Original Articles

Melatonin reduces the expression of chemokines in rat with trinitrobenzene sulfonic acid-induced colitis

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ABSTRACT

الأهداف: دراسة أثر الميلاتونين على التهاب القولون لدى الجرذان المصابة بالتهاب القولون وتحديد فيما إذا كان هذا التأثير مصحوب بحصر الجزيئات الجاذبة للكيماويات (IL-8) و (MCP-1).

الطريقة: صممت الدراسة ونفذت في مستشفى الشعب رقم 1 – جنغ مين بمقاطعة هيو باي – الصين، خلال الفترة مابين مايو 2006م وحتى أبريل 2007م. شملت الدراسة 72 جرذا تم تقسيمها إلى ست مجموعات، كل منها تتألف من 12 جرذا: المجموعة الطبيعية، والمجموعة النموذجية، ومجموعة حامض 5-أمينوساليسيليكليك، ومجموعة الميلاتونين (10.0mg/kg ,2.5,5.0). تم تحديد مجموعة نموذج التهاب القولون بحقنة من حامض 2، 4، ترينيتروبنزيت–6 سولفونيك (TNBS). تم فحص بروتينات (IL–8) و (MCP-1) في أنسجة القولون بواسطة الفحص الكيميائي المناعي النسيجي والويسترن بلوت. كما تم تحديد مظاهر الحركيات الكيميائية من خلال تحليل التفاعل التسلسلي للبوليميراس.

النتائج: أدت حقنة حامض 2، 4، ترينيتروبنزيت–6 سولفونيك (TNBS) في تغيرات مرضية ظاهرة في غشاء القولون في مجموعة الجرذان النموذجية، والتي كانت متفقة مع ارتفاع كبير في نشاط القولون (MPO). كما ارتفع مستوى مظاهر الحركيات الكيمياوية في التهاب القولون. قلل العلاج بالميلاتونين من آفات القولون، وحسن من أعراض التهاب القولون، وقلل من مظاهر البروتين والجزيئات الجاذبة للكيمياويات (IL-8) و (MCP-1) بشكل كبير في أنسجة القولون لدى الجرذان المصابة بالتهاب القولون.

خاتمة: تعتبر الحركيات الكيميائية (IL-8) و (MCP-1) مرتفعة في الأنسجة الغشائية في حالات التهاب القولون، وتلعب دورا مهما في إدامة العمليات الالتهابية المدمرة للأنسجة، بينما يقلل الميلاتونين من الإصابة الالتهابية في القولون لدى الجرذان المصابة بالتهاب القولون وذلك من خلال التقليل من مظاهر الحركيات الكيميائية. ومن ثم يمكن اعتبار الميلاتونين بديلا علاجيا حديثا لعلاج أمراض الأمعاء الالتهابية (IBD).

Objectives: To investigate the effect of melatonin on the colon inflammatory injury of rats with colitis and determine whether this effect is associated with inhibition of chemoattractant molecules interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1.

Methods: The study was designed and implemented in JingMen No.1 People's Hospital, HuBei Province, from May 2006 to April 2007. It involved 72 animals divided into 6 groups of 12 each: normal group, model group, 5-aminosalisalicylic acid group, and melatonin group (dose of 2.5, 5.0, and 10.0mg/kg). Rat colitis model was established by 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) enema. Interlukin-8 and MCP-1 proteins in colon tissue were examined by immunohistochemistry and western blot. The messenger-RNA expressions of chemokines were determined by reverse transcription polymerase chain reaction analysis.

Results: Trinitrobenzene sulfonic acid enema resulted in pronounced pathological changes of colonic mucosa in model rats, which were in accordance with the significantly elevated myeloperoxidase activity. Expressions of chemokines were up-regulated in colitis. Melatonin treatment reduced colonic lesions and improved colitis symptom, and decreased the protein and mRNA expressions of IL-8 and MCP-1 significantly in colon tissues of rats with colitis.

