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• BASIC RESEARCH •

Effects of melatonin on the expression of iNOS and COX-2 in rat models of colitis

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Abstract

AIM: To investigate the effects of melatonin (MT) on the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in rat models of colitis.

METHODS: Healthy adult Sprague-Dawlay (SD) rats of both sexes, weighing 280±30 g, were employed in the present study. The rat models of colitis were induced by either acetic acid or 2,4,6-trinitrobenzene sulfonic acid (TNBS) enemas. The experimental animals were randomly divided into melatonin treatment and model control group that were intracolicly treated daily with melatonin at doses of 2.5, 5.0, 10.0 $mg \cdot kg^{-1}$ and equal amount of saline respectively from 24 h following induction of colitis in rats inflicted with acetic acid enema and the seventh day in rats with TNBS to the end of study. A normal control group of rats treated with neither acetic acid nor TNBS but saline enema was also included in the study. On the 28th day of the experiment, the rat colon mucosal damage index (CDMI) was calculated, and the colonic prostaglandin E_2 (PGE_2), nitric oxide (NO), as well as the iNOS and COX-2 expression were also determined biochemically or immunohistochemically.

RESULTS: CDMI increased to 2.87±0.64 and 3.12±1.12 respectively in rats treated with acetic acid and TNBS enema, which was in accordance with the significantly elevated colonic NO and PGE_2 contents, as well as the up-regulated colonic iNOS and COX-2 expression in both of the two rat models of colitis. With treatment by melatonin at the doses of 5.0 and 10.0 $mg \cdot kg^{-1}$, CDMI in both models of rat colitis was significantly decreased (P<0.05-0.01), which accorded synchronously and unanimously with the reduced colonic NO and PGE_2 content, as well as the down-regulated expression of colonic iNOS and COX-2.

CONCLUSION: Melatonin has a protective effect on colonic injury induced by both acetic acid and TNBS enemas, which is probably via a mechanism of local inhibition of iNOS and COX-2 expression in colonic mucosa.

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INTRODUCTION

Inflammatory bowel disease (IBD) consists of a group of illnesses with chronic inflammation of the gastrointestinal tract, which causes life-impairing symptoms, necessitates long-term dependence on powerful drugs, and often results in debilitating surgery and even death. Although the etiology remains unclear, IBD appears to result from a dysregulated immune response. In recent years, plenty of studies have shown that nitric oxide (NO) and prostaglandin (PG) as the main inflammatory mediators take part in the pathogenesis of inflammatory bowel disease, with enhanced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the morbid colonic mucosa[1,2]. Meanwhile, it has been noted melatonin (MT) normally produced mainly in the gastrointestinal tract besides the pineal gland bears a number of beneficial properties including anti-oxidation, anti-inflammation and immunoregulation[3-15], and could alleviate colonic injury caused by both dextran sulfate sodium[16] and dinitrobenzene sulfonic acid[17] in rats. It is reasonable to extrapolate that the protective roles of melatonin might be related to its effect on the expression of iNOS and COX-2 in local tissue. We therefore performed the present study in an attempt to confirm this hypothesis.

MATERIALS AND METHODS

Animals

Healthy adult Sprague-Dawlay (SD) rats of both sexes, weighing 280±30 g, were employed in the study. They were purchased from the Experimental Animal Center, Anhui Medical University, housed in a temperature conditioned room (22-24 ℃) with a 12 h light-dark cycle, allowed access to standard rat chow and water ad libium, and acclimatized to the surroundings for one week prior to the experiments. The study protocol was in accordance with the guideline for animal research and was approved by the Ethical and Research Committee of the hospital.

Reagents

Melatonin, TNBS, and N-1-naphthylenediamine hydrochloride came from Sigma Corp. Acetic acid was purchased from Bangbu Chemical Corp. PGE_2 assay kit was from Radio-immunity Institute of PLA General Hospital. Immunohistochemical assay kits for iNOS and COX-2 were provided by Beijing Zhongshan Reagent Corp. Other reagents used in the present study were all with a quality of analytical grade.

Experimental protocol

Rat model of colitis induced with either acetic acid or TNBS enema was described in the literature[18, 19]. According to different treatment regimens, the experimental animals were randomly divided into melatonin treatment and model control group that were intracolicly treated under anesthesia with melatonin at doses of 2.5, 5.0, 10.0 $mg \cdot kg^{-1}$ and equal amount of saline respectively and daily (8:00 am) from 24

h following induction of colitis in rats inflicted with acetic acid enema and the seventh day in rats after TNBS treatment to the end of the experiment. A normal control group of rats treated with neither acetic acid nor TNBS but saline enema was also included in the study. On the 28th day of the experiment, the animals were killed and the colon mucosal damage index (CDMI) was evaluated with the methods reported elsewhere[19, 20]. At the same time, colon tissue prostaglandin E_2 (PGE_2) and nitric oxide (NO), as well as the expression of iNOS and COX-2 were determined biochemically or immunohistochemically.

