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Original Research Article

Effect of atractylenolide III on interstitial cells of Cajal and C-kit/SCF pathway of rats with loperamide-induced slow transit constipation

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Abstract

Purpose: *To determine the effect of atractylenolide-III (ATL-III) on loperamide-induced slow transit constipation (STC) in a rat STC model, and to elucidate the mechanisms involved.*
Methods: *Male Wistar rats were divided into five groups (n=6 per group): normal control group (NG), model group, and three STC rat groups treated with different doses of ATL-III, viz, 5, 10 and 15 mg/kg. The rats were treated for 15 days. Feed consumption, fecal excretion and intestinal transit rate were determined. Nitric oxide synthase (NOS), somatostatin (SS), serotonin (5-HT), and vasoactive intestinal peptide (VIP) were measured with enzyme-linked immunosorbent assay (ELISA). The protein and mRNA expressions of C-kit, SCF, PKC, and PI-3K were assayed using Western blot analysis and real-time reverse transcription polymerase chain reaction (RT-PCR), respectively.*
Results: *The amount, weight, and moisture content of stool, and water consumption were significantly higher in ATL-III-treated groups than in the untreated (model) group ($p < 0.05$), whereas no difference was observed in feed intake. Intestinal transit rate was higher in the ATL-III-treated groups ($p < 0.05$). Decreased NOS, SS and VIP levels and increased 5-HT level were seen in the ATL-III-treated groups ($p < 0.05$). ATL-III treatment also induced increases in smooth muscle cells, neuronal cells, and mucous layer ($p<0.05$). Results from RT-PCR and Western blot revealed that ATL-III–treated groups had elevated c-kit, SCF, PKC, as well as PI-3K mRNA and protein expressions ($p < 0.05$).*
Conclusion: *These results suggest that ATL-III mitigates loperamide-induced STC in rats via stimulation of NOS, SS, VIP, and 5-HT secretions. It also increases smooth muscle cells, neuronal cells, and mucous layer, and regulates the signaling pathways involving PKC, PI3K, SCF, and c-kit.*

Keyword: *Slow transit constipation, Atractylenolide III, Interstitial cells of Cajal, Smooth muscle cells, Neuronal cells, Mucous layer*

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INTRODUCTION

Constipation is a global concern of public health, particularly affecting elderly population [1]. Slow transit constipation (STC) is caused by a disorder in colon transmission, and it is characterized by symptoms including infrequent and difficult defecation, accompanying with a sensation of

incomplete bowel evacuation. The prevalence of STC in chronic constipation is about 46 % [2]. The cure rate is low due to persistence of the clinical symptoms. Thus, the disease is often recrudesced, and may even induce colorectal tumors, prostatic hypertrophy and myocardial infarction.

Various chemical drugs such as cisapride, mosapride, and prucalopride are used for STC therapy, although their applications are restricted by high costs and various adverse effects [3]. Research in recent years have led to greater understanding of the pathogenesis of STC. In particular, studies on gastrointestinal nervous system, interstitial cells of Cajal (ICC) and proteomics have provided new perspectives for research on STC, as well as a scientific basis for research on new drugs for treatment of the disease. The interstitial cells of Cajal (ICC) are pacemaker cells which mediate neuromuscular transmission from nerve endings to smooth muscle cells, and regulate slow wave activity in the gastrointestinal tract [4,5]. It has been demonstrated that, in STC patients, the colonic ICC density is remarkably lower than in that in normal patients. Thus, restoration of ICC may be crucial in the cure for STC.

Using traditional Chinese herbal medicine formulas, scientists have opened new avenues in the treatment of chronic constipation in recent years [6,7]. According to the Chinese Pharmacopoeia, atractylodes is beneficial for spleen and stomach, and of clinical importance in the treatment of edema and diarrhea [8]. However, the effect of atractylodes on STC remains unclear. Atractylenolide III (ATL-III) has been reported as an important active constituent of atractylodes. It has good protective activity against corticosterone-induced damage to PC12 cells through a mechanism related to inhibition of neuronal apoptosis [9]. In addition, ATL-III prevents neuron loss and ameliorates cognitive impairment caused by chronic homocysteine (Hcy) administration in rats [10]. Thus, it can be speculated ATL-III may be the active principle involved in the treatment of STC with atractylodes. In this study, we aimed to determine the role of ATL-III on loperamide (Lop)-induced STC in rats, and the underlying mechanism.