Conclusion: Chemokines IL-8 and MCP-1 are elevated in mucosal tissues in colitis and play an important role in the perpetuation of tissue destructive inflammatory processes; melatonin reduces colonic inflammatory injury of rats colitis through down-regulating the expressions of chemokines. Melatonin can be considered as a novel therapeutic alternative for the treatment of inflammatory bowel disease.

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Inflammatory bowel disease (IBD), consisting of ulcerative colitis (UC) and Crohn's disease (CD), are chronic relapsing inflammatory conditions of colon with unknown aetiology. It is characterized by continuous infiltration of affected tissues by inflammatory cells from the circulation, which results in the further upregulation and exacerbation of destructive processes within the intestine.[1] The recruitment and activation of leukocytes in inflamed tissues is a complex process, and many studies have shown that chemokines, proinflammatory cytokines and adhesion molecules exhibit a key role in the process that involves inflammatory cell adhesion and locomotion.[2,3] Chemokines can rapidly induce an inflammatory cell response and mediate the recruitment and activation of immunocytes. In recent years, plenty of studies have described the increased expression of CXC and CC chemokines including interleukin (IL)-8 and monocyte chemo-attractant protein (MCP)-1 in diseased tissues of patients with IBD.[4-6] Interlukin-8 is a C-X-C chemokine predominantly chemotactic for neutrophils, and MCP-1 is a C-C chemokine predominantly chemotactic for monocytes; they elicit the infiltration of neutrophils and monocytes into the colonic mucosa of patients with UC, which is thought to be an important factor in the pathophysiology of intestinal inflammation.[6-8] Therapy of UC is difficult on account of the complex etiology of disease. At present, medical treatment of UC relies mainly on traditional drugs: aminosalicylates, corticosteroids and immunosuppressants. These drugs, including glucocorticoids and aminosalicylates, reduce inflammatory injury and attenuate the expression of some proinflammatory molecules,[9,10] but their side effects and systemic action are so hard that disturb the life quality of patients severely, particularly during long term treatment, which limit their use. And so, it is very important to find an optimal therapy with fewer adverse reactions for UC. Melatonin, which mainly produced in the pineal gland, has various biological activities and different pharmacologic effects, including antioxidation, anti-inflammatory and modulation of immune response. Gastrointestinal tract is a rich source of extrapineal melatonin.[11] Some studies have demonstrated that melatonin exerts as an antioxidant and scavenger of free radicals to reduce the severity of colitis.[12] And the other data have also shown that melatonin can alleviate colonic injury in experimental colitis induced by both dextran sulfate sodium and dinitrobenzene sulfonic acid in rats,[12,13] but the mechanism that exogenous melatonin improves inflammatory injury still remains unclear. This therapeutic effects of melatonin rely at least partially on immunomodulatory function and antioxidation, anti-inflammatory activities in inflammatory tissues.[14]

In the present study, we observed the protective effect of melatonin on inflammatory injury in 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, investigated its effects on the production of chemokines, IL-8 and MCP-1, and explored the probable mechanisms that melatonin ameliorates inflammatory injury in colitis.

Methods. Animals Purebred Sprague-Dawlay (SD) rats of both gender, weighing 250 ± 30 gm, were purchased from the Experimental Animal Center of Wuhan University (Wuhan, China). Animals were allowed to adapt to our laboratory environment for one week before beginning the experiment, and they were housed in a standard cages with free access to tap water and maintained in a room under standard conditions of feeding and temperature with a 12-hour light-dark cycle. The study protocol was approved by the Ethical and Research Committee of the hospital.

