Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) cause damage to the small intestine. Many clinical and experimental investigations have documented a propensity of diclofenac and other NSAIDs to injure the small intestine, resulting in small intestinal perforation, ulcers, and strictures requiring surgery. A recent report on 312 patients who received NSAIDs for rheumatologic conditions, including 166 who received diclofenac, showed a 44% prevalence of enteropathy. Capsule endoscopy presented evidence of macroscopic injury to the small intestine resulting from 2 weeks of slow-release diclofenac ingestion in up to 68% of volunteers. NSAIDs have been linked to the development of serious gastrointestinal side effects, and numerous strategies have been employed to reduce the likelihood of mucosal damage. Various approaches, including proton pump inhibitors and prostaglandin analogues, have been used for this purpose. However, none of these approaches has solved the problem of NSAID-induced gastrointestinal damage.

The pathogenesis of NSAID-induced enteropathy is a multistage process involving specific biochemical and subcellular organelle damage followed by an inflammatory tissue reaction. Increased intestinal permeability has been implicated in the pathogenesis of NSAID-induced enteropathy. This altered permeability allows for dietary macromolecules, bile acids, pancreatic juices, bacteria, and other intra-luminal toxins to access the usually intact intestinal epithelium. The increased intestinal permeability is presumably related to cellular damage resulting from energy depletion. Glutamine, a major energy source for the intestinal epithelium, has been

A protective effect of melatonin on intestinal permeability is induced by diclofenac via regulation of mitochondrial function in mice

Qiao MEI*, Lei DIAO, Jian-ming XU, Xiao-chang LIU, Juan JIN

Department of Gastroenterology, First Affiliated Hospital of Anhui Medical University, Key Laboratory for Digestive Diseases of Anhui Province, Hefei 230022, China

Aim: This study investigated the effect of intragastrically administered melatonin on intestinal mucosal permeability induced by diclofenac in mice.

Methods: Intestinal mucosal permeability was induced in mice by intragastric administration of diclofenac (2.5 mg/kg). Melatonin was given intragastrically (10 mg/kg) once per day for 3 d after diclofenac administration. The small intestine was examined macroscopically and microscopically for pathologic injury to the intestinal mucosa. Intestinal mucosal permeability was evaluated by Evans blue and FITC-dextran methods. Mitochondrial functional parameters, including mitochondrial membrane potential, mitochondrial ATPase and succinate dehydrogenase (SDH) activity, were assessed. The malondialdehyde (MDA) and myeloperoxidase (MPO) levels were determined from small intestinal mucosal homogenates.

Results: As compared with control mice, the permeability, pathologic score, MDA and MPO levels and ulceration of the intestinal mucosa were increased significantly by diclofenac treatment, and a broadened junctional complex and enlarged intercellular space were observed by transmission electron microscopy (TEM). Melatonin treatment significantly reduced the intestinal mucosal permeability, pathologic score, MDA, and MPO levels and ulceration of the intestinal mucosa. By TEM, the small intestine villus morphology and intercellular spaces were nearly normal in melatonin-treated mice. At the level of the mitochondria, melatonin treatment significantly restored the activities of ATPase and SDH.

Conclusion: The intestinal damage and increased intestinal permeability induced by diclofenac in mice was limited by melatonin; moreover, melatonin preserved several aspects of mitochondrial function.

Keywords: melatonin; intestinal mucosal permeability; mitochondria; diclofenac

Original Article

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shown to play an important role in the protection of the intestinal mucosa against exogenous substance-mediated injury[6]. Thus, it has been hypothesized that glutamine might prevent NSAID-induced changes in intestinal permeability.

Melatonin plays a fundamental role in the neuroimmune-endocrine system[7]. The levels of melatonin and melatonin receptors are highly concentrated in the intestine[8]; melatonin concentrations in the gut are 10–100 times greater than those found in the plasma[9]. Moreover, it has been suggested that the intestine is the major site for extra-pineal melatonin production[10]. Melatonin was found to have a beneficial effect in experimental models of gastric injury, such as ischemia-reperfusion (I/R), ethanol and indomethacin[11–14]. This protection was attributed to melatonin’s function as an antioxidant[15–17]; therefore, melatonin may play an important role in the regulation of the intestinal mucosa. Additionally, melatonin has significant anti-apoptotic effects, which could protect the gastric mucosa from NSAID-induced apoptosis and gastropathy and make it useful as a potential therapy against the gastric damage that occurs with NSAID treatment[18]. There is also evidence that melatonin exerts some effect against the pathogenesis associated with NSAID-induced enteropathy[14]. Most previous studies have focused on the protection provided by melatonin against NSAID-related gastric mucosal injury, but there is a shortage of data as to whether melatonin can improve NSAID-related small intestinal mucosa injury. Thus, in the current study, we investigated the protective ability of melatonin in a mouse model of diclofenac-induced enteropathy.