Determination of NO and PGE_2

Colonic specimen was prepared to a concentration of 20 g·L^{-1} by adding dehydrated alcohol-saline (1:4), and then centrifuged at 4 000 g for 30 min, 4 ℃. Two milliliters of the supernatant were added into 0.1 ml of HCL (0.1 mol·L^{-1}) and further adjusted pH to 3.5 by 0.05 mol·L^{-1} of NaOH. After mixed with ethyl acetate 5 ml for 2 min, they were centrifuged at 1 500 g for 15 min. Repeat the procedures above and the sample solution was evaporated to dryness by N_2 and stored at -20 ℃ until analysis. The samples was dissolved to 1 ml of phosphate buffered saline, from which 0.1 ml of specimen was taken to perform the PGE_2 measurement following the manufacturer's instruction of the assay kit[21]. The colonic tissue NO was detected as described in the literature[22].

Immunohistochemistry detection

The expression of iNOS and COX-2 in colon tissue was exhibited immunohistochemically as reported before[23], in which the employed first polyclonal antibody was 1:80 rabbit-anti-rat-iNOS and 1:50 goat-anti-rat-COX-2, and the second antibody was biotinylation goat-anti-rabbit IgG and biotinylation rabbit-anti-goat IgG respectively. The iNOS or COX-2 negatively expressed cells were manifested as blue-stained nuclei and the positive cell was with brown-yellow cytoplasma or nuclear membrane. The expression of target protein was further semiquantitated according to the percentage of positively-stained cells, in which positive cells appeared less than 5 %, from 6 % to 30 %, from 31 % to 70 % and morn than 71 % were scored as 0, 1, 2 and 3 respectively.

Statistical analysis

Experimental results were analyzed by ANOVA and *t*-tests for multiple comparisons between groups. Data were finally expressed as mean ± standard error of the mean. *P* value less than 0.05 was considered statistically significant.

RESULTS

Protective effects of melatonin on rat colonic lesion

Pronounced pathological changes of colonic mucosa similar to that in human IBD were observed in rats with colitis induced by both acetic acid and TNBS enema, which were in accordance with the colon mucosal damage index that was significantly increased in these experimental animals compared with normal controls ($P<0.01$). Local treatment with different doses of melatonin by enema could effectively reduce the severity of gut injury and the CMDI was significantly decreased in a dose dependent manner in rats treated by melatonin compared with that in model control animals ($P<0.05$-0.01, Table 1).

Effects of MT on NO content and iNOS expression

In normal controls, colonic iNOS expression was mainly observed on histocytes, neutrophils and smooth muscle cells with a sparse distribution in epithelial cells. The NO content and the expression of iNOS in colonic tissue were synchronously and significantly increased in rats inflicted with both acetic acid and TNBS enema compared with that of the normal controls ($P<0.01$), which were significantly inhibited by different doses of melatonin employed in the present study ($P<0.05$-0.01 *vs.* Model control, Table 2, 3, Figure 1).

Table 1 Effects of MT on CMDI in rats with experimental colitis ($\bar{x}$±s, n=8)

Group	Doses (mg · kg^{-1})	CMDI	
		Acetic acid	TNBS
Normal control		0.0±0.0	0.0±0.0
Model control		2.87±0.64[a]	3.12±1.12[a]
Melatonin	2.5	2.12±0.83[b]	2.33±0.51
Melatonin	5.0	1.75±0.88[c]	1.66±0.81[b]
Melatonin	10.0	1.12±0.35[c]	1.75±0.88[b]

[a]$P<0.01$ *vs.* Normal control; [b]$P<0.05$, [c]$P<0.01$ *vs.* Model control.

Table 2 Effects of MT on the colonic NO and PGE_2 levels in rats with experimental colitis ($\bar{x}$±s, n=8)

Group	Doses (mg·kg^{-1})	NO (μmol·g^{-1} tissue)		PGE_2 (ng·g^{-1} tissue)	
		Acetic acid	TNBS	Acetic acid	TNBS
Normal control		0.174±0.044	0.287±0.069	43.1±32.1	43.1±32.1
Model control		0.327±0.090[b]	0.533±0.068[b]	184.5±96.3[b]	181.3±51.7[b]
Melatonin	2.5	0.230±0.017[d]	0.403±0.042[d]	89.1±59.1[c]	109.0±33.3[d]
Melatonin	5.0	0.218±0.018[d]	0.380±0.029[d]	76.4±23.6[c]	89.8±37.7[d]
Melatonin	10.0	0.189±0.029[d]	0.340±0.019[d]	57.1±23.2[d]	85.9±39.2[d]

[a]$P<0.05$, [b]$P<0.01$, *vs.* normal control; [c]$P<0.05$, [d]$P<0.01$ *vs.* model control.

