

# The inhibitory effect of melatonin on colonic motility disorders induced by water avoidance stress in rats

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**Abstract. – OBJECTIVES:** Psychological stress is involved in the etiology of functional gastrointestinal disease (FGID). Accumulations of chronic continuous stress associate with the symptoms of the gastrointestinal tract (GIT) including abdominal discomfort, pain, constipation or diarrhea. Melatonin (MT) is a neurohormone which mainly synthesized in the pineal gland and GIT, has been shown to alleviate the stress and regulate the intestinal motility. Although melatonin attenuated abnormal defecation induced by stress, the mechanisms of the effect are not fully elucidated.

This study sought to investigate the effect of melatonin (MT) on colonic smooth muscle contractility disorders induced by chronic water avoidance stress (WAS).

**MATERIALS AND METHODS:** 36 adult Wistar rats were divided into three groups [Control+V (vehicle solution), WAS+V, WAS+MT]. The WAS+V and WAS+MT groups were suffered from WAS for one hour daily for 10 days. Melatonin was given 30 min before stress session in the WAS+MT group. Colonic motility was assessed using the numbers of fecal pellet output. The concentrations of melatonin and serotonin (5-HT) were measured through ELISA method. The contractions of isolated colonic strips were assessed through isometric force transducer.

**RESULTS:** The numbers of fecal output was increased in the WAS+V group, and melatonin attenuates the increased fecal pellet output induced by WAS. The concentrations of 5-HT was increased by WAS, and melatonin decreased the augment in the concentration of 5-HT. In the part of contraction recording of colonic smooth muscle, melatonin inhibited the fortified spontaneous contraction induced by WAS, and decreased the amplitude of spontaneous contraction stimulated by Ach and KCl after WAS treatment.

**CONCLUSIONS:** Melatonin could inhibit WAS-induced fecal output pellets by inactivating the 5-HT pathway, may interact with calcium channel and inhibit the influx of calcium.

## Key Words:

Melatonin, Stress, Serotonin, Colon, Contraction.

## Abbreviations

Ach = acetylcholine  
CRF = corticotropin releasing factor  
FGID = functional gastrointestinal disease  
GIT = gastrointestinal tract  
HPA = hypothalamic-pituitary-adrenal;  
MT = Melatonin  
5-HT = serotonin  
WAS = water avoidance stress

## Introduction

In modern society, all individuals often suffer from various kinds of stress in their daily life. Accumulations of chronic continuous stress associate with the symptoms of gastrointestinal (GI) tract including abdominal discomfort, pain, constipation or diarrhea<sup>1,2</sup>. In addition, functional gastrointestinal disorders (FGID) such as irritable bowel syndrome (IBS) are combine with stress-associated symptoms included anxiety and depression<sup>3,4</sup>. Psychological stress has been suggested to play an important role in developing of FGID<sup>1,5,6</sup>. Some studies showed that cold or wrap-restraint stress facilitates colonic transit and induces defecation<sup>7</sup>. But the mechanisms underlying the colonic response to stress are not fully elucidated. The release of corticotropin releasing factor (CRF) induced by stress in the brain stimulates vagal efferent, resulting in the colonic hyperkinesis<sup>8</sup>. The activation of colonic cholinergic myenteric neurons induced by water avoidance stress stimulates fecal pellet output<sup>9</sup>. And the colonic 5-HT concentrations are associated with the colonic response to stress<sup>10</sup>.

Melatonin is known as a neurohormone which mainly synthesized in the pineal gland and the GIT. It has regulatory effects on a series of physiological processes including season and circadi-

