Protective effect of melatonin against oxidative stress on adhesion formation in the rat cecum and uterine horn model

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Abstract

This experimental study was designed to evaluate the degree of adhesion formation and peritoneal tissue levels of malondialdehyde (MDA), reduced glutathione (GSH) and nitric oxide (NO) and the effect of melatonin on these metabolites in a postoperative intraperitoneal adhesion formation model in rats. Thirty adult female Wistar albino rats were subjected to standardized lesions by cecal and uterine horn abrasion and were randomly divided into three groups. Control rats were treated with 5% ethanol. Melatonin treated rats received 4 mg/kg melatonin before closure and for 10 consecutive days intraperitoneally after surgery. Rats in the sham operation group underwent a surgical procedure similar to the other groups however the peritoneal abrasion was not performed. On postoperative day 10 relaparatomy was performed. After the assessment of the adhesions, the rats in each group were sacrificed and peritoneal tissues were harvested to determine the tissue levels of MDA, GSH and NO activity. Adhesion formation scores in the melatonin group were significantly lower than that of control and sham group ($p < 0.01$ and $p < 0.02$, respectively). Tissue levels of MDA and NO were significantly lower in the melatonin treated rats when compared with control and sham groups. The levels of GSH in the melatonin treated rats were significantly higher than those of control and sham groups ($p < 0.01$). The results demonstrate that in this experimental model, intraperitoneal administration

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of melatonin decreases the extent of peritoneal adhesions and causes a decrease in MDA and NO and an increase in GSH levels.

Introduction

Postoperative abdominal adhesion formation is an important clinical problem and a common cause of complications such as intestinal obstruction, infertility and chronic pain, often requiring additional surgery (Menzies, 1993; Ellis, 1997). Although the peritoneum normally possesses some preventative physiological properties for adhesion formation, mechanical trauma, tissue ischemia, thermal injury, foreign materials and peritonitis of infectious origin are the most important causes of adhesion formation (Sahin and Saglam, 1994; Thompson and Whawell, 1995). Numerous substances have been tested for their ability to reduce postoperative adhesion formation, including L-arginine and pentoxifylline (Kaleli et al., 1998), melatonin (Ozcelik et al., 2003), methylene blue (Kluger et al., 2000) and vitamin E (Sanfilippo et al., 1995). However, satisfactorily effective adjuvants have not been found. The inflammatory response has long been recognized as a common denominator in all pathways for adhesion formation. Recent studies have shown that leukocyte dependent inflammatory reactions may increase cellular and tissue injury through the actions of oxygen-derived free radicals and metabolites (Fantona and Ward, 1982). Numerous mediators of inflammation such as arachidonic acid, cytokines, nitric oxide and oxygen-derived free radicals may participate in postoperative formation of adhesions (Galili et al., 1998). A recent study using dietary vitamin E supplementation showed a significant reduction in postoperative peritoneal adhesions in rats (Hemadeh et al., 1993). In another study, intraperitoneally injected superoxide dismutase and catalase, known to block the effect of oxygen free radicals and reactive oxygen species, have been shown to reduce the inflammatory reaction and thereby the adhesion formation in an endometriosis animal model (Portz et al., 1991).

Melatonin, a pineal secretory product, which is formed from L-tryptophan via the serotonin pathway, functions not only as a direct free radical scavenger, i.e. a scavenger of various oxygen free radicals, especially ·OH and peroxyl radical, but also as an indirect antioxidant through the enhancement of antioxidant enzyme activities in many tissues (Pieri et al., 1994; Reiter, 1997; Reiter et al., 1999, 2000; Allegra et al., 2003; Rodriguez et al., 2004).

The aim of this study is to evaluate whether melatonin administration would protect against adhesion formation in an experimental peritoneal adhesion model in rats. To assess the ability of melatonin to function as an antioxidant in rats with peritoneal adhesion, we measured the levels of GSH, MDA and NO. Also we examined histopathological changes of cecum.