EXPERIMENTAL

Animals

Thirty male Wistar rats (150 ± 30 g) were purchased from Shanghai Laboratory Animal Research Center. All rats were maintained in standard cages with 12h/12h light/dark cycle at room temperature and humidity. The access to food and drinking water were unlimited. This research was approved by the Animal Ethical Committee of Department of Anorectal Surgery, Nanjing University of Chinese Medicine, Qinhuai District, Nanjing City, China (approval no. 201713696), and performed according to NIH laboratory animal guidelines [11].

Experimental design and treatment

The thirty rats were divided into five experimental groups randomly (n=6 per group): group A: normal control group without any treatment (NG); group B: STC model group with mock treatment (Lop + vehicle-treated group); group C: STC group with low-dose ATL-III treatment (Lop + LDATL-III-treated group), group D: STC group with medium-dose ATL-III treatment (Lop + MDATL-III-treated group), and group E: STC group with high-dose ATL-III treatment (Lop + HDATL-III-treated group). Rats in group A were injected with physiological saline, while the others were treated with subcutaneous injection of Lop at the concentration of 4 mg/kg diluted in physiological saline. The injections were performed twice per day and lasted for 3 days. The doses of ATL-III were selected on the basis of previous studies [10–13]. Groups C, D, and E were treated with 5, 10, and 15 mg/kg ATL-III in water by oral administration, respectively. The oral administration was performed once a day from day 4 to day 18. Rats in groups A and B received equivalent volume of water in place of ATL-III via gavage.

Assessment of water consumption and feed intake

During the experimental period, alterations in water consumption and feed intake were observed and recorded every day. All measurements were performed in three independent experiments.

Measurement of stool parameters

The pellets excreted by each rat were collected at 10:00 am. The pellets were counted and stool weight was obtained three times per sample. Stool water content was calculated. All measurements were performed in three independent experiments.

Assessment of intestinal transit time

After 18 days treatment, all animals were subjected to 24 h fasting but with unlimited drinking water. Each rat was administered 0.5

mL of ink, and a timer was immediately switched on. After 30 min, all rats were sacrificed by abdominal injection of 200 mg/kg pentobarbital, and whole intestines from the rectum to the pylorus were taken out and straightened. Then, the entire length of the intestine and the distance the ink was propelled in the intestine were measured to calculate the intestinal transit rate, using Eq 1.

$$ITR\ (\%) = D/(IL) \quad \text{..... (1)}$$

ITR: intestinal transit rate; D: distance traveled by the ink (in cm); IL: intestinal length (in cm). All measurements were performed in triplicate.

Assessment of neurotransmitter levels in blood

Before the rats were sacrificed, blood samples were obtained from their tail veins. Serum was obtained from blood samples by centrifugation at 3500 rpm for 15 min. The serum concentrations of nitric oxide synthase (NOS), somatostatin (SS), serotonin (5-HT), and vasoactive intestinal peptide (VIP) were measured with ELISA using commercially available kits. All measurements were performed in three independent experiments.

Hematoxylin and eosin (H & E) staining

Colons were collected from control and experimental animals, and then fixed with 10 % formalin for 12 h, embedded in paraffin wax, followed by sectioning in 5 µm thickness. Sections were then stained with hematoxylin and eosin (H & E, Sigma-Aldrich, MO, USA). The images were acquired with Leica Application Suite (Leica Microsystems, Switzerland). All measurements were performed in three independent experiments.