Experimental protocol. The study took place from May 2006 to April 2007 and was conducted on 72 rats which were randomly divided into 6 groups of 12 each: normal group, model group, 5-aminosalicylic acid (5-ASA) group, and melatonin treatment group (low, middle and high dose group). Rat model of colitis induced with 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma Co) enema was described in the literature.[15] The animals were intracolicly treated under anesthesia with saline, TNBS/40% ethanol (150mg/kg), 5-aminosalicylic acid (100mg/kg), and melatonin (Sigma Co) at doses of 2.5, 5.0, 10.0 mg/kg enema respectively (once a day, from the 24-hour after colitis was established to the end of experment). At the end of 4 weeks, the animals were sacrificed and the colon samples were collected. The tissue of colon 8 cm proximal to anus was excised, opened longitudinally, and washed in saline buffer for macroscopic score. Colon tissue was fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded, processed, and sectioned in 4 μm thick sections, and stained with haematoxylin and eosin. The colon mucosal macroscopic and histological damage indices were evaluated with the methods reported previously.[14,15]

Determination of myeloperoxidase The colon tissue was rinsed and weighed, and homogenized in a solution prepared from the assay kit (Nanjing Jiancheng Bioengineering Co. Ltd, China), and homogenates of 5% were obtained and was measured by the assay kit according to its provider's instructions. One unit of MPO activity was defined as the quantity of enzyme that degraded one μmol H_2O_2 at 37^0C per g wet tissue. Immunohistochemistry detection tissue sections were baked at 65^0C until get deparaffinage and hydrated by a series of ethanol solutions; microwave repairs the antigen. The slides were incubate in 1% hydrogen dioxide approximately 20 min to inactivate the

endogenous peroxidase. Washed by phosphate buffer solution (PBS), the sections were further blocked by normal goat serum for 15 minutes to reduce non-specific antibody binding and then incubated with the primary antibodies of IL-8 (diluted to 1:50, rat monoclonal, Santa Cruz Biotechnology) or MCP-1 (diluted to 1:100, rat polyclonal, Santa Cruz Biotechnology) at 37°C for 60 minutes. Washed by PBS, the slides were incubated with biotin-labeled anti-rat IgG for one-hour at room temperature and developed in 0.05% freshly prepared diaminobenzedine solution for several minutes, and then counterstained with hematoxylin. Phosphate buffer solution was used as first-antibody and second-antibody in negative control. The positive cells were observed and evaluated by 2 independent observers. The results were evaluated semi-quantitatively according to the percentage of positive cells in 10 randomly selected fields under high-power microscope (400-fold magnification) for each sample.

Western blot analysis. Western blotting was performed as described previously[16] with minor modification. Colon tissue was pounded to pieces in liquid nitrogen, then minced and homogenized in 400 µL of hypotonic lysis buffer. The homogenates were separated by centrifugation (0°C, 14000 r/min, 10 min) and the supernatants were collected. Proteins were measured with a Bradford protein assay. Equivalent amounts of proteins (40ug) was loaded on each lane of a 12% denaturing polyacrylamide gel and separated by polyacrylamide gel electrophoresis, then transferred to a nitrocellulose membrane. The membranes were blocked in buffer for one-hour at room temperature and incubated with the primary antibody (anti-IL-8, anti- MCP-1 1:1000 diluted in blocking buffer, Sigma Corp) on a shaker at 4^0C overnight. The membranes were then treated with a peroxidase conjugated secondary antibody (diluted to 1:2000, Santa Cruz Biotechnology). Western blotting luminol reagent (Cell Signal Corp) was used to detect antibody binding. The membranes were exposed to x-ray film several minutes and the bands were quantified by densitometry. The expression of ß-actin was used as a normalization control for protein loading.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from a fresh colon sample with Trizol reagent (Sigma, Co) by the single-step method.[17] An aliquot of total RNA was reverse transcribed and amplified using moloney murine leukemia virus (MMLV) reverse transcriptase and Taq DNA polymerase (Promega, Southampton, UK), respectively. Transcripts of the gene for ß-actin were used as an internal control. Reactions were carried out under the following conditions: 3 minutes at 94°C for one cycle; denaturation for 30 minutes at 94°C, annealing for 45 minutes at 55°C, and extension for one minute at 72°C for 30 cycles; 7 minutes at 72°C for one cycle. The rat specific primers (sense and antisense primers) for IL-8 and MCP-1, and ß-actin were 5'-GGGTAGAAACTCCTAGGCTTC-3' and 5'-TTAGCGTTTCTTACCTGGTTA-3' (IL-8,360 bp); 5'-CACCTGCTGCTACTCATTCACT-3' and 5'-GTTCTCTGTCATACTGGTCACTTC-3' (MCP-1,349bp); 5'-ATGGATGACGATATCGCTG-3' and 5'-ATGAGGTAGTCTGTCAGGT-3' (ß-actin, 568bp), respectively. Polymerase chain reaction products were electrophoresed on 15g/L agarose gels and stained with ethidium bromide.