an rhythms<sup>11,12</sup>. In addition, melatonin has therapeutic effects on sleep-disorders<sup>13</sup>, pain<sup>14</sup>, inflammation<sup>15</sup> and depression<sup>16</sup>. In the GIT, melatonin is secreted by enterochromaffin cells as well as 5-HT and the concentrations of melatonin are many folds higher than that in the plasma and the pineal gland<sup>17</sup>. The expression of melatonin receptors including MT<sub>1</sub> and MT<sub>2</sub> has been proven in the GIT<sup>18,19</sup>. Many studies showed that melatonin has inhibitory and excitatory effects on the intestinal motility<sup>20,21</sup>. In rats, melatonin increased intestinal transit in small dose, but reduced intestinal transit and the force of spontaneous contraction in high concentrations<sup>20,22</sup>. The mechanisms of melatonin effects on the intestinal motility are incompletely elucidated. Some animal model studies showed that melatonin inhibits the nicotinic channels in the sub mucous plexus to regulate the cholinergic transmission and interacts with small conductance K<sup>+</sup>-channels to relax the contraction<sup>23,24</sup>. These data suggested that melatonin may be involved in the regulation of intestinal motility. Previous studies have been proven that melatonin attenuated abnormal defecation induced by acute wrap-restraint stress because of its inhibitory effects on augment of serotonin and CRF secretion<sup>25</sup>. But, the effect of melatonin on bowel dysfunction induced by chronic stress was puzzled.

In the present work, we investigated the effect and mechanisms of melatonin on colonic smooth muscle contractility disorders induced by chronic continuous stress.

## Materials and Methods

### Animals

Male Wistar rats weighting 200-250 g were employed in this experiment and were housed in the standard controlled environment (22±2°C; 12/12-hr light/dark cycle: dark period: 7PM-7AM). Equal food (pre-weight) and water were given to each rat every day. Rats were habituated to single housing in controlled environment for one week before the experiment. All empirical procedures of this study were approved by the Institutional Animal Care and Use Committee of Wuhan University.

### Experiment Procedure and Melatonin Administration

To avoid the effects of circadian rhythms of melatonin, the experiments started at the same

time (8AM). The animals were randomly divided into 3 groups: 1) control group (Control+V group) (n=12): control rats were injected with vehicle solution; 2) water avoidance stress group (WAS+V group) (n=12): rats were suffered from WAS for 10 days; 3) water avoidance stress exposed, melatonin injected (WAS+MT group) (n=12): rats were subjected to chronic WAS after the injection of 10 mg.kg<sup>-1</sup> for 10 days. Melatonin was purchased from Sigma (Sigma Chemical Co. St Louis, MO, USA) which freshly dissolved in saline containing 5% ethanol. Rats were given 10 mg.kg<sup>-1</sup> of melatonin by intraperitoneal injection daily before the stress carried out 30 min or Vehicle for 10 days. Melatonin has different effects at different concentrations, so the dose was chosen to avoid its excitatory effects<sup>22</sup>.

### Water Avoidance Stress (WAS)

The stress procedure was slightly modified according the previous methods<sup>26</sup>. Briefly, rat was placed on a platform (10×8×8 cm) attached to the bottom of a plastic tank (45 cm length × 25 cm width × 25 cm height). The tank was filled with warmed water (25°C) within 1 cm of the top of the block. Rat was stand on the platform to avoid water stimulus for one hour during 10 consecutive days. Control rats were kept individually in their standard housing cages without water or food during the same period (sham stress).

### Measurement of Fecal Pellet Output

Baseline 24h fecal pellet output of each rat was monitored for at least three consecutive days before WAS treatment. On the day of the experiment, each rat was exposed to WAS for one hour; the fecal pellets expelled during the period of WAS were counted. After WAS, each rat was returned to the individual standard housing cage, and 24h fecal pellet output of each rat were collected.

### Measuring the Concentrations of Serum Hormone

Rats were anaesthetized by 10% chloral hydrate and then killed (24 h after the final day of stress). Their heart blood was collected for the hormonal measurement. Blood samples were centrifuged at 1500 g at 4°C for 20 min. The serum was collected and stored at -80°C until the assay. The concentrations of melatonin and 5-HT were determined using an enzyme immunoassay (ELISA) kit (R&D Systems, Hercules, CA, USA).