Immunohistochemistry assays

Unstained sections were cut from the paraffin blocks which were prepared as described above. The sections were incubated for 1 h with PBST containing 5 % BSA. Thereafter, they were incubated overnight at 4 °C with primary anti-c-kit (1:1000 dilution). Subsequently, the secondary antibody was used for 1 h at room temperature, followed by amplification with DAB Vectastain ABC kits (Vector Laboratories). The tissue sections were then examined under an upright Leica microscope, and the staining intensity was quantified using Image-Pro Plus 6.0 software (Mdia Cybernetics, Silver Springs, MD). All measurements were performed in triplicate.

Western blotting

Proteins from the transverse colon of all rats were subjected to 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were blocked with 5 % skim milk in 50 mM Tris-buffered saline containing 0.1% Tween 20 (TBST), and were incubated separately with the primary antibodies anti-c-kit (#37805, 1:1000 dilution); anti-SCF (#2093, 1:1000 dilution); anti-PI3K (#4249, 1:1000 dilution); anti-PKC (#59754, 1:1000 dilution); Cell Signaling Technology Inc., Cambridge, MA, USA,), and anti-GAPDH (G9545, Sigma-Aldrich, Saint Louis, MO, USA, 1:2000 dilution) at 4°C overnight. Then the PVDF membranes were washed with TBST and incubated with horse radish peroxidase-conjugated secondary antibody [goat-anti-rabbit IgG (A32732, Zymed Laboratories, South San Francisco, CA, USA)] at a dilution of 1:5000, at room temperature for 1 h. Finally, the signals were detected by ECL reagents, then images were acquired and analyzed by a Tanon image system. All measurements were performed in three independent experiments.

Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR)

Total RNA was extracted from transverse sections of colon using TRIzol (Invitrogen, Grand Island, NA, USA), according to the manufacturer's instruction. The total RNA samples (1-µg) were added to 12 µL Oligo (dT) (10 µM), and made up to a volume of 20 µL with appropriate amount of water. The samples were heated at 70 °C for 10 min and then chilled on ice, and then reverse-transcribed to cDNA for 55 min at 42 °C using SuperScript™ Pre-amplification System for First Strand cDNA Synthesis (GIBICOL). Real-Time RT-PCR was performed using ABI StepOne Plus System (ABI). The cycling protocol was listed as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 sec, 63 °C for 20 sec, and 68 °C for 40 sec. After all amplification cycle, the melting curve was evaluated to ensure the specificity of PCR products. Each sample was treated in triplicate. The PCR products were detected using SYBR Green (QPK201, Toyobo, Japan). GAPDH gene was used as internal control. The sequences of the primers used were as follows:

c-kit: 5′-CTGGCTGCCAA ATCTCTGTGAA-3′; 5′-AGATGACGAGCTGGCTCTGGA-3′;
SCF: 5′-GGAGATGGCAGTTGTGACTA-3′; 5′-CATGCTTTAAGGCCTTTGTCACGA-3′;

PI3K: 5′-TGGGCACAGGGAAGACAA-3′; 5′-ACCAGTTGGCTCGGCATA-3′;
PKC: 5 -AAGTGAGAAACCCCGGCTAT-3′; 5′-AGGCAAATCCCTTCCAGTCT-3′;
GAPDH: 5′-TGATTCTACCCACGGCAAGTT-3′; 5′-TGATGGGTTTCCCATTGATGA-3′.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed with ANOVA followed by a Bonferroni's test using Prism software (Graphpad Software, CA, USA). Results at $p<0.05$ level were considered significant.

RESULTS

Feed intake and fecal excretion of loperamide-induced STC rats

Rats with STC had comparable feed intake with rats in the other groups. However, the Lop + ATL-III–treated groups showed less water consumption than the STC model group (Figure 1 B and C). Moreover, the quantity and weight of stool, as well as their moisture content were decreased in STC model group, comparing with corresponding values in the normal control. However, ATL-III treatment reversed these changes (Figures 1 D, E and F). These results show that ATL-III treatment (Figure 1 A) did not alter feed intake, water consumption and fecal excretion of the STC rats.