Materials and methods

Mice

Male Kunming mice weighing 20±2 g were supplied by the Experimental Animal Center of Anhui Medical University. The mice were maintained in a controlled environment at (20±1) °C for 1 week with a 12-h light/dark cycle and 50%±5% relative humidity throughout the experimental period. All mice were allowed free access to water and chow diet. The experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

Reagents

Melatonin, diclofenac, FITC-D, rhodamine 123, and succinate were all purchased from Sigma (Saint Louis, Missouri, USA). Glutamine and acetylcysteine were obtained from Beijing Solarbio Science & Technology Co, Ltd (Beijing, China). Succinate dehydrogenase (SDH), ATPase and kits for assaying malondialdehyde (MDA) and myeloperoxidase (MPO) were obtained from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). The other reagents were all of analytical grade.

Induction of small intestinal mucosal injury with diclofenac

Mice were administered diclofenac (2.5 mg/kg) dissolved in 0.2% methylcellulose daily by oral gavage for 3 d. Controls received only the methylcellulose vehicle by gavage.

Treatment schedule

The animals were randomly divided into four groups: the control group, diclofenac-treated group, the glutamine-treated group (1000 mg/kg) and the melatonin-treated group (10 mg/kg). Melatonin and glutamine were given intragastrically (ig) once per day for 3 d beginning 4 h after diclofenac administration. In the control and diclofenac-treated groups, saline was given instead of melatonin. At the end of the experiment, mice were decapitated, and their plasma and intestines were collected for biochemical analyses.

Morphological analyses

The small intestine was removed and fixed in 2% neutral buffered formalin for 24 h and then transferred to 70% ethanol. The mucosa of the small intestine was exposed by cutting along the anti-mesenteric side and pinning it out on a piece of cork for assessment of macroscopic damage. The number and size of ulcers (longest diameter ≥5 mm) and the areas (mm²) of visible lesions were measured[19]. A piece of ileum initially fixed in 10% neutral buffered formalin was embedded in paraffin for histological analysis. Full-thickness (5-μm) sections were stained with hematoxylin and eosin and examined microscopically by a pathologist blinded to the treatment groups. The severity of intestinal injury was graded on a scale of 0–4 and expressed as the pathological index according to a standard scoring system[20]. A portion of ileum approximately 0.5 cm from the ileocecal junction that was approximately 1 cm in length was excised with a scalpel. Specimens processed for TEM were fixed in 2.5% glutaraldehyde for 4 h at 4 °C followed by fixation in osmic acid and embedding in Epon. Ultrathin sections were examined with a Hitachi transmission electron microscope.

Determination of intestinal permeability using fluorescein isothiocyanate dextran (FD-4)

Intestinal mucosal permeability to the fluorescent tracer fluorescein isothiocyanate dextran (4000 Da) was determined using the method previously described by Chen et al. A 5-cm segment of the ileal sac was ligated beginning at 3 cm proximal to the ileocecal valve, 0.2 mL of PBS (pH 7.4) containing 25 mg/mL FD-4 was injected into the ileal sac with a syringe, and the abdomen was sutured closed. After 30 min, a blood sample (100 μL) was collected from the portal vein and immediately diluted with 1.9 mL of 50 mmol/L Tris (pH 10.3) containing 150 mmol/L chloride natrium. The diluted plasma was centrifuged at 3000×g for 10 min, and plasma FD-4 concentrations were determined by fluorescence spectrophotometry at an excitation wavelength of 480 nm and an emission wavelength of 520 nm[21].

Determination of intestinal permeability by Evans blue

Small intestine sacs were prepared as previously described[22]. Briefly, small intestine was incised, and the fecal contents were washed out gently with 2–3 mL of PBS. The proximal and distal intestines were ligated, and 0.2 mL of 1.5% (w/v) Evans blue (EB) in PBS was infused into the lumen. The intestinal...
sacs were incubated in 20 mL of Krebs buffer at 95% O₂ and 37 °C. They were removed 30 min later, washed three times in 6 mmol/L acetylcysteine, dried on filter paper at 37 °C for 24 h and then incubated with 1 mL of formamide at 50 °C for 24 h. The amount of dye eluted was estimated using a wavelength of 655 nm. The amount of EB permeating into the intestinal wall was calculated based on a standard curve of EB in formamide.