### Preparation of Rat Colonic Smooth Strip

The proximal colon was removed and placed in  $\text{Ca}^{2+}$ -free physiological saline solution (PSS) which was constantly oxygenated. The colon was cut along the mesenteric border and cleaned by  $\text{Ca}^{2+}$ -free PSS. After the mucosa was carefully dissected away, the smooth muscle strips were obtained.

### Contraction Recording of Colonic Smooth Muscle Strips

About 3 mm×10 mm fresh longitudinal muscle strip was mounted in a 5 ml Tyrode's solution and connected to an isometric force transducer (JZJOIH, Chengdu, China). Tyrode's solutions were continuously warmed by a circulating water jacketed at 37°C and bubbled with carbogen (95%  $\text{O}_2$ +5%  $\text{CO}_2$ ). The muscle strips were placed under 0.5 g load tension and allowed to equilibrate for 60 min with solution change every 20 min. Frequencies of contraction were calculated by counting the contraction waves per minute.

### Reagents and Solutions

The  $\text{Ca}^{2+}$ -free physiological saline solution (PSS) contained (in mM): NaCl 135, KCl 5.0,  $\text{MgCl}_2$  1.2, Glucose 10.0, and HEPES10 (pH adjusted to 7.4 with NaOH). Tyrode's solution contained (in mM): NaCl 147.0, KCl 4.0,  $\text{CaCl}_2$  2.0, and  $\text{NaH}_2\text{PO}_4$  0.42,  $\text{Na}_2\text{HPO}_4$  2.0,  $\text{MgCl}_2$  1.05,  $\text{CaCl}_2$  2, glucose 5.5 (pH adjusted to 7.35 with NaOH). All these chemicals were purchased from Sigma Chemical, Co, (St Louis, MO, USA).

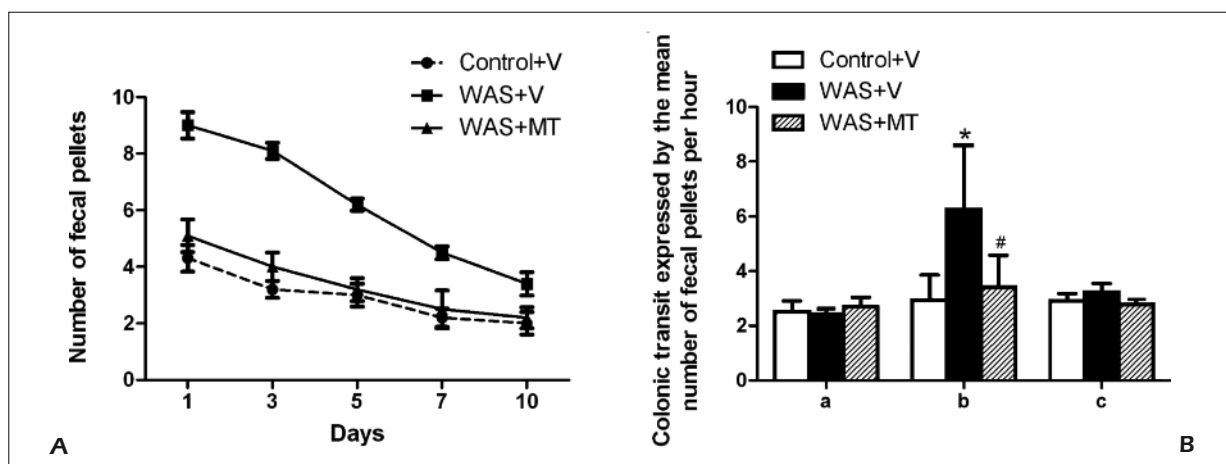
### Statistical Analysis

Data were expressed as Mean±SEM. Student's *t* test and one-way ANOVA were used according to the different data. A probability of  $p < 0.05$  was accepted as the level of statistical significant.