Effect of ATL-III on intestinal transit rate in loperamide-induced STC rats

As shown in Figure 2 A, the intestinal transit rate in STC model group was significantly lower when comparing to untreated control. The intestinal transit rates in STC model groups were recovered after ATL-III treatment in a dose-dependent manner. Thus, ATL-III treatment improved intestinal transit rate in loperamide-induced STC rats.

Effect of ATL-III on serum NOS, VIP, SS, and 5-HT of loperamide-induced STC rats

The serum levels of NOS, VIP, SS, and 5-HT from loperamide-induced STC rats were assayed with ELISA. The levels of NOS, VIP, and SS were remarkably induced by loperamide, but downregulated by ATL-III treatment (Figures 2 B, D, and E). In contrast, the level of 5-HT in STC model group was inhibited, while ATL-III treatment eliminated the inhibitory effect (Figure 2 C).

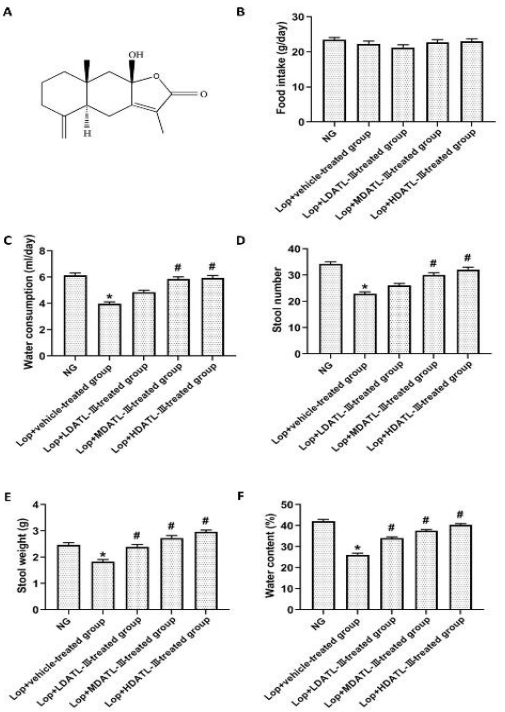

Figure 1: Effect of ATL-III on feed intake and fecal excretion in loperamide-induced STC rats; *p < 0.05, Lop+vehicle-treated group was compared with normal control group (NG); #p < 0.05, Lop+ATL-III-treated groups (three doses) were compared with Lop+vehicle-treated group

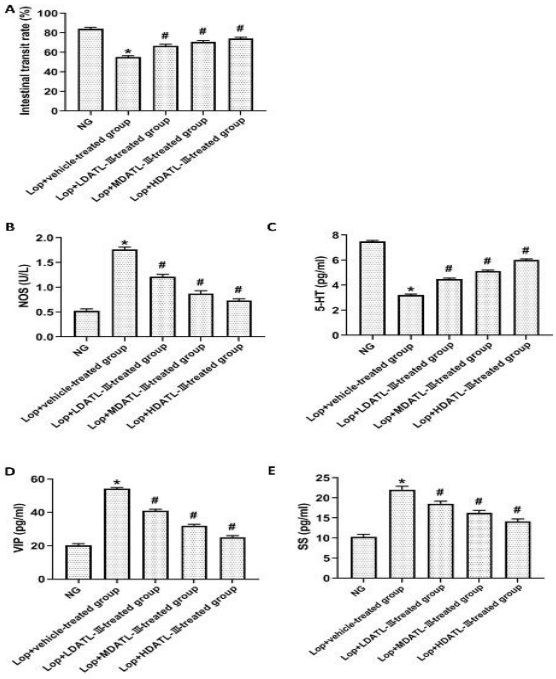

Figure 2: Effect of ATL-III on intestinal transit rate and neurotransmitter levels in blood in loperamide-induced STC rats. (*p < 0.05, Lop+vehicle-treated group was compared with normal control group (NG); #p < 0.05, Lop+ATL-III-treated groups (three doses) were compared with Lop+vehicle-treated group)

Histological alterations in colon tissue

To investigate the effect of ATL-III on structural alterations in colon tissue, smooth muscle cells, neuronal cells, and mucous layer were examined in rat transverse colons after H & E staining (Figure 3). In the model group, the smooth muscle cells were atrophied and disorderly arranged; the neuronal cells of the colon tissue were reduced in population, and there was evidence of inflammatory cell infiltration in the mucosa. However, the colon smooth muscle cells and neuron cells of the rats in the Lop + ATL-III-treated groups appeared normal and similar to those of the control group, and the infiltration of inflammatory cells in the colon tissue was decreased.