Materials and methods

Animals and treatment

A total of 30 three-month-old Wistar albino rats weighing 300–350 g were included in the study. They were housed in groups of six on a 12 : 12 light / dark schedule with lights on at 06:00 hours.

Rats were obtained from Firat University, Animal Laboratory (Elazig, Turkey). Experiments were performed at the Inonu University Experimental Research Center. All experiments in this study were performed in accordance with the guidelines for Animal Research from the National Institute of Health and were approved by the Committee on Animal Research at Inonu University.

Rats were kept in stainless steel cages, given food and water ad libitum and quarantined 14 days before surgery. Food was withheld 8 h prior to surgery, but they had free access to water. Rats were divided into three equal groups: Rats in the sham operation group underwent a surgical procedure similar to the other groups but the cecum and uterine horn were not abraded. The second group that was treated with 4 mg/kg melatonin intraperitoneally (i.p) before closure and for 10 consecutive days after surgery. For the third control group, 1 ml dilution vehicle (NaCI) containing 5% ethanol was given before closing the abdomen and for 10 consecutive days after surgery.

Melatonin® (Sigma Chemical Co, St Louis, MO, USA), used in this study, was dissolved in ethanol and diluted in saline to give a final concentration of 5% ethanol.

Surgical technique

All surgical procedures were performed while the rats were under intraperitoneal ketamine (50 mg/kg) and xylazine HCl (10 mg/kg) anesthesia. Before incision, the abdomen was shaved and prepared with betadine (Poviiodeks, Kim-Pa Corporation, Istanbul, Turkey).

In order to remove powder particles, operation gloves were washed thoroughly with saline. The same investigator performed all surgical procedures. A midline laparotomy was performed under clean surgical conditions. The cecum was indentified and exteriorized, and an area of serosa measuring approximately 1 cm^2 on the antimesenteric surface was gently abraded along the longitudinal axis using a small brush until bleeding was observed from the serosal surface. In addition, surgical trauma was performed on the left uterine horn of each rat. It was subjected to a standardized lesion by denuding the serosa on the proximal antimesenteric area with a scalpel until macroscopic punctate bleeding was observed over the most of the uterine horn surface. The defect in the cecum and uterine horn was closed with continuous no. 4/0 delayed absorbable sutures (Vicryl, Ethicon and Cologne, Germany). All animals were maintained under the same conditions after surgery. To eliminate complications arising from diurnal effects, all rats were sacrificed on the postoperative day 10, by ketamine overdose at the same time of the day and peritoneal tissues and any adherent material was harvested. During relaparatomy, the presence and degree of adhesions were graded independently by two investigators who were blinded to the therapy used. The extent and severity of adhesions in the operation site were evaluated using an established scoring system (Linsky et al., 1987). According to this system the extent of adhesions was evaluated as follows:

0, no adhesion;
1, 25% of traumatized area;
2, 50% of traumatized area;
3, total involvement.

A part of the cecum was preserved in 10% formalin for histological examination, and a part of the peritoneum was stored at -30 °C for the analyses of Malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH) levels.

Hematoxylin and eosin (H and E) stained tissues were examined by a pathologist blinded to all the study groups using light microscopy to evaluate the presence and degree of neutrophilic infiltration.