## Results

### Effects of Melatonin on Fecal Pellet Output

There was no significant difference in fecal output number per hour between the WAS+V and Control+V groups before WAS treatment. The number of fecal pellets of three groups in 1, 3, 5, 7, 10 days during the sham-WAS or WAS treatment was shown in Figure 1A. Water avoidance stress significantly increased fecal pellet output during a one hour period compared with the Control+V groups (Control+V group,  $3.62 \pm 1.46$  vs WAS +V group,  $5.75 \pm 0.40$ ;  $p < 0.05$ ). In addition, the WAS induced-increase of fecal output number was obviously attenuated by melatonin at 10 mg.kg<sup>-1</sup> (WAS +V group,  $5.75 \pm 0.40$  vs WAS +MT group,  $2.80 \pm 0.57$ ;  $p < 0.05$ ), and also decreased the dry weight of stool. The dry weight of stool of WAS +V group and WAS+MT group were  $0.59 \pm 0.03$  g and  $0.29 \pm 0.07$  g, respectively ( $p < 0.01$ ). But, there was no statistically significant difference between the WAS +V and WAS +MT groups during the 24h after last WAS treatment ( $p > 0.05$ ), as shown in Figure 1B.



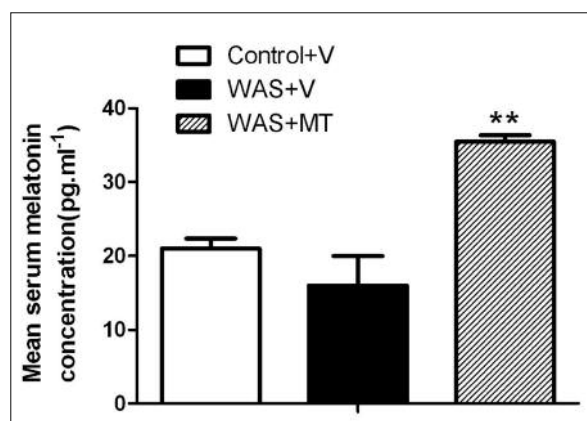
**Figure 1.** Water avoidance stress-induced alterations in defecation and the effect of melatonin on defecation. During each water avoidance stress session, colonic motility was assessed using the numbers of fecal output pellet numbers. **A**, An analysis of the 1, 3, 5, 7, 10 day defecation in the Control+V, WAS+V, WAS+MT groups. **B**, The mean fecal pellet output per hour before (a), during (b) and 24h after last WAS (c). Data are expressed as mean±SEM; n=12. (Control+V group vs WAS+V group, \* $p < 0.05$ ; WAS+V group vs WAS+MT group, # $p < 0.05$  by two-way ANOVA)

### Effects of Melatonin on Concentrations of 5-HT in Serum

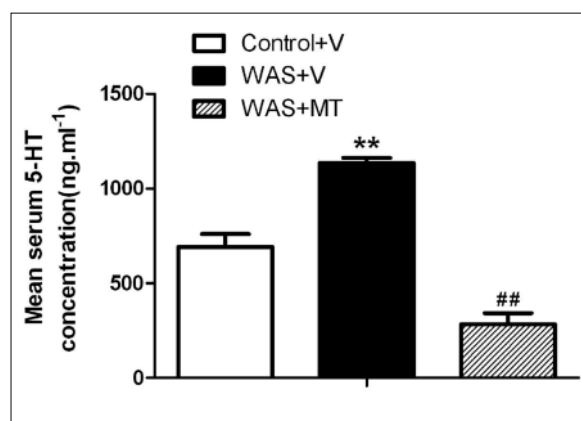
In the WAS+V group, the concentration of melatonin were seem to less than that in the Control+V group. But there are no significantly difference between them ( $p > 0.05$ ). The level of melatonin was increased in the WAS +MT group compared to the WAS+V group and the Control+V group (WAS +MT group,  $35.45 \pm 6.89$  pg mL<sup>-1</sup> vs WAS+V and Control+V group,  $16.03 \pm 3.96$  pg mL<sup>-1</sup> and  $20.99 \pm 1.36$  pg mL<sup>-1</sup>; all  $p < 0.01$ ), as shown in Figure 2. 5-HT is also secreted by enterochromaffin cells. Melatonin may regulate the intestinal motility through 5-HT pathway. As shown in Figure 3, compared with the Control+V group, WAS significantly stimulated serum 5-HT concentration in rats (Control+V group,  $692.72 \pm 67.03$  ng mL<sup>-1</sup> vs WAS +V group,  $1135.35 \pm 26.28$  ng mL<sup>-1</sup>;  $p < 0.01$ ). This effect was inhibited by intraperitoneal administration of melatonin (WAS+MT group,  $282.66 \pm 58.59$  ng mL<sup>-1</sup>;  $p < 0.01$ ).