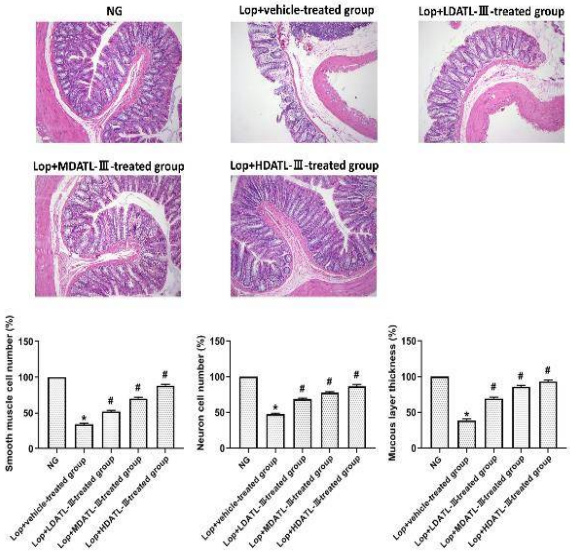

Figure 3: Effect of ATL-III on structural alterations of colon tissue in loperamide-induced STC rats. *p < 0.05, Lop+vehicle-treated group was compared with normal control group (NG); #p < 0.05, Lop+ATL-III-treated groups (three doses) were compared with Lop+vehicle-treated group

Effect of ATL-III on expression of C-Kit, SCF, PKC, and PI-3K

As a specific marker of ICC, the immunoreactivity of C-kit was measured using immunohistochemistry. After staining with DAB, brown stained particles appeared in the cytoplasm. The expression of C-kit in colon tissue dramatically decreased to a basal level after loperamide treatment, and the distribution was sparse (Figure 4 A). In contrast, the expressions of C-kit in colon tissues of Lop + ATL-III-treated groups were increased to various degrees, and the cells were densely distributed.

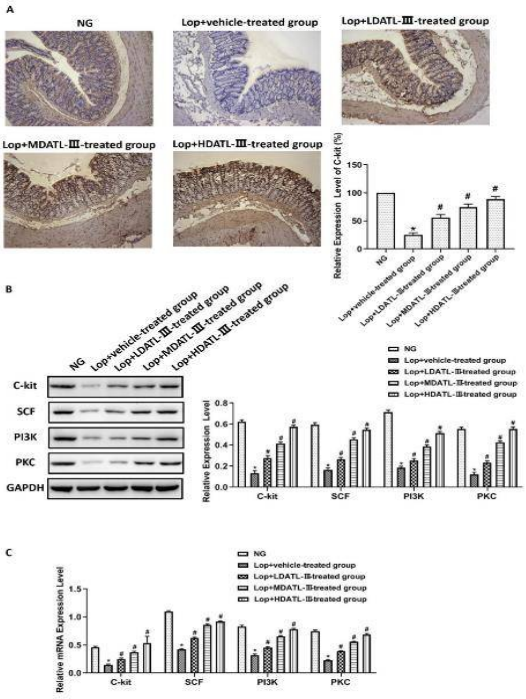

Figure 4: Effect of ATL-III on expression of C-kit, SCF, PKC, and PI-3K in the colon of Lop-induced STC rats. The immunoreactivity of c-kit was measured by immunohistochemistry, and expressions of c-Kit, SCF, PKC, and PI3K of the colon tissue were measured by Western blot and real-time RT-PCR; *p < 0.05, Lop+vehicle-treated group was compared with normal control group (NG); #p < 0.05, Lop+ATL-III-treated groups (three doses) were compared with Lop+vehicle-treated group