Biochemical analyses

The harvested tissues were homogenized in ice-cold 0.1 M Tris–HCl buffer (pH 7.5) with a homogenizer (IKA Ultra Turrax T 25 Basic) at 16,000 r.p.m. for 3 min. The homogenates were used to measure the levels of MDA, GSH, and NO. All procedures were performed at 4 °C. MDA levels were assayed spectrophotometrically at 535 and 520 nm according to the method of Mihara and Uchiyama (1978). Results are expressed as nanomoles per gram wet tissue. GSH levels were measured by the method of Ellman (Ellman, 1959). GSH is reacted with 5, 5 dithiobis-2-nitrobenzoic acid (DTNB) resulting in the formation of a product which has a maximal absorbance at 410 nm. Results are expressed as nanomoles per gram wet tissue. NO levels were measured according to the method of Ozbek et al. (2000), with minor modifications. Results are expressed as nanomoles per gram wet tissue.
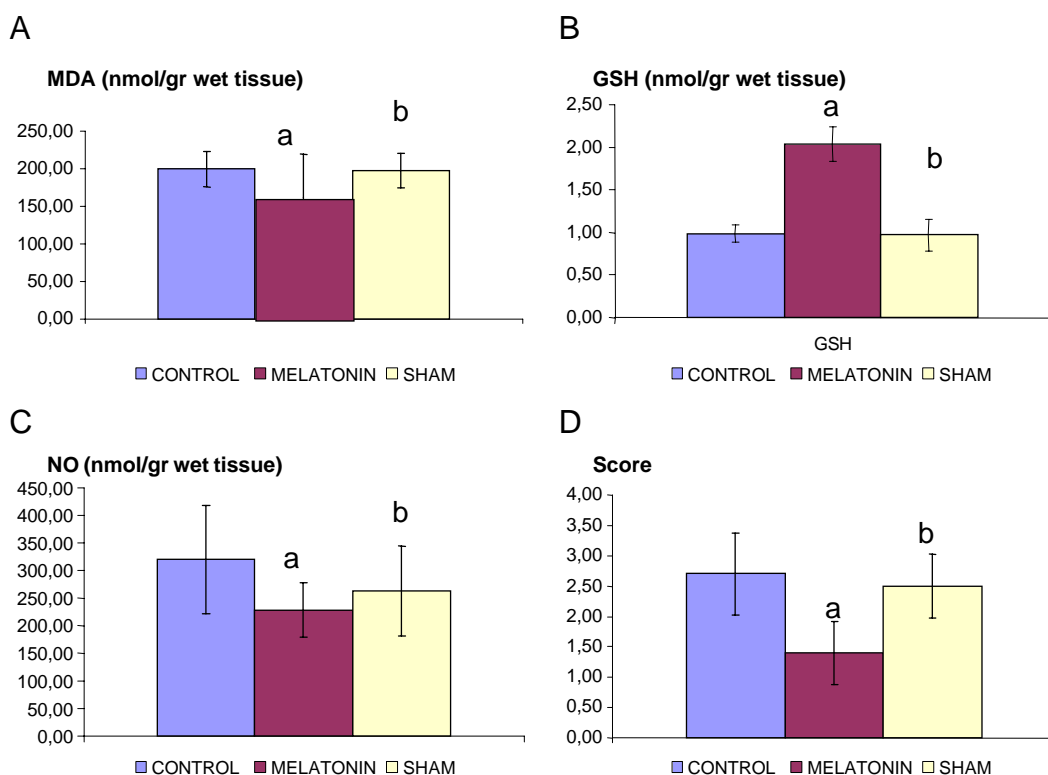

Fig. 1. Graphic appearance of MDA, GSH, NO levels and adhesion scores of all groups. A: MDA levels in all groups. [a]Statistically different from control ($p < 0.02$), [b]statistically different from melatonin ($p < 0.02$). B: GSH levels in all groups. [a]Statistically different from control ($p < 0.01$), [b]statistically different from melatonin ($p < 0.01$). C: NO levels in all groups. [a]Statistically different from control ($p < 0.02$), [b]statistically different from melatonin ($p < 0.01$). D: Adhesion scores in all groups. [a]Statistically different from control ($p < 0.01$), [b]statistically different from melatonin ($p < 0.02$).

Statistical analyses

The statistical package for social sciences (SPSS) version 10.0 was used for statistical analysis. Individual group parameters were assessed using the Mann–Whitney U test. The results are given in the text as means $\pm$ SEM. For all comparisons, statistically significant difference was defined as $p < 0.05$.