### Effect of Melatonin on Spontaneous Contraction of Colonic Smooth Muscle Strips

The smooth muscle strips isolated from rat proximal colon manifested spontaneous contraction with a tension and frequency. Compared with the Control+V group, the baseline amplitude of spontaneous contraction was significantly increased in the WAS+V group. As shown in Fig-



**Figure 2.** The concentrations of melatonin in serum in all groups. Rats were placed in WAS for one hour a day during 10 consecutive days. The concentration of melatonin was analyzed 24h after the last session of stress. There was no significantly difference between the Control+V and the WAS+V groups ( $p > 0.05$ ). And the level of melatonin was increased in the WAS+MT groups (\*\* $p < 0.01$  by two-way ANOVA). Data are expressed as mean $\pm$ SEM; n = 12.



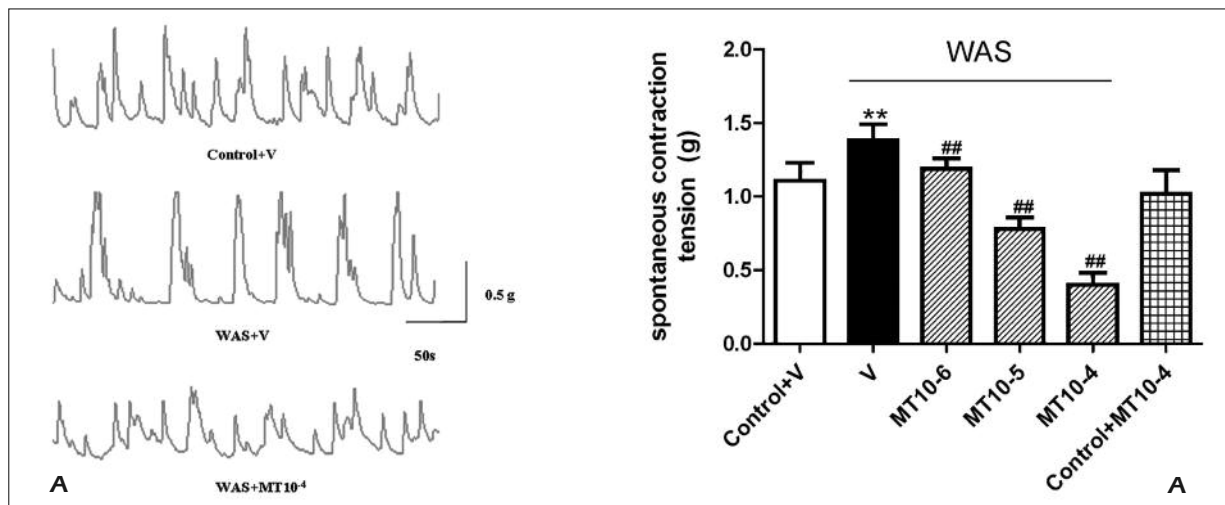
**Figure 3.** Effects of melatonin on the concentrations of 5-HT in serum in all groups. Rats were placed in WAS for one hour a day during 10 consecutive days. The concentration of melatonin was analyzed 24h after the last session of stress. The WAS+V groups showed a significantly increase in serum 5-HT concentration after chronic stress in comparison with the Control+V group, and melatonin reversed this augment in serum 5-HT concentration induced by chronic stress (Control+V group vs WAS+V group, \*\* $p < 0.01$ ; WAS+V group vs WAS+MT group, ## $p < 0.01$  by two-way ANOVA). Data are expressed as mean $\pm$ SEM; n = 12.

ure 4A, the mean tension of spontaneous contraction in the WAS +V group was significantly higher than that in the Control+V group (WAS +V group,  $1.39 \pm 0.10$  g vs Control+V group,  $1.11 \pm 0.12$ ;  $p < 0.01$ ). But there was no significantly difference between the Control+V and the WAS +V groups in the frequency of spontaneous contraction.

