Mei Q *et al* / Acta Pharmacol Sin 2002 Oct; 23 (10): 882-886

ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Melatonin reduces colon immunological injury in rats by regulating activity of macrophages

MEI Qiao, YU Jie-Ping, XU Jian-Ming[1,2], WEI Wei[3], XIANG Li[2], YUE Li[3]

Renmin Hospital of Wuhan University, Wuhan 430060; [2]First Affiliated Hospital and [3]Clinical Pharmacology Institute of Anhui Medical University, Hefei 230032, China

KEY WORDS melatonin; colon; macrophages; inflammatory bowel diseases; interleukin-1; tumor necrosis factor; nitric oxide

ABSTRACT

AIM: To investigate the effects of melatonin on the colon immunological injury of rats and the role of macrophages in this process. **METHODS:** The rats colitis was established by intrarectal injection with 2,4,6-trinitrobenzenesulfonic acid (TNBS) and ethanol. The animals were randomized into 6 groups: normal group, model group, 5-aminosalicylic acid group (100 mg/kg), and melatonin group (2.5, 5.0, and 10.0 mg/kg), treated intrarectally with saline, saline, 5-aminosalicylic acid, and melatonin, respectively (once a day, from d 7 after colitis established to d 28). At the end of the experiment, the colon mucosa damage index (CMDI), the score of histology (HS), the level of myeloperoxidase(MPO), and the sore of occult blood test (OBT) were evaluated. Meanwhile, the activity of interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), and nitric oxide (NO) were also detected. **RESULTS:** After treated with TNBS and ethanol, the extents of CMDI, HS, OBT, and the level of MPO in model group were more higher than that in normal group. Melatonin could alleviate the colon injury, and reduce the level of MPO and the degree of OBT. The activity of IL-1, TNF-α, and NO which released mainly from macrophages was elevated remarkably. Melatonin could depress all this parameters. **CONCLUSION:** Melatonin could reduce the colon damage in the colitis rats by regulating macrophage activity.

INTRODUCTION

Inflammatory bowel disease (IBD) was a chronic interstinal inflammation which includes Crohn's disease and ulcerative colitis, but their etiology and pathophysiology still remain unknown. Now the incidence and prevalence of IBD increase in China, and the quality of life for IBD patients was severely disturbed by the absence of very effective drugs. So finding an optimal therapy for IBD was important but mainly blocked by its unknown etiology. Many studies have illustrated that the central role in the pathogenesis of IBD was the immunoregulation dysfunction[1], and macrophage (M_Φ) may be involved in this process. The hypothetic mechanism was that M_Φ triggered the "wrong attack" to the self colonic mucosal epithelial cells and initiate the colon injury[2]. M_Φ in colitis was becoming an precise cellular target for the IBD therapy.

Melatonin, the major product of the pineal gland, had multiple fundamental physiological role in sleep, immunoregulation, and aging. Melatonin and its synthase have been demonstrated in the gastrointerstinal tract, and there are a lot of melatonin receptors located

[1] Correspondence to Prof XU Jian-Ming.
Phn 86-551-363-3411. E-mail meiqiao@hotmail.com
Received 2001-08-13 Accepted 2002-06-05

in colon[3]. Many data have shown that the actions of melatonin be highly relative with colon disease[4]. But the effects of exogenous melatonin against colitis and its relation with M_Φ have scarcely been reported. On the model of rats colitis induced by 2,4,6-trinitrobenzene-sulfonic acid (TNBS) and ethanol, we investigated the effects of melatonin against this colitis, and elucidated the role of M_Φ in this process.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats (Grade II, Certificate № 002), weighing (280±20) g, C57BL/6J mice (Grade II, Certificate № 004), weighing (20±2) g, supplied by the Experimental Animal Center of Anhui Medical University. Animals were maintained in the controlled enviroment (20±1) ℃ for 1 week and under the light/dark (12 h:12 h) cycle.