Detection of intestinal homogenates
Dissected intestine were excised, all fat and mesenterium was removed and intestinal mucosal specimens were scraped and cut into pieces using sharp scissors and forceps. The tissue was gently washed and weighed (all samples were approximately 100 mg), homogenated in physiologic saline and temporarily stored at -20 °C prior to detection of MDA content and MPO activity with their respective kits according to the manufacturer’s instructions (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China). Protein content was determined by the Lowry method[23].

Preparation of liver mitochondria
Mouse liver mitochondria were isolated and used for these studies because it is exceedingly difficult to obtain a high yield of mitochondria from intestinal tissue[24]. A liver mitochondrial fraction was prepared according to the method described by Schneider and Hogeboom[25]. Briefly, the liver was rapidly removed and placed in ice-cold 0.9% saline, cut into approximately 1-cm³ pieces and homogenized in 50 mL of ice-cold homogenizing solution [250 mmol/L sucrose, 10 mmol/L Tris-HCl buffer (pH 7.4), 0.5 mmol/L EDTA] by six strokes with a tight-fitting Teflon pestle. The homogenate was then centrifuged at 600×g for 10 min, and the resulting supernatant was further centrifuged at 15000×g for 10 min. Finally, the pellet was removed and resuspended in 10 mL of homogenizing solution and centrifuged for 10 min at 15000×g to give the resulting mitochondrial-enriched pellet used for the experiments. All procedures were performed at 4 °C. Mitochondrial protein concentration was determined using Lowry’s method. The pellets were resuspended in mitochondria separating medium (pH 7.4) to make a final suspension containing 5 mg/mL mitochondrial proteins, which was stored at -20 °C.

Determination of mitochondrial membrane potential (MMP)
MMP was evaluated from the uptake of rhodamine 123[26], which electrophoretically accumulates into energized mitochondria in response to their internal negative membrane potential. First, 1800 μL of phosphate buffer (250 mmol/L sucrose, 5 mmol/L KH₂PO₄, pH 7.2 at 25 °C), 3 mmol/L succinate and 0.3 μmol/L rhodamine 123 were added to the cuvette, and the scanning fluorescence of the rhodamine 123 was monitored using a fluorescence spectrometer at excitation and emission wavelengths of 503 and 527 nm, respectively. After 30 s, mitochondria (0.5 mg/mL) were added. Finally, the fluorescence intensity of the mitochondria suspension was recorded continuously at 25 °C for 5 min. Each measurement was expressed as a relative value as compared with the baseline intensity.

Measurement of mitochondrial swelling
Mitochondrial swelling was assessed by measuring the change in absorbance of the suspension at 520 nm (ΔA₅₂₀) over time according to the procedure described by Hernández-Muñoz et al[27]. The standard incubation conditions used for the swelling assay were 250 mmol/L sucrose, 10 mmol/L Tris (pH 7.4), 5 mmol/L succinate, and 0.3 mmol/L CaCl₂. Mitochondria (0.5 mg) were suspended in 3.6 mL of phosphate buffer. A quantity of 1.8 mL of this suspension was added to both sample and reference cuvettes; then 6 mmol/L succinate was added to the sample cuvette only, and the A₅₂₀ scanning was started and recorded continuously at 25 °C for 10 min. The extent of the swelling of the mitochondria was evaluated according to the decrease in values obtained at 520-nm absorption.

Determination of mitochondrial succinate dehydrogenase (SDH) and ATPase activity
The activities of mitochondrial SDH and ATPase were detected according to the instructions provided with their respective kits[28,29]. The quantity of Pi produced was representative of the activities of the ATPase, with the active unit mmol (Pi)/(h·mg) protein. SDH activity was expressed as units/mg protein.

Statistical analysis
All results are expressed as mean±SEM. Statistical comparisons were made using a one-way analysis of variance (ANOVA) test. The level of significance was set at P=0.05. All tests were two-sided.

Results
Effect of melatonin on intestinal mucosal injury induced by diclofenac in mice
As compared with the control group, intestinal mucosal injuries, such as edema and small ulcers, were observed macroscopically (P<0.05), and microscopic assessment of the intestinal mucosa showed disarranged villi, epithelial exfoliation, inflammatory cell infiltration and ulceration in the diclofenac group. In contrast, melatonin reduced the observed intestinal mucosal injury both macroscopically and microscopically. Histological scoring based on the Chiu scale showed that melatonin decreased the intestinal damage score significantly in comparison with the diclofenac group (4.8±0.4 vs 1.8±0.4). Therefore, melatonin administration protected mice from diclofenac-induced intestinal inflammation and injury (Figure 1).