Results

Ascites in the abdomen at laparatomy was found in the non-melatonin treated group. In these rats, adhesions were also found between the depersonalized antimesenteric surface of the cecum, adjacent small or large bowels and the left uterine horn. The mean score of adhesions in control group was 2.70 ± 0.67. In the melatonin treated rats, the mean score was 1.00 ± 0.67, and adhesion formation was significantly less than the control group ($p < 0.01$). The results for MDA, GSH and NO were shown in Fig. 1. In the melatonin treated rats, the MDA and NO levels were significantly lower than in the untreated animals ($p < 0.02$). On the other hand, the levels of GSH in the melatonin injected rats were significantly higher than in the non-treated group ($p < 0.01$). Total scores of adhesion and levels of MDA and NO in melatonin treated rats were significantly lower when compared with sham group ($p < 0.02$,
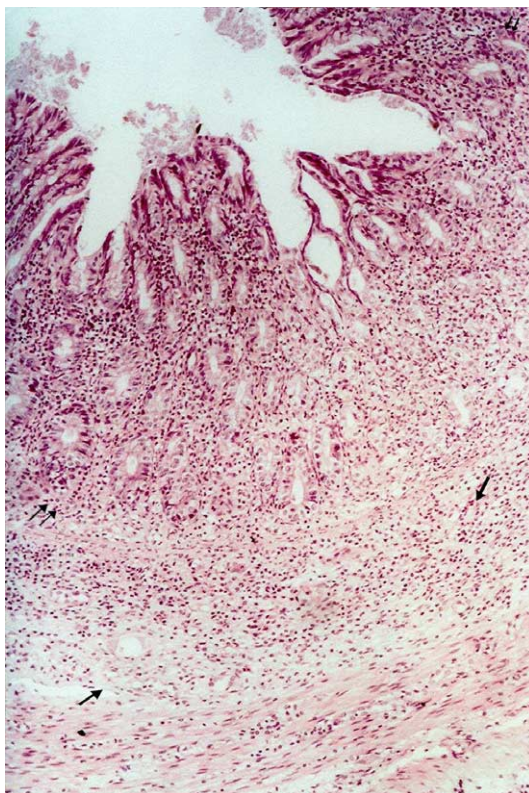

Fig. 2. Prominent transmural neutrophilic infiltration of cecum in the control group. Severe inflammation has partially distorted the mucosal glandular architecture (H and E $\times$ 100).
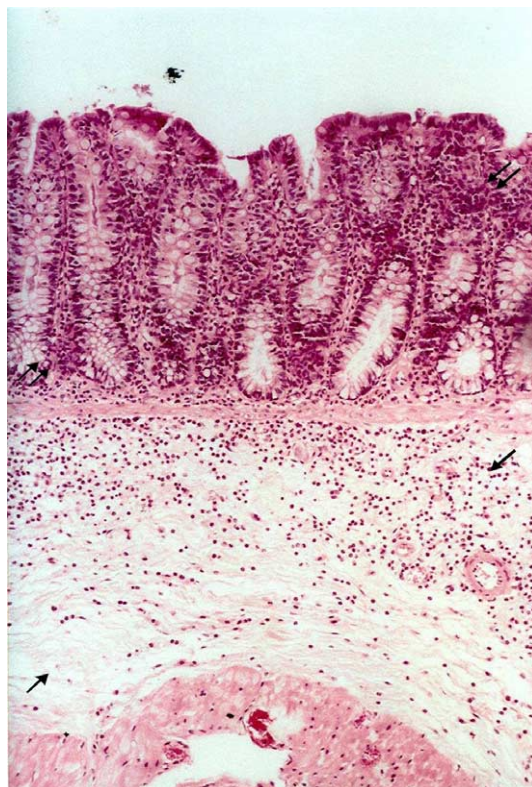
Fig. 3. Neutrophilic infiltration limited to the mucosa and submucosa of cecum in the melatonin group. Note the inflammation is less severe and the muscularis propria has no inflammation (H and E × 100).

$p < 0.02$ and $p < 0.01$, respectively). In the melatonin treated rats, levels of GSH were significantly higher than that of sham group ($p < 0.01$).