Different concentrations of melatonin were given to the bath to observe their effects on the colonic longitudinal muscle spontaneous contractions in the Control+V and the WAS +V groups. Melatonin ( $10^{-6}$ M- $10^{-4}$ M) alone decreased the amplitude of spontaneous contractions in the WAS +V group ( $p < 0.01$ ), did not change the frequency ( $p > 0.05$ ). But this effect was not observed in the Control+V group, as shown in Figure 4B.

### Effect of Melatonin on Ach-Induced Colonic Contraction

Compared with the Control+V group, the  $10^{-5}$ M Ach-induced contractions of the WAS +V group were significantly increased (WAS +V group,  $1.63 \pm 0.17$  g vs Control+V group,  $1.32 \pm 0.16$  g;  $p < 0.05$ ). Melatonin ( $10^{-6}$ M- $10^{-4}$ M) decreased Ach-induced contractions in the all groups ( $p < 0.01$ ). But melatonin elicited the greater inhibitory effect on the amplitude of spontaneous contractions in the Control+V group



**Figure 4.** The effect of different dose of melatonin on the colonic motility in WAS+V rats comparing to the Control+V group. **A**, Representative traces of the effect of WAS or WAS+MT10-4M on the spontaneous contraction of colonic smooth muscle. **B**, Summarized results of the effect of WAS or WAS+MT [ $10^{-6}$ M- $10^{-4}$ M] on the spontaneous contraction of colonic smooth muscle. The spontaneous contraction tension was increased by WAS. The inhibitory effect of different dose of melatonin were shown on the spontaneous contraction of colon of the WAS+V rats (Control+V group vs WAS+V group,  $^{**}p < 0.01$ ; WAS+V group vs WAS+MT [ $10^{-6}$ M- $10^{-4}$ M] groups by t-test, all  $^{##}p < 0.01$ ). Data are expressed as mean $\pm$ SEM; n = 12.

than that in the WAS+V group ( $p < 0.05$ ), as shown in Figure 5. And melatonin was did not change the frequency of spontaneous contractions in all groups ( $p > 0.05$ ).

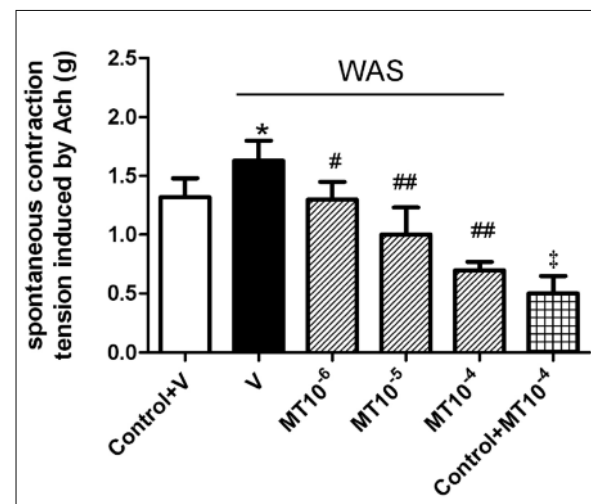
#### Effect of Melatonin on KCl-Induced Colonic Contraction

In the Tyrode's solution, 30 mM KCl induced an augment on the amplitude of spontaneous contraction of colonic smooth muscle. The muscle strips manifested more sensitive to KCl in the WAS+V group, compared with the Control+V group (WAS+V group,  $1.54 \pm 0.15$  g vs Control+V group,  $1.29 \pm 0.16$ ,  $p < 0.05$ ). After the concentrations of  $10^{-5}$ ,  $10^{-4}$  M melatonin added, the amplitude of contraction decreased in the all groups ( $p < 0.01$ ), as shown in Figure 6. But there was no significantly difference between the WAS+V group and the Control+V group in the inhibitory effect of melatonin ( $p > 0.05$ ). The frequency of spontaneous contractions was no significantly difference between the two groups ( $p > 0.05$ ).