Effects of melatonin on PGE_2 content and COX-2 expression

Compared with normal group, the content of PGE_2 and the expression of COX-2 in rat colitis, the number of positive granules and the degree of staining were enhanced significantly. The content of PGE_2 was decreased after different doses of melatonin were given by enema. The expression of COX-2 was inhibited by melatonin, which proved that melatonin decreased the synthesis of PGE_2 and it might be related with inhibition of the expression of COX-2 (Figure 2, Table 2, 3).

Table 3 Effects of MT on the colonic expression of iNOS and COX-2 semi-quantitated in rats with experimental colitis ($\bar{x}$±s, n=6)

Group	Doses (mg·kg^{-1})	iNOS		COX-2	
		Acetic acid	TNBS	Acetic acid	TNBS
Normal control		0.3±0.5	0.3±0.5	0.2±0.4	0.2±0.4
Model control		2.2±0.9[b]	2.3±0.8[b]	1.8±0.9[b]	2.2±0.9[b]
Melatonin	2.5	1.3±0.5[c]	1.5±0.5[c]	1.2±0.4	1.7±0.8
Melatonin	5.0	0.5±0.5[d]	1.2±0.8[c]	1.0±0.6	1.3±0.8
Melatonin	10.0	0.3±0.5[d]	0.7±0.8[d]	0.5±0.5[c]	0.8±0.8[c]

[a]$P<0.05$, [b]$P<0.01$ *vs.* Normal control; [c]$P<0.05$, [d]$P<0.01$ *vs.* Model control.