Statistical analysis. Data were presented as mean ± standard deviation. All statistical analyses were performed using the Statistical Package for Social Sciences Version 11.5. Student's t test and one-way analysis of variance (ANOVA) were used to compare continuous variables among groups. Probability value less than 0.05 was considered statistically significant.

Results.

Protective effects of melatonin on colonic lesion. The colonic mucosa of rats with colitis was hyperemic, edematous, and granular, and some small punctate ulcers were visible, which is similar to that in human IBD. Most animals inflicted with TNBS enema had disease limited to the rectum and rectosigmiod, some had disease extending beyond the sigmoid or had a total colitis. And rats with TNBS-induced colitis showed a number of neutrophils, macrophages, lymphocytes and eosinophil infiltration in mucosa and submucosa. The colon mucosal macroscopic and histological injury indices, and MPO activity were significantly increased in these experimental animals compared with normal controls. No macroscopic and histological damage was seen in normal controls. Treatment with melatonin significantly reduced the severity of colonic lesion score and alleviated the colitis symptoms.

Immunohistochemistry. Compared with the normal controls, the protein expressions of IL-8 and MCP-1 in colon tissues were significantly increased in rats inflicted with TNBS enema. The positive cells of IL-8 and MCP-1 were predominantly located within the mucosa and mucosa lamina propria with brown-yellow cytoplasm. or nuclear membrane (**Figures 1 & 2**). Administration of 5-ASA resulted in a significant reduction of colon IL-8 and MCP-1 levels. And the expression of IL-8 and MCP-1 decreased dose-dependently in rats with melatonin treatment. The effect of 10mg/kg melation is similar to that of 5-aminosalicylic acid (100mg/kg) (**Table 1**).

Western blotting. The Western blotting results showed that the expressions of IL-8 and MCP-1 in rat colitis were significantly increased compared with normal controls (p=0.000, p=0.000). Melatonin

