1} doses enhances intestinal transit in rats, while higher doses (i.e. 1000 μg kg^{-1}) appear to be reversing that effect[37]. Ozacmak *et al.* showed that melatonin's actions were likely not mediated via receptors but rather by receptor independent scavenging actions. They also stated that the only high dose of melatonin (50 mg kg^{-1}) restored the contraction response may result from dose dependent antiinflammatory effect of melatonin besides its antioxidant and scavenging effects[11]. Paskaloglu *et al.* showed that treatment with melatonin restored the contractility of the ileal and bladdary tissues[12].

In the present study we studied relaxation responses of ileal tissue and showed that relaxation responses were lower in ischemia and IR groups and statistically different compared with those of control and sham-operated groups. Relaxant responses in the melatonin treated ischemia and IR groups were different from those of untreated ischemia group ($p<0.05$ at a dose of 10^{-7} M;

$p<0.01$ at the other doses) and of the untreated ischemia-reperfusion group ($p<0.05$).

Paskaloglu *et al.* studied the role of melatonin in protecting the intestinal and bladder tissues against damage in a rat model of sepsis. Sepsis was induced by cecal ligation and perforation. Melatonin (10 mg kg^{-1}, intraperitoneal) was given 30 min prior to and 6 h after the operation. Contractile responses to carbachol of ileal and bladdary tissues was examined and found that treatment with melatonin restored the contarctility of the tissues[12].

Surgical manuplation of gastrointestinal tract (i.e. laparatomy, replacement of intestine and exteriorisation) by itself results in not only reduced ileal contractility, enhanced MDA level and decreased GSH content[38,39] but also damage of the intestinal brush border membrane[40]. Thus, intestinal manuplation plays an essential role in the initiation of intestinal inflammation. Our study has a group named sham-operated group which was not different statistically compared with those of control group. But, relaxant responses, MDA levels and semiquantitative histologic grading were not as the same as the results of those of control group. So, our results were in accordance with the results of above-mentioned studies.

Lipid peroxidation, mediated by free oxygen radicals, is believed to be an important cause of destruction and damage to cell membranes, since polyunsaturated fatty acids of the cellular membranes are degraded by this process with consequent disruption of membrane integrity. Membrane peroxydation can lead to changes in membrane fluidity and permeability nd also enhanced rates of protein degradation and these will eventually lead to cell lysis[41]. In the present study, the levels of MDA, an end product of lipid peroxydation, are significantly increased in ischemia and IR groups. This increase is higher in IR group. This observation is in agreement with the previous studies[1,10,12,42]. Melatonin-treated groups (IM and IRM groups) were statistically indistinguishable from the control and sham-operated groups in terms of tissue MDA content ($p>0.05$). The comparison of ischemia group with ischemia-reperfusion and ischemia+melatonin groups were statistically significant ($p<0.01$). Similarly, the comparison of IR and IRM was found to be statistically significant ($p<0.01$). These results demonstrated that treatment with melatonin inhibits MDA elevations significantly and reverses back to control and sham-operated levels. Thus, melatonin has a protective effect against organ damage by preserving the cellular integrity.

Based on our histopathological examination and semiquantitative grading, we observed that there is no change in control group, grading as 0. In sham-operated group, changes was minimal and not different compared with those in control group. The most extensive changes in morphology were detected in I/R group. In sections from this group, there is a severe damage and necrosis: Inflammation and necrosis in areas throughout the thickness of the intestinal wall (Grade 3.5±0.189) as seen Fig. 2b. The treatment with melatonin had a healing effect on mucosal damage ($p<0.001$). These results was consistent with the previously reported studies[1,10-12].

In summary, the present study shows that melatonin restores the reduction of ileum smooth muscle responses to adrenalin in intestinal I/R model. Our results suggest that lower dose of melatonin treatment (10 mg kg^{-1}) beforehand may ameliorate structural and functional damage observed in an experimental I/R, due mainly to reducing inflammation, inhibiting lipid peroxidation. These results confirms that surgical manuplation of the intestinal system has some adverse affect as seen in sham-operated group. Additionally, our study confirms that ischemia-induced tissue damage is milder than the damage occuring after reperfusion.

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