Effect of melatonin on intestinal mucosal barrier function induced by diclofenac in mice
As compared with the control group (Figure 2A), the intestinal mucosa in the diclofenac group showed a focal reduction in the thinning of the microvillous carpet and the disarrangement of the epithelial surface as viewed by TEM. In addition,
we observed irregular widening of the intercellular space, decortated and broadened junctional complexes and partially damaged surface epithelium in the diclofenac group (Figure 2B). Melatonin-treated intestine exhibited an attenuation of the surface epithelial damage that was observed in the diclofenac-treated intestine along with a regular microvillous carpet and an improved tight junction structure (Figure 2C). Accordingly, the amount of EB permeating into the intestinal wall and the plasma FD-4 concentrations in diclofenac group were much greater than those recorded in the control group \((P<0.01)\); in contrast, the increases in dye movements were suppressed effectively by melatonin, indicating that melatonin significantly decreased the permeability of the intestinal mucosa (Figure 3).

**Effect of melatonin on the MDA and MPO levels in intestinal mucosal homogenates**

As shown in Figure 4, the MDA levels of intestinal mucosal homogenates increased in the diclofenac group \((1.65\pm0.32 \text{ nmol/mg protein})\), whereas melatonin significantly decreased MDA production \((1.12\pm0.22 \text{ nmol/mg protein})\). Correspondingly, MPO activity was also increased in the intestinal mucosal homogenates obtained from the diclofenac group \((0.236\pm0.027 \text{ U/g homogenate})\). Melatonin treatment effec-
Effect of melatonin on liver mitochondrial swelling and MMP in the diclofenac mouse model of intestinal mucosal injury

Mitochondrial permeability transition (MPT) occurrence was assessed by measurement of the resulting large-amplitude swelling. After energizing with succinate, the swelling rate of mitochondria isolated from the diclofenac group was less than that of the control group. The administration of melatonin partly prevented the diclofenac effect on mitochondrial volume. Mitochondria isolated from the control group had a significant reduction in fluorescence intensity by 30 s after adding the reaction solution. The fluorescence intensity of mitochondria isolated from the diclofenac group decreased less than did that of the control group, and melatonin effectively restored the decrease in fluorescence intensity induced by diclofenac. This observation shows that melatonin improved the impaired mitochondrial function that occurred as a result of diclofenac treatment and maintained a normal MMP (Figure 5).

Effect of melatonin on liver mitochondrial SDH and ATPase activities in diclofenac-treated mice

As shown in Figure 6, as compared with the values of the control group, marked decreases in the activities of ATPase and SDH in mitochondria were found in the diclofenac group (P<0.01). Melatonin clearly restored the activities of ATPase and SDH in mitochondria and, thus, contributed to maintenance of a normal energy metabolism.

Discussion

NSAID enteropathy is a frequent complication that occurs upon treatment with diclofenac and other NSAIDs. Newer imaging modalities, such as capsule endoscopy studies, suggest that small bowel erosions may be common in nonselective NSAID users. In two retrospective studies of 268 and 188 patients, hospitalization due to intestinal perforations or hemorrhage were twice as likely to develop in patients on NSAIDs than in controls[30]. Bjarnason et al[31] reported that up to 70% of patients receiving long-term NSAID therapy, including diclofenac therapy, developed small intestinal NSAID enteropathy. Numerous studies have demonstrated that an increase in intestinal permeability is the central mechanism that translates biochemical damage to tissue damage in the pathogenesis of NSAID enteropathy. NSAIDs increase intestinal permeability in the human within 24 h of ingestion, and this increase is equally evident when NSAIDs are taken long term[32]. As an alternative to intestinal ulceration, intestinal permeability has been investigated as a method for measuring the level of NSAID-induced gut damage in the rat and
then developed as a method for routine measurement. Consequently, it has become a potential therapeutic target for the prevention of NSAID enteropathy.

Several recent publications have presented evidence that melatonin has a significant protective action against gastrointestinal damage. Furthermore, other studies have shown that melatonin has a beneficial effect in experimental models of gastric injury\cite{33, 34}. This protective effect of melatonin, which is believed to be related to its antioxidant activity, has also been demonstrated in the rat small intestine experimental model of I/R. However, it was not clear whether melatonin could improve NSAID-related small intestinal mucosal injury. In the current study, we found that melatonin treatment protected mice from the intestinal mucosal injury induced by diclofenac. Mice treated with melatonin exhibited significantly reduced numbers of and areas of intestinal ulceration, and histological staining showed that melatonin administration strongly attenuated intestinal inflammation. The beneficial effect of melatonin appeared to be related to its reinforcement of intestinal barrier function, as demonstrated by the attenuation of the diclofenac-induced intestinal permeability and the improvement in the ultrastructure observations of the intestinal mucosa in the melatonin-treated group as compared with the diclofenac-treated group.