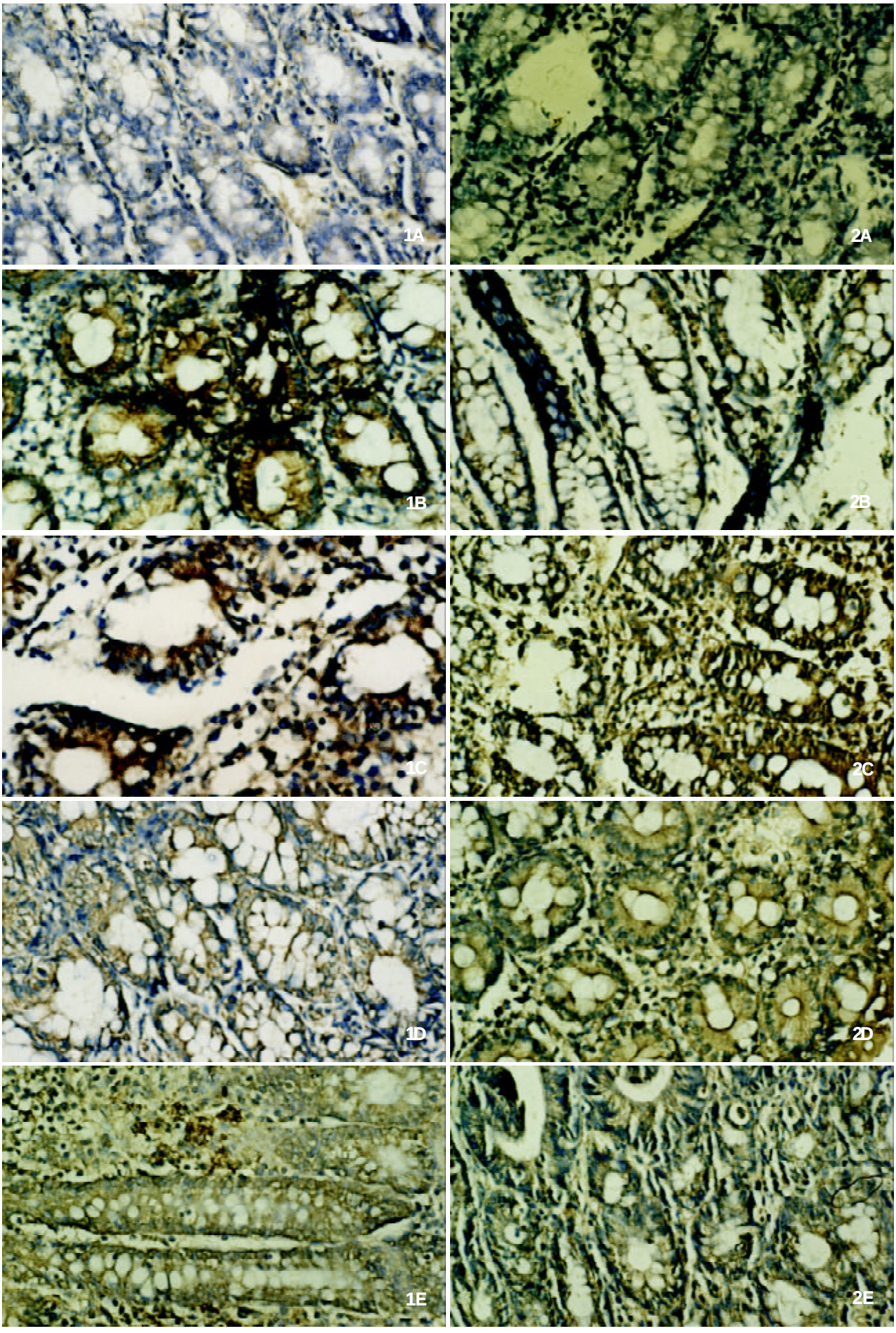

Figure 1 Abnormal expression of iNOS in colonic tissue of rats with cloitis induced by both acetic acid and TNBS enemas and its

improvement by melatonin. A. Immunohistochemical localization of iNOS in normal control, which was manifested as fine brown granules distributed mainly in the cytoplasm of histocytes, neutrophils and smooth muscle cells. B. Positively stained granules for iNOS were significantly increased in both number and intensity in colonic tissue of model control rats. (a) Acetic acid treated rats; (b) TNBS treated rats. C. The colonic iNOS expression was significantly reduced in both acetic acid (a) and TNBS (b) treated rats after intervened with 10.0 $mg \cdot kg^{-1}$ of melatonin.

Figure 2 Abnormal expression of COX-2 in colonic tissue of rats with colitis induced by acetic acid or TNBS enema and its improvement by melatonin, which was in accordance with the observation in clonic iNOS expression. A. Positively stained COX-2 granules in colonic tissue of normal control rats. B. iNOS expression was significantly increased as manifested by the augmented and intensified positively stained granules in colonic tissue of model control rats. (a) Acetic acid treated rats; (b) TNBS treated rats. C. The colonic COX-2 expression was significantly reduced in both acetic acid (a) and TNBS (b) treated rats after intervened with 10.0 $mg \cdot kg^{-1}$ of melatonin.

DISCUSSION

Although the etiology of IBD remains unclear, dysregulated immune response has been widely accepted as a possible mechanism in the pathogenesis of inflammatory bowel disease. Numerous reports have revealed that certain local bioactive agents including NO and PGE_2 are involved in colonic injury by various inducers[1, 2]. Meddleton and other authors[24, 25] found NO concentration was rather higher in ulcerative colitis patients with obviously strengthened iNOS activity. As an important inflammatory mediator, NO could react with superoxide anion to form more poisonous nitrite anion, which then disturbs the function of inflammatory cells and further impairs the colonic mucosa[26, 27]. PGE_2, another major local inflammatory mediator that might come from activated eosinophils and monocytes[28], is also considered as a marker of colitis. In the two rat models of colitis respectively induced by acetic acid and TNBS in the present study, the mucosal NO and PGE_2 contents in the morbid colon were significantly increased with enhanced expression of iNOS and COX-2, which was in accordance with the previous reports.

Melatonin, a major hormone produced in pineal gland, was also found in recent years to be secreted for a certain amount from gastrointestinal tract and played an important role in the adjustment of gastrointestinal function[7-14]. As a potent antioxidant agent that could clear oxygen-derived free radicals, inhibit the activation of NF-κB and reduce inflammatory response, melatonin has been widely used to treat inflammatory bowel diseases[29, 30]. Pentney and his coworker[16] have shown melatonin could reduce the severity of dextran-induced colitis in mice. Protective effects of melatonin on dinitrobenzene sulfonic acid induced colitis have been proved by Cuzzocrea and his colleagues[17]. In the present investigation, melatonin was demonstrated to reduce colonic lesions induced by acetic acid and TNBS enemas, which combined with the reports above, suggested that the protective effect of melatonin on the induced colitis might be universal. The present study also revealed the improvement of colonic lesions by melatonin accorded synchronously and unanimously with the decrease of colonic NO and PGE_2 content, as well as the down-regulated expression of colonic iNOS and COX-2, which indicates the improvement is probably via a mechanism of local inhibition of iNOS and COX-2 expression in the colonic mucosa. Further studies are needed to explore other mechanisms involved in the protection of colonic mucosa by melatonin.

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