Reagents and chemicals Melatonin, TNBS, *N*-1-naphthylenediamine hydrochloride, *O*-dianisidine, concanavalin A, lipopolysaccharide, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide were all purchased from Sigma Co; 5-aminosalicylic acid (5-ASA) was provided by Guoyi Pharmaceutical Ltd; hexadecyltrimethylammonium bromide was purchased from Xizhong Chemical Factory; bovine serum was provided by Department of Microbiology, Anhui Medical University, and was heat-inactivated at 56 ℃ before use; RPMI-1640 medium was purchased from Gibco Co; rhIL-1 and rhTNF-α were all provided by Bang-Ding Biotech Co; actinomycin D was provided by Hua-Mei Biotech Co; L929 cells was provided by Prof CHEN Ming-Zhu in Anhui Medical University; the other reagents were all of analytical pure grade.

Colitis induction According to the reference[5], a flexible plastic rubber catheter with an outside diameter of 2 mm was inserted rectally into the colon such that the tip was 8 cm proximal to the anus. TNBS (100 mg/kg) dissolved in 50 % ethanol (v/v) were instilled into the colon lumen through the rubber catheter (the final volume 0.25 mL), saline was instilled as control.

Experiment protocol The animals were randomly divided into 6 groups: normal group, model group, 5-aminosalicylic acid group (100 mg/kg), and melatonin group (2.5, 5.0, and 10.0 mg/kg), treated intrarectally with saline, saline, 5-aminosalicylic acid, and melatonin 2.5, 5.0, and 10.0 mg/kg, respectively (once a day, from d 7 after colitis was established to d 28).

Assessment of colon macroscopic damage At the end of experiment, the tissue of colon 10 cm proximal to anus was excised, opened longitudinally, washed in saline buffer, and pinned out on a wax block. Each colon was observed and evaluated by two independent observers. Then colon tissue samples from the site of TNBS/ethanol application were quickly excised for the histology and the other measurements. The assessment criteria of the colon mucosa damage index (CMDI) was according to previous reports[6]: 0=no damage; 1=mild hyperaemia and edema, no erosion or ulcer existing in the mucosa surface; 2=moderate hyperaemia and edema, erosion existing in the mucosa surface; 3=severe hyperaemia and edema, necrosis and inflammation and ulcer existing in the mucosa surface, area of the major ulceration extending less than 1 cm; 4=severe hyperaemia and edema, necrosis and ulcer on mucosa, area of the major ulceration extending more than 1 cm.

The colon tissue samples taken for histology were fixed overnight in 4 % neutral buffered formalin, processed, sectioned (4-μm thick), and stained with haematoxylin and eosin. Each colon was observed and evaluated by two independent observers. The assessment criteria of the histopathological score (HS) were modified according to the reference[7]: (1) the infiltration of acute inflammatory cells: 0=no, 1=mild increasing, 2=severe increasing; (2) the infiltration of chronic inflammatory cells: 0=no, 1=mild increasing, 2=severe increasing; (3) the deposition of fibrotin protein: 0=negative, 1=positive; (4) the submucosa edema: 0=no, 1=patchy-like, 2=fusion-like; (5) the epithelium necrosis: 0=no, 1=limiting, 2=widening; (6) the epithelium ulcer: 0=negative, 1=positive.

Occult blood test detection At the end of experiment, the rat feces were collected for occult blood test (OBT) detection under the guide of instruction.

Determination The colon tissue samples taken for the detection of MPO were homogenized (50 g/L) in ice-cold potassium phosphate buffer 50 mmol/L (pH 6.0) containing 0.5 % hexadecyltrimethylammonium bromide. The homogenate was frozen and thawed three times, then centrifuged at 4000×*g* for 20 min at 4 ℃. The level of MPO in supernatant was measured according to Daniel *et al*[8].

The colon tissue samples were homogenized in ice-cold phosphate buffer saline (pH 7.4). The homogenate were centrifuged at 40 000×*g* for 10 min at 4 ℃, and the supernatant was stored at -80 ℃ until determination for IL-1, TNF-α, and NO. The activity

of IL-1 was measured by the C57BL/6J mice thymocytes[9]; the activity of TNF-α was measured by L929 cells[10]; the content of NO was assessed by colorimetry[11].

Data analysis Data were reported as the mean± SD, differences were analyzed using *t*-test.