The expressions of C-kit, SCF, PKC, and PI-3K were measured at both protein and mRNA levels, and the results are shown in Figure 4 B and C. The expression of C-kit, SCF, PKC, and PI-3K at protein levels were remarkably lower after loperamide treatment. In addition, Lop + ATL-III–treated groups had elevated c-kit, SCF, PKC, and PI-3K protein expression levels. Furthermore, the pattern of changes in mRNA expression levels was very similar to that of protein expression levels. Thus, ATL-III accelerated the transmission of electrical excitation and enhanced colon movement by increasing the expressions of c-kit, SCF, PKC, and PI-3K.

DISCUSSION

Slow transit constipation (STC) is due to various factors such as delayed colon movement and reduced fecal function. Surgical treatment does not fundamentally cure STC, and the side effects of drug treatment are enormous. The disease causes physical and mental agony to patients, while bringing them huge economic burdens. In recent years, Chinese medicinal herb and its

extracts have been found to be effective in the treatment of STC, and they have attracted the attention of researchers. Atractylenolide-III (ATL-III) is a key active component of *Atractylodes lancea.* Therefore, the effect of ATL-III on loperamide-induced STC in rats was investigated in the present study. Feed intake, water consumption and fecal excretion are main factors when evaluating the severity of constipation and drug efficacy [14].

In this study, feed intake was not affected by ATL-III. In contrast, the amounts of feces and their moisture contents were decreased in the model group, while water consumption and urine volume were increased. However, these indices were gradually and dose-dependently improved by ATL-III to the levels of control group. *Aloe ferox* Mill. is widely used for its healing effects, and also effective in treating loperamide-induced constipation in rats, consistent with present study [15]. Furthermore, the intestinal transit rate was decreased in the model group, while it remained normal in low-dose, medium-dose, and high-dose ATL-III groups. These results demonstrate that ATL-III treatment mitigates the changes in feed intake, water consumption, fecal excretion, and intestinal transit rate of loperamide-induced STC rats.

Intestinal motility is regulated by a complex neuroendocrine network composed of multiple neuropeptides, neurotransmitters and their receptors [16,17]. Neurotransmitters related to the pathogenesis of STC include nitric oxide (NO) [18], vasoactive intestinal peptide (VIP) [19], somatostatin (SS) [20], and serotonin (5-HT) [21]. Abnormalities in the expressions of these neurotransmitters may be involved in the neuropathological mechanism of STC. Being the main suppressive transmitters of colonic motility, the concentrations of NO, VIP, and VIP in the colon tissue of ATL-III groups were understandably lower than those in the colonic tissue of the model group. Moreover, the excitable neurotransmitter 5-HT was increased in the ATL-III groups when compared with the STC model group, indicating that the inhibitory role of ATL-III on colonic movement was relatively weak.

The Cajal interstitial cells (ICC) are special cells that produce slow waves and regulate neurotransmitters and conduction of electrical excitability. They are located between gastrointestinal smooth muscle cells (SMC) and nerve cells, and are closely linked with gastrointestinal motility [22,23]. The specific marker of ICC is the proto-oncogene C-kit of the gastrointestinal tract, which is distributed on the surface of the cells [24]. After combining with its ligand stem cell factor (SCF), a chain reaction of intercellular signaling effectors is triggered by phosphorylation, resulting in the regulation of several signaling pathways, involving phosphatidylinositol-3 kinase (PI-3K) and other downstream substrates. The end-result is the activation of the cytosolic transcription factor which regulates gene expression and activation, and control cell proliferation, differentiation and growth [25,26].

ENS is a complete intramural nerve system in the gastrointestinal tract that regulates the alimentary movement independently [27]. At present, it is generally believed that ENS, ICC and SMC form functional network connections. As a basic functional unit of gastrointestinal motility, this functional network effectively transmits nerve impulse to the SMC around them, resulting in gastrointestinal motility. The present study