The severity of the inflammation in the cecum tissue was more prominent in the control group (Fig. 2) compared with the sham and melatonin injected rats. In addition, the inflammation in the cecum tissue was transmural in the control group while limited to the mucosa and submucosa in the melatonin group (Fig. 3).

Discussion

Postoperative intraperitoneal adhesion formation is part of the normal healing process and it continues to be a source of great concern to the surgeon (Ellis, 1997; Risberg, 1997). Many approaches have been taken to prevent postoperative intraperitoneal adhesion formation. Postoperative adjuvants, including antibiotics, corticosteroids and intraperitoneal placement of high-molecular-weight dextran, all have served as time-honored means of preventing adhesion formation. Even with the utilization of minimally invasive and laparoscopic surgery, postoperative adhesion has been reported (Ellis, 1997). Any circumstances that produce ischemia with reduction in tissue oxygenation are known to result in adhesion formation (Drollette and Badawy, 1992). Notably, during the first 5

min of ischemia, there is already a significant production of free radicals, which are able to react rapidly with oxygen, exacerbating the oxygen deficit (Bertuglia et al., 1993). Locally generated free radicals such as superoxides, peroxides and hydroxyl radicals are potential oxidizers of polyunsaturated fatty acids (Kagoma et al., 1985). Lipid peroxidation of cellular membranes occurs and increased vascular permeability, leads to the formation of serosanguineous exuda, which in turn initiates adhesion formation. These adhesions are generally lysed within 72 h after formation by the endogenous fibrinolytic activity and much of the healing is complete within 5 days (Gutmann et al., 1995; Hellebrekers et al., 2000a). If there is an imbalance between fibrin deposition and dissolution, deposited fibrin may persist and fibrinous adhesions may develop (Vipond et al., 1990; Hellebrekers et al., 2000b). In the melatonin treated rats, adhesion scores were significantly lower than untreated rats. Our results are in agreement with previous studies reporting on antioxidant agents (Galili et al., 1998; Kagoma et al., 1985; Sanfilippo et al., 1995). A recent study using i.p melatonin administration showed a significant reduction in postoperative peritoneal adhesions in rats (Ozcelik et al., 2003). As an addition to previous study, we examined histopathological changes of cecum and we measured the levels of MDA, GSH and NO as well as adhesion scores in the present study. Another difference of our study was the administration of melatonin for 10 days compared with 5 days in the previous study.

MDA, a product of lipid peroxidation, is generated as a result of toxic effects of active oxygen radicals which destroy unsaturated fatty acids in the membranes (Parlakpınar et al., 2002). Locally generated free radicals such as superoxides, peroxides and hydroxyl radicals are potential oxidizers of polyunsaturated fatty acids, and therefore are postulated to induce peritoneal adhesions through damage to cellular membranes. In the present study, levels of MDA in the melatonin treated rats were significantly lower than in the control group. Although tissue MDA levels were clearly decreased by melatonin, its exact mechanism is not clean. Since indirect antioxidant and direct free radical scavenger properties of melatonin are well documented (Allegra et al., 2003; Rodriguez et al., 2004), reductions in MDA levels in the melatonin treated rats is probably due to melatonin's antioxidant and free radical scavenging effects. Melatonin is readily absorbed when it is administered via any route and it seems to enter traumatized peritoneal tissues with ease where it prevents oxidative damage, preserves mitochondrial function (Leon et al., 2004), and has low toxicity. It is possible that the interference of melatonin with free radical generation may be related to a decline in peritoneal adhesion formation.

NO is a free radical synthesized from L-arginine by nitric oxide synthase (NOS) in biological systems (Palmer et al., 1988). Depending on its redox state (Lipton et al., 1993) and cellular source (Iadecola, 1997) NO may be toxic or protective. During ischemia and reperfusion, increased NO formation occurs, and this can interact with xantine oxidase-derived O_2, leading to the formation of peroxynitrite (ONOO) (Reiter et al., 2000). ONOO is a potent oxidant that can attack a wide variety of biological molecules and is produced in diverse inflammatory and pathological processes including post ischemic injury (Wang and Zweier, 1996).