RESULTS

Effects of melatonin on rats colitis induced by TNBS and ethanol About d 10 after rats received TNBS and ethanol, the observation of fluid-like feces and positive OBT in rats indicated the appearance of diarrhea and colon mucosa hemorrhage. The lines-like ulcers were surrounded by thickening inflammed edema mucosa, and seemed to down-develop into the serosal layers, but the mucosa between two ulcerations remained normal figuration, this changes were characterized as "skipping segment ulceration". The whole colon wall was becoming thicken grossly. All this profiles were very similar to the clinical changes of IBD. The microscopic features of colon include the transmural infiltration of acute and chronic inflammatory cells, noticeable edema in the submucosa layer, and occurrence of necrosis and ulceration. So the parameter (CMDI, HS, and MPO, which reflect the degree of colon injury and inflammation) in model group, were all higher than that in normal group.

Administration with melatonin intrarectally at different dosage (2.5, 5.0, and 10.0 mg/kg) could inhibit the extents of inflammation, prevent the mucosa injury, minimize the ulceration area, and alleviate the colitis symptoms. It was supported by the reduction of the values of CMD, HS, and the levels of MPO in colon. Melatonin 10.0 mg/kg could reduce the degree of the OBT in the colitis rats (Tab 1, 2).

Effect of melatonin on the action of M_F in colon of the colitis rats The activity of IL-1, TNF-α, and the content of NO in plasma and colon were all elevated sharply (Tab 3). Melatonin could suppress the elevation of these indicators completely. This suggested that melatonin could obliterate the colitis, which might be associated with the down-regulation of M_Φ activity.

Tab 1. Effects of melatonin (MT, 2.5, 5.0, and 10.0 mg/kg) and 5-aminosalicylic acid (5-ASA, 100 mg/kg) on the value of the colon mucosa damage index (CMDI) and the score of histology (HS) in the colitis rats treated with TNBS (100 mg/kg) and ethanol. *n*=8. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* model group.

Group	Dose/mg·kg^{-1}	CMDI	HS
Normal	–	0.0±0.0^c	0.7±1.2^c
Model	–	3.1±1.1	9.7±0.5
5-ASA	100	1.7±0.5^c	5±3^c
MT	2.5	2.3±0.5	4±3^c
	5.0	1.7±0.8^b	3.2±0.9^c
	10.0	1.8±0.9^b	2.1±0.8^c

Tab 2. Effects of melatonin (MT, 2.5, 5.0, and 10.0 mg/kg) and 5-aminosalicylic acid (5-ASA, 100 mg/kg) on the score of occult blood test(OBT) in feces and the level of myeloperoxidase (MPO) of colon homogenates in the colitis rats treated with TNBS (100 mg/kg) and ethanol. *n*=8. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* model group.

Group	Dose/mg·kg^{-1}	OBT	MPO/U·g^{-1} wet weight
Normal	–	0.0±0.0^c	29±20^c
Model	–	2.0±0.9	191±33
5-ASA	100	0.8±0.8^b	111±12^c
MT	2.5	1.3±1.0	176±11
	5.0	1.3±1.0	139±37^c
	10.0	0.8±0.8^b	117±28^c

DISCUSSION

Progress in IBD research has been hampered by the lack of reproducible and available animal model of chronic colon inflammation. TNBS model developed by Morris *et al* was regarded as a classic model for colon immunological injury[5]. This model shares many of the histopathological and clinical features of human IBD and is very useful for the study of the etiopathogenesis of chronic colon inflammation as well as providing an inexpensive model suitable for assessing therapeutic agents. The whole process was summarized as following: after the destroy of mucosa integrity by ethanol (the barrier breaker), the hapten TNBS binded to colon tissue protein, and changed into a modified protein compounds, which recognized by M_Φ as abnormal antigen and presented quickly to the sensitized T lymphocyte. So the specific attack to the normal colonic muscoa epithelial cells were mediated, and the severe colon inflammation were initiated subsequently.