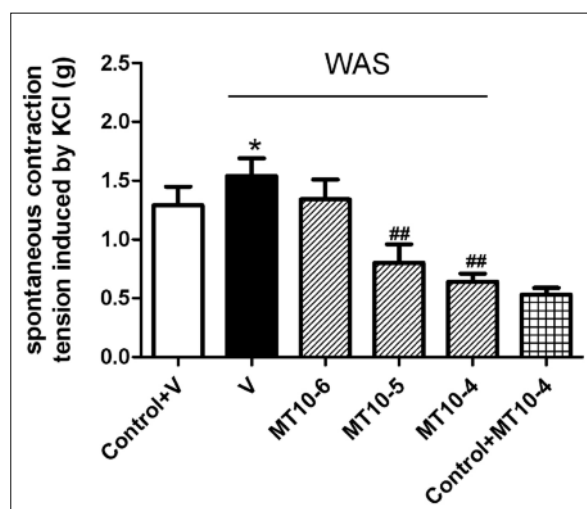
#### Discussion

A series of studies have demonstrated that melatonin is involved in the regulation of GI motility in many species<sup>20,24</sup>, and it has inhibitory effect on the accelerated colonic transit induced

by acute stress<sup>25</sup>, but the mechanisms is not completely understood. This study demonstrates that melatonin attenuates the increased fecal pellet



**Figure 5.** Permeated with Ach, the effect of different dose of melatonin on the colonic motility in WAS+V rats comparing to the Control+V group. The colonic response to Ach was increased in the WAS+V group. The inhibitory effect of different dose of melatonin were shown on the spontaneous contraction of colon of the WAS+V rats and the Control+V group (Control+V group vs WAS+V group,  $^{*}p < 0.05$ ; WAS+V group vs WAS+MT [ $10^{-6}$ M- $10^{-4}$ M] groups by t-test,  $^{#}p < 0.05$ ,  $^{##}p < 0.01$ ). But the inhibitory effect of melatonin was higher in the Control+MT10<sup>-4</sup> group than that in the WAS+MT10<sup>-4</sup> group (WAS+MT10<sup>-4</sup> group vs Control+MT10<sup>-4</sup> group,  $^{‡}p < 0.05$ ). Data are expressed as mean $\pm$ SEM; n = 12.



**Figure 6.** Permeated with KCl, the effect of different dose of melatonin on the colonic motility in WAS+V rats comparing to the Control+V group. The colonic response to KCl was increased in the WAS+V group. The inhibitory effect of different dose of melatonin were shown on the spontaneous contraction of colon of the WAS+V rats and the Control+V group (Control+V group vs WAS+V group,  $*p < 0.05$ ; WAS+V group vs WAS+MT [ $10^{-6}$ M- $10^{-4}$ M] groups by t-test,  $##p < 0.01$ ). Data are expressed as mean  $\pm$  SEM;  $n = 12$ .

output induced by chronic water avoidance stress, and the concentrations of MT and 5-HT associate with the colonic response to stress. In the part of contraction recording of colonic smooth muscle, melatonin inhibited the fortified spontaneous contraction induced by WAS, and decreased the amplitude of spontaneous contraction stimulated by Ach and KCl after WAS treatment.