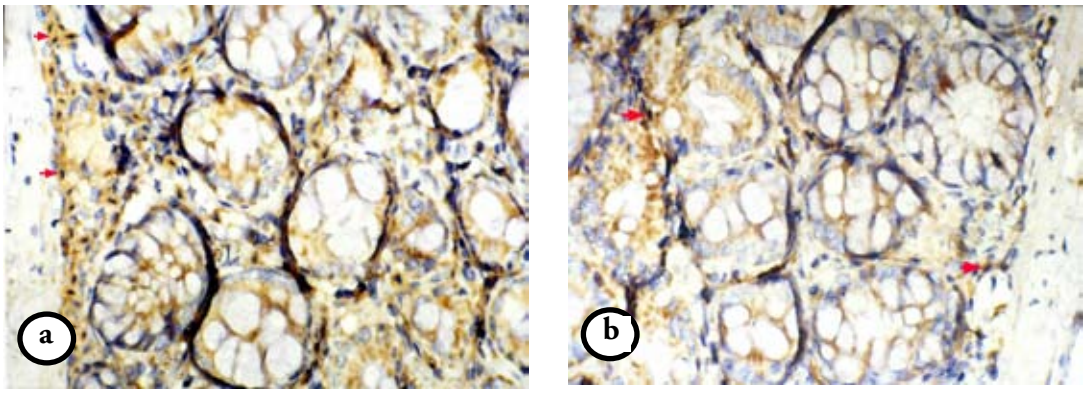

Figure 1- Immunohistochemical staining of interleukin-8 (IL-8) in colon tissues of model rats **a)** and rats with melatonin treatment (10 mg.kg-1) **b)** SP × 400. Interlukin-8 protein is mainly expressed in the cytoplasmic and nuclear membrane accumulation of IL-8 is also detected and positively stained granules distributed mainly in mucosa and mucosa lamina propria. Interlukin-8 expression decreases dramatically in melatonin group.

Table 1 - Effects of melatonin and 5-aminosalicylic acid (5-ASA) on protein expression of IL-8 and MCP-1 in colon tissue (n=12).

Group	Doses (mg.kg^{-1})	IL-8	MCP-1
Normal		12.15±5.38*	17.28±4.57*
Model		80.27±13.64	87.23±7.36
5-ASA	100	25.38±8.25*	29.61±9.27*
Melatonin low	2.5	72.53±14.87	79.29±11.38
Melatonin middle	5.0	60.86±10.26*	52.16±9.33*
Melatonin high	10.0	33.51±9.31*	37.73±7.24*

*p<0.01 versus model group

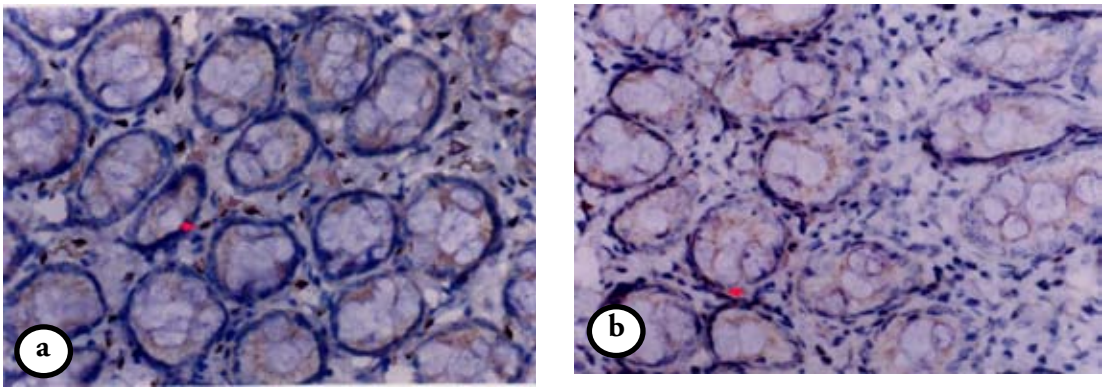

Figure 2 -Immunohistochemical staining for monocyte chemoattractant protein (MCP-1) protein expression. SP×400 **a)** MCP-1 protein expression in model group. **b)** MCP-1expression in melatonin group (10mg.kg^{-1}). Positively stained granules for MCP-1 were significantly increased in colonic tissue of model control rats. The colonic MCP-1 expression was significantly reduced in melatonin group.

treatment decreased the expressions of IL-8 and MCP-1 in a dose-dependent manner, the inhibition effect of melatonin was most obvious at a concentration of 10 mg/kg. Five-aminosalicylic acid also reduced the expressions of IL-8 and MCP-1 significantly (p=0.006, p=0.008, **Figure 3**).