We firstly reported the effects of melatonin given

Tab 3. Effects of melatonin (MT 2.5, 5.0, and 10.0 mg/kg) and 5-ASA (100 mg/kg) on the activity of interleukin-1(IL-1), tumour necrosis factor-a (TNF-a), and the content of nitric oxide (NO) in plasma and colon in the colitis rats treated with TNBS (100 mg/kg) and ethanol. *n*=5. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* model group.

Group	Dose/mg·kg^{-1}	IL-1/kU·g^{-1}		TNF-α/kU·g^{-1} wet weight		NO	
		Plasma	Colon	Plasma	Colon	Plasma/ kU·L^{-1}	Colon/μmol·g^{-1} wet weight
Normal	–	0.125±0.003^{c}	0.27±0.05^{c}	16±4^{c}	30±11^{c}	15.2±1.9^{c}	0.29±0.07^{c}
Model	–	0.148±0.015	0.42±0.05	45±20	84±20	19±4	0.53±0.07
5-ASA	100	0.113±0.008^{c}	0.31±0.02^{c}	19±6^{c}	47±25^{c}	21±4	0.38±0.02^{c}
MT	2.5	0.133±0.009^{b}	0.36±0.06^{b}	37±12	59±23^{b}	17.2±0.7	0.40±0.04^{c}
	5.0	0.124±0.014^{c}	0.32±0.06^{c}	30±15	61±35	16.6±0.5	0.38±0.03^{c}
	10.0	0.119±0.007^{c}	0.24±0.03^{c}	28±8^{b}	54±17^{c}	16.9±2.2	0.34±0.02^{c}

by intrarectal administration against rats colitis induced by TNBS and ethanol. Our results showed that melatonin could attenuate the colitis symptoms and reverse the mucosa damage, this may be partly due to the modulation of melatonin on the aberrant colon immunological function. In addition, melatonin could be metabolized quickly when it was pulled through the upper gastrointerstinal tract given by intragastric way, which lead to a limited concentration of drug in inflamed colon. Administration of melatonin intrarectally could avoid this disadvantage and attain an effective level to treat rats colitis. This method can deliver melatonin directly into the inflamed mucosa to protect the colon damage[12].

Control of immune-regulating cells in the colonic mucosa is important in the treatment of IBD patients. M_{Φ} plays a key role in the onset and enlargement of the colon inflammation[13-16]. Primarily, M_{Φ} in the colon could present the abnormal antigen and aggravate the Th_1 type response predominantly, then it secreted pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α and killing free radicals such as NO. These cytokines introduce the generation of "inflammation cascade" and exaggerate the colon immunological injury. At the same time, excessive M_{Φ} from vessels are recruited into colon to participate the inflammation. So the activity of IL-1 and TNF-α and the content of NO are often used as sensitive indicators for evaluating the colitis severity. Several studies have reported that inhibition of pro-inflammatory cytokines from M_{Φ} was helpful to repair the colon damage in many experimental colitis. This may be associated with the activity of M_{Φ}, which can normalize the unbalance between pro- and anti-inflammatory cytokines. This approach was considered a very precise and valuable therapeutic strategy for the IBD patients and be accepted popularly.

Data obtained in this study indicated that the activity of IL-1 and TNF-α and the content of NO in plasma and colon of the colitis rats were elevated markedly, administration of melatonin intrarectally can correct this aberrant immunological status. Many previous data suggested that there were a lot of melatonin receptors located in M_{Φ}, and melatonin could inhibit the action of M_{Φ} in inflammatory tissues. Melatonin can repress the expression of inducible nitric oxide synthase (iNOS) by suppressing the activity of nuclear factor (NF) kappa B in M_{Φ}, and NF kappa B is a critical transcription factor which regulates the expression of pro-inflammatory cytokines and iNOS genes, the therapeutic properties of melatonin rely at least in part on the inhibition of NF kappa B activation , resulting in the suppression of pro-inflammatory cytokines genes expression in the inflamed mucosa[17-20]. In summary, we found that melatonin could reduce the colon damage effectively by modulating the action of M_{Φ}. Now despite the variety of medical therapies available for the treatment of IBD, none is ideal. Research of melatonin against colitis, as well as a novel agents with low toxicity, combining with the well-established drugs such as 5-ASA, may contribute to an optimal therapy of IBD in the near future.