Lipid peroxidation is considered a major mechanism of oxygen free radical attack and an indicator for oxidative stress and reactive oxygen species production. Experimental evidence has been presented that connects melatonin to the prevention or treatment of gastrointestinal disorders with the scavenging properties of active oxygen\cite{35}. In separate experiments, cotreatment of rats with melatonin and ranitidine or omeprazole was found to protect against stress ulceration in doses at which none of these alone could protect the stomach. These findings raise the possibility that melatonin, although an effective gastroprotective agent individually, may function even better when used in conjunction with ranitidine or omeprazole. Melatonin likely exerts its gastroprotective effects through direct and indirect antioxidant activities\cite{36–38}. Accordingly, our results demonstrate that melatonin treatment decreased intestinal mucosal oxidative injury induced by diclofenac.

NSAID-induced enteropathy has now been shown to be multifactorial, involving a combination of biochemical events that direct mucosal toxicity, mitochondrial damage, and the breakdown of intercellular integrity. This allows mucosal exposure to luminal damaging agents (bacteria and their degradation products, bile acids, etc) with predictable inflammatory reactions. This inflammation varies in intensity from mild to that producing erosions and ulcers. NSAIDs were recently reported to cause mitochondrial injury, resulting in dissipation of the mitochondrial transmembrane potential and induction of a mitochondrial permeability transition pore, which liberates cytochrome c\cite{39}. Cytochrome c generates reactive oxygen species and thereby triggers the caspase cascade and cellular lipid peroxidation, resulting in cellular apoptosis. The pathogenesis of mitochondrial damage induced by NSAIDs alters intestinal permeability with consequent intestinal damage. Accordingly, our results indicate that melatonin strongly protects against intestinal mucosal injury induced by diclofenac in mice. This protective effect of melatonin appears to be mediated by its action on mitochondria. Melatonin protects against alterations in various mitochondrial bioenergetic parameters associated with NSAIDs. Specifically, we found that melatonin significantly restored the activities of ATPase and SDH in mitochondria, thus helping to maintain a normal energy metabolism. This observation suggests that melatonin improved the impaired mitochondrial function induced by diclofenac and maintained a normal MMP.

A role for melatonin in the improvement of mitochondrial function and an increase in ATP production in different experimental conditions has been widely reported. The results of Kim et al\cite{40} suggest that melatonin ameliorates I/R-induced hepatocyte damage by inhibiting the level of oxidative stress and the induction of the apoptotic pathway. The degree of mitochondrial swelling, which reflects the extent of the MPT was greater after 5 h of reperfusion, but this increase was attenuated by melatonin treatment. In this experiment, melatonin limited the release of cytochrome c into the cytosol and the activation of caspase-3 observed in I/R rats. Thus, melatonin protected against mitochondrial injury by reducing mitochondrial oxidative stress and improving I/R-induced hepatic energy metabolism\cite{41} and by directly inhibiting formation of the mitochondrial permeability transition pore and reducing the membrane potential\cite{42, 43}. Martin et al\cite{44} showed that melatonin counteracted mitochondrial oxidative stress and increased the activity of the mitochondrial oxidative phosphorylation enzymes both in vivo and in vitro. Melatonin also increased the production of ATP in control mitochondria. Acuña-Castroviejo et al documented that melatonin counteracted mitochondrial oxidative damage induced by t-butyl hydroperoxide, recovering glutathione levels and ATP production\cite{45, 46}. These data, along with other findings, suggest that melatonin regulates mitochondrial homeostasis\cite{47}.

In conclusion, our results demonstrate that pharmacological concentrations of melatonin strongly protect against the intestinal mucosal injury and ameliorate the increased intestinal permeability induced by diclofenac. The mechanism of protection is likely to be due, at least in part, to the preservation of mitochondrial function mediated by melatonin.

**Author contribution**

Qiao MEI and Jian-ming XU designed research; Lei DIAO, Xiao-chang LIU, and Juan JIN performed research; Qiao MEI, Lei DIAO and Xiao-chang LIU analyzed data; Lei DIAO wrote the paper.

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