Reverse transcriptase-polymerase chain reaction analysis revealed the mRNA levels for IL-8 and MCP-1 in colon tissues. Compared with the normal controls, the mRNA levels of IL-8 and MCP-1 of the model group showed a significantly high expression (p=0.000, p=0.000). The mRNA expressions were inhibited dose-dependently when animals were treated with melatonin and 5-ASA. Maximum inhibition effect was observed with melatonin at a concentration of 10mg/kg. These results were in accord with immunohistochemical

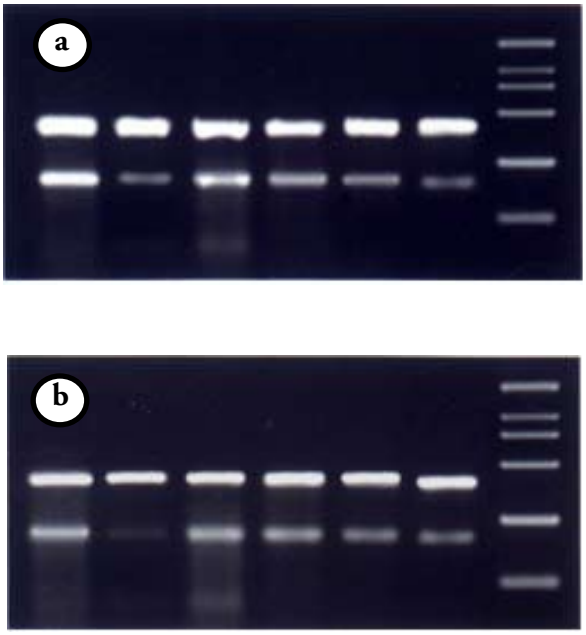

Figure 3 - The mRNA expressions of **a**) interleukin-8 (IL-8) and **b**) monocyte chemoattractant protein (MCP)-1 were assessed using reverse transcriptase-polymerase chain reaction standardized by coamplifying the housekeeping gene ß-actin. Lanes 1-7: model, normal, melatonin (2.5, 5.0, 10.0 $mg.kg^{-1}$), and 5-aminosalicylic acid (5-ASA) marker.

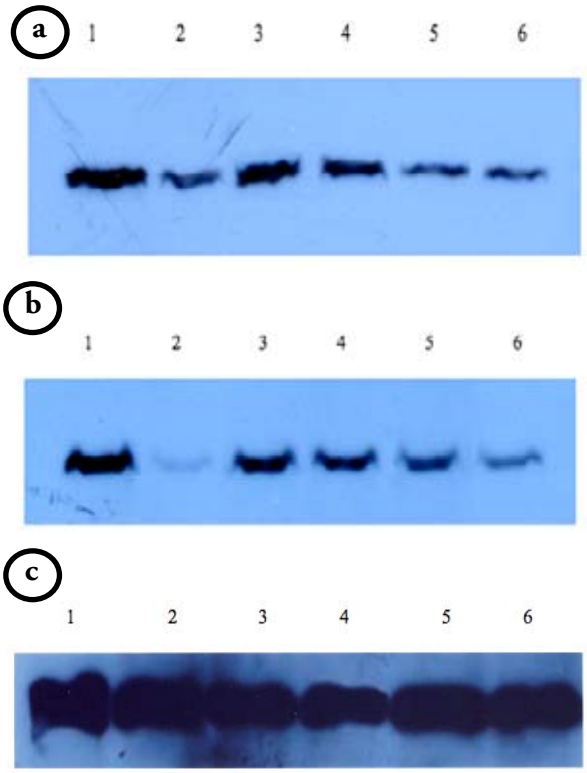

Figure 4 - Western-blotting showed levels of **a**) interleukin-8 (IL-8) and **b**) monocyte chemoattractant protein (MCP)-1 in colon tissue of rats. Lanes 1-6: model, normal, melatonin (2.5, 5.0, 10.0 $mg.kg^{-1}$) and 5-aminosalicylic acid (5-ASA).

and Western blotting analysis of molecule expressions (**Figure 4**).