REFERENCES

1 Sartor RB. Pathogenesis and immune mechanisms of chronic inflammatory bowel disease. Am J Gastroenterol 1997; 92: 5-11.

2 Mahida YR. The key role of macrophages in the immuno-

pathogenesis of inflammatory bowel disease. Inflamm Bowel Dis 2000; 6: 21-33.
3 Poon AM, Mak AS, Luk HT. Melatonin and [^{125}I] iodomelatonin binding sites in the human colon. Endocr Res 1996; 22: 77-94.
4 Pentney PT, Bubenik GA. Melatonin reduces the severity of dextran-induced colitis in mice. J Pineal Res 1995; 19: 31-9.
5 Morris GP, Beck PI, Herridge MS. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 1989; 96: 795- 803.
6 Deng CS, Xia B, Chen DJ, Zhou Y. The mucosa protective effects of superoxide dismutase on rats colitis induced by acetic acid. Chin J Pathophysiol 1994; 10: 23-5.
7 Millar AD, Rampton DS, Chander CL. Evaluating the antioxidant potential of new treatments for inflammatory bowel disease using a rat model of colitis. Gut 1996; 39: 407-15.
8 Daniel R, Philip LS, Lester WS, Don EG, Joseph DF, Martin AW. Inflammatory mediators of experimental colitis in rats. Gastroenterology 1989; 97: 326-37.
9 Liang JS, Wei W, Zhou AW, Chen MZ, Xu SY. Method for measuring IL-1 and effects of total glucosides of paeony on IL-1 production. Chin Pharmacol Bull 1989; 5: 354-7.
10 DiGiovine FS, Nuki G, Duff GW. Tumor necrosis factor in synovial exudates. Ann Rheu Dis 1988; 47: 768-72.
11 Shechter H, Gruener N, Shuval HI. A micromethod for nitrite in blood. Anal Chem Acta 1972; 60: 93-9.
12 Nakase H, Okazaki K, Tabata Y. Development of an oral drug delivery system targeting immune-regulating cells in experimental inflammatory bowel disease: a new therapeutic strategy. J Pharmacol Exp Ther 2000; 292: 15-21.
13 Hoshi O, Iwanaga T, Fujino MA. Selective uptake of intraluminal dextran sulfate sodium and senna by macrophages in the cecal mucosa of the guinea pig. J Gastroenterol 1996; 31: 189-98.
14 Rugtveit J, Brandtzaeg P, Halstensen TS. Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. Gut 1994; 35: 669-74.
15 Murch SH. Local and systemic effects of macrophage cytokines in intestinal inflammation. Nutrition 1998; 14: 780-3.
16 McAlindon ME, Hawkey CJ, Mahida YR. Expression of interleukin 1 beta and interleukin 1 beta converting enzyme by intestinal macrophages in health and inflammatory bowel disease. Gut 1998; 42: 214-9.
17 Cutolo M, Villaggio B, Candido F. Melatonin influences interleukin-12 and nitric oxide production by primary cultures of rheumatoid synovial macrophages and THP-1 cells. Ann N Y Acad Sci 1999; 876: 246-54.
18 Garcia-Perganeda A, Guerrero JM, Rafii-El-Idrissi M. Characterization of membrane melatonin receptor in mouse peritoneal macrophages: inhibition of adenylyl cyclase by a pertussis toxin-sensitive G protein. J Neuroimmunol 1999; 95: 85-94.
19 Gilad E, Wong HR, Zingarelli B. Melatonin inhibits expression of the inducible isoform of nitric oxide synthase in murine macrophages: role of inhibition of NF kappaB activation. FASEB J 1998; 12: 685-93.
20 Raghavendra V, Agrewala JN, Kulkarni SK. Melatonin reversal of lipopolysacharides-induced thermal and behavioral hyperalgesia in mice. Eur J Pharmacol 2000; 395: 15-21.
21 Cuzzocrea S, Mazzon E, Serraino I, Lepore V, Terranova ML, Ciccolo A, *et al*. Melatonin reduces dinitrobenzene sulfonic acid-induced colitis. J Pineal Res 2001; 30: 1-12.