Stress is an important factor in the pathophysiology of GI dysfunction. Some studies reported that acute wrap-restraint stress inhibited small intestine transit and promoted large intestinal transit, but did not affect gastric emptying<sup>7</sup>. The activation of hypothalamic-pituitary-adrenal (HPA) axis and release of CRF contributed to the GI response to stress<sup>7</sup>. In this work, the fecal output pellets were increased during WAS treatment, but there was no significantly difference between the Control+V and WAS+V groups 24 h after last stress. These suggested that adaptation may be involved in the colonic response to chronic stress. Previous reports showed that melatonin attenuated abnormal defecation induced by acute wrap-restraint stress<sup>2</sup>. The same effect of melatonin was observed in chronic stress. Melatonin decreased the fecal pellets output stimulated by WAS. 5-HT is secreted by enterochromaffin cells

and enteric neurons, plays an important role in the regulation of intestinal motility<sup>27</sup>. Some investigations have shown that chronic stress stimulated fecal pellets and increased the colon 5-HT concentration in C57BL/6 mice<sup>10</sup> and the counterbalancing effects between melatonin and 5-HT involved in the inhibitory effect of melatonin on acute stress<sup>25</sup>. In this study, the concentration of 5-HT increased in the WAS+V group, and melatonin inhibited this augment. The results indicated that melatonin could inhibit WAS-induced fecal output pellets by inactivating the 5-HT pathway. In addition, some studies reported that melatonin has direct effect on the smooth muscle contraction<sup>20,24</sup>.

Melatonin may exert its effect on the GI system through both central and peripheral mechanisms in rats. Despite there was no difference between the Control+V and the WAS+V groups in the fecal pellets output 24h after last stress, but colonic smooth muscle of the WAS+V groups manifested over-reaction in spontaneous contraction. The over-reaction of colonic contraction associated with the function of corticotrophin-releasing hormone, Ach and 5-HT in stress. A research has proven that WAS activates a population of cholinergic myenteric neurons in the colon which associated with propulsive motility in female rats<sup>9</sup>. Ach stimulated the colonic contraction through the muscarinic receptors. In this report, melatonin inhibited the amplitude of colonic smooth muscle contraction of the WAS+V group, and reduced the amplitude the colonic smooth muscle contraction of the WAS+V group which permeated with Ach or KCl. Previous studies reported that melatonin inhibited the contraction of large intestine, but its action may not be directly on smooth muscle contraction<sup>20</sup>. Melatonin also inhibited the spontaneous contraction induced by Ach, KCl and 5-HT in colon and bladder<sup>21,28</sup>. Mechanisms underlying the colonic motor response to melatonin may involve the calmodulin/CaMKII system and voltage-dependent calcium channels<sup>29</sup>. In this work, the inhibitory of melatonin on colonic contraction of the WAS+V group may be associated with the activation of Ach and 5-HT induced by stress. Melatonin inhibited the nicotinic channels in the sub mucous plexus to regulate the cholinergic transmission interacted with small conductance  $K^{+}$ -channels to relax the contraction<sup>23,24</sup>. Except for the interaction with membrane receptors  $MT_1$  and  $MT_2$ , melatonin can bind with the intracellular protein such as calmodulin because

of its permeability of cell membrane. Melatonin can interact with  $\text{Ca}^{2+}$ -activated calmodulin with high affinity and prevent it from activating myosin light-chain kinase, inducing to decreased muscle contraction. Be well known, the contraction induced by high  $\text{K}^{+}$  was attributed to the influx of  $\text{Ca}^{2+}$  through calcium channel. Melatonin inhibited the colonic contraction of the WAS+V group after KCl pretreatment. These suggested that melatonin may interact with calcium channel and inhibit the influx of calcium.

Irritable Bowel Syndrome (IBS) is common functional gastrointestinal disorders. The precise study found that IBS patients have the different melatonin secretion and metabolism, but there are no differences between C-IBS (constipation-IBS) and (diarrhoea-IBS) D-IBS patients<sup>30</sup>. Melatonin improved abdominal pain and reduced rectal pain sensitivity by modifying visceral pain perception<sup>31</sup>. Melatonin could modulate gastrointestinal motility in IBS patients<sup>32</sup>.

## Conclusions

This study shows that melatonin inhibits the colonic smooth muscle contractility disorders induced by water avoidance stress in rats. The central and peripheral mechanisms may be involved in the effect of it. These may afford the some evidences for the treatment of IBS.

## Acknowledgements

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## Conflict of Interest

The Authors declare that they have no conflict of interests.

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