In the present study, the levels of NO in melatonin treated rats were significantly lower than in untreated rats. Several mechanisms may contribute to reduced levels of NO in melatonin injected rats. For instance, melatonin may be capable of reducing peroxynitrite generation due to its ability to scavenge nitric oxide (Noda et al., 1999) and to inhibit one isoform of NOS (Pozo et al., 1994). Additionaly, recent reports have shown that melatonin directly neutralizes ONOO (Gilad et al., 1997; Blanchard et al., 2000). The studies on the role of NO in adhesion formation are limited. Galili et al.

(1998) reported that the NOS inhibition causes severe peritoneal adhesions in more than 95% of rats. Ozden et al. (1999) showed that administration of L-arginine increased levels of nitric oxide and reduced adhesion formation in rat uterine horn with the parietal peritoneal defect model. On the other hand, NO inhibitor and the free radical scavenger agent, methylene blue was found to be effective in the prevention of adhesion formation but this activity was attributed to the inhibition of free radical generation and not to an NO action (Galili et al., 1998). Possible explanations for our findings include reduced excessive nitric oxide formation, and/or neutralization of NO, once formed. This would lower oxidative damage and, therefore, allow melatonin to reduce peritoneal adhesion formation in rats.

All cells contain some enzymatic and non-enzymatic antioxidant defense mechanisms to protect themselves from hazardous effects of oxidative attacks. Levels of GSH, an essential component of the cellular defense mechanisms against radical-mediated tissue injury, has been used as an indicator of oxidative stress induced by reactive oxygen species during of ischemia reperfusion (Gibson et al., 1993). The enzyme glutathione peroxidase (GSH-Px) utilizes GSH, an intercellular thiol that is typically in millimolar concentrations, as a substrate. Maintaining high intracellular concentrations of GSH seems also to be a function of melatonin since this indole stimulates the activity of its rate limiting enzyme, gamma-glutamylcysteine synthase (Urata et al., 1999). When GSH is metabolized by GSH-Px, a reaction that also requires H_2O_2 or other hydroperoxides, it is converted to oxidized glutathione (GSSG). Within cells the GSH:GSSG ratio is normally greatly in favor of the former, and to maintain this ratio GSSG is rapidly metabolized back to GSH by GSH-Rd. Recent studies have shown that melatonin also promotes the activity of GSH-Rd, thereby helping to maintain high levels of reduced glutathione (Hara et al., 2001). In the present study, significantly higher GSH levels were detected in melatonin treated rats. This increased GSH is consistent with the protective effects of melatonin against oxidative damage, which is expected to reflect the fact that peritoneal tissue is better protected by melatonin against oxidative damage.

Adhesion formation begins with the formation of fibrin matrix, which is then gradually organized and replaced by tissue containing fibroblasts, macrophages, and giant cells (Raftery, 1976). In our study, when melatonin was administered, lower clinical adhesion scores and less proven inflammation were detected (Fig. 2). Intraperitoneal melatonin enters traumatized peritoneal tissues where it may cause fibroblasts to proliferate more slowly, synthesize less collagen and reduce adhesions. Inhibition of fibroblastic proliferation, once it is established, has been described as one of the major attacks in the prevention of peritoneal adhesions.

A major clinical problem related to peritoneal repair is the formation of intraabdominal and pelvic adhesions. Considerable effort has been made to better understand the pathogenesis and underlying mechanisms of adhesion formation and to develop anti-adhesion treatments.

Conclusion

In the current study, intraperitoneal administration of melatonin significantly inhibited adhesion formation in an experimental peritoneal adhesion model in rats. Considering the reduced oxidative damage because of melatonin treatment, melatonin's protective actions in the current study are believed to be a consequence of direct and indirect antioxidative activities. Finally, melatonin could be useful in treating intraabdominal and pelvic adhesions and possibly other clinical conditions involving excess free radical production.

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