Discussion. A number of animal models of acute and chronic colon inflammation have been developed to mimic human IBD. Trinitrobenzene sulfonic acid-induced colitis model was widely adopted to observe the effects of drugs, because this model is well known to have similarity to human IBD and the availability of a quantitative scoring system. The present study demonstrated that melatonin in a dose-dependent manner improved the inflammation damage and inhibited the infiltration of inflammatory cells in TNBS-induced colitis in rats. Ulcerative colitis is characterized by chronic inflammation of colon with unknown aetiology, and during chronic inflammation of the intestine, there is a continuous migration of large numbers of activated granulocytes, macrophages, and lymphocytes from the circulation into the mucosa. Infiltration and persistence of inflammatory cells within tissues are hallmarks of inflammation.[18-22] And the infiltration of leukocytes leads to the further upregulation and perpetuation of inflammatory destructive processes and is primarily caused by the presence of a wide variety of different chemoattractant molecules.[23,26] Chemokines are produced by resident cells such as tissue macrophages, mast cells, fibroblasts, and endothelial and epithelial cells, but also by infiltrating inflammatory cells. And the secretion of chemokine is low or non-existent in resting cells but rapidly becomes up-regulated during inflammation. Numerous studies have described that the expressions of IL-8 and MCP-1 were extensively detected in diseased tissues.[27-30] Interlukin-8 is the major attractant and activator of neutrophils, and MCP-1 attracts monocytes via receptors CCR1.[6-8] These chemokines were chosen because the inflammatory cells activated by them are frequently present in inflamed tissues. Our studies showed that colon tissue obtained from rats with TNBS-induced colitis exhibited significantly more IL-8 and MCP-1 expressions than normal controls, which supported the idea that chemokines molecule participate in the occurrence and development of ulcerative colitis. Melatonin, a major hormone produced in pineal gland, bears a number of beneficial properties including antioxidation, antiinflammation and immunoregulation.[11,31-32] The previous studies have showed that melatonin could improve the colonic injury and reduce the severity of

dextran-induced colitis in mice. And several papers have also reported that melatonin could clear oxygen-derived free radicals, inhibit the activation of proinflammatory cytokines and reduce inflammatory response.[11-14] These data suggested that the protective effect of melatonin on the induced colitis might be universal. In present study, we found that melatonin inhibited protein expression of IL-8 and MCP-1 dose-dependently. In addition, melatonin could reduce the levels of MPO, a marker for neutrophils infiltration. Our results also showed that melatonin could attenuate the colitis symptoms such as rectal bleeding and occult blood, and reduce the frequency and severity of mucosa damage dramatically. These significant protective effects may be partly due to its effect on inhibition of expression of chemokines including IL-8 and MCP-1. In order to elucidate the mechanism of action of melatonin on chemokines, we investigated the mRNA expression of IL-8 and MCP-1 in colon tissues of rats and determined the influence of melatonin treatment on their mRNA production. We found that in accord with the results of immunohistochemistry detection and westblot assay, RT-PCR analysis revealed increased mRNA levels for IL-8 and MCP-1 in colon tissues of model group. Similarly, melatonin had a dose-dependent effect in decreasing mRNA expression of chemokines. Since the chemokines have been showed to attract different types of leukocytes in the process of inflammatory response, we presumed that the effect of melatonin on colonic injury and infiltration of inflammatory cells is probably via the mechanism of inhibition of IL-8 and MCP-1 mRNA and protein expression in the colonic mucosa.

Ulcerative colitis afflicts more and more people in China now, and affects the patient's quality of life seriously. However, there is no specific treatment was available. Despite a variety of new therapies such as anti-TNF-α antibodies have been applied in the treatment of colitis, none is ideal due to their high price and the safety needed to be further identified.[33,34] And so, melatonin will be an interesting alternative approach for the treatment of UC for its significant protective effects in animals experiments. Our study did not investigate the side effects and systemic action of melatonin in the experiment and safety of melatonin, its use is still unknown especially when it was used in clinical treatment of UC. Further sufficient preclinical and clinical studies should be conducted to prove its effects on colitis and the toxicology experiments should be carried out soon. In any case, melatonin combined with the established drugs is a promising strategy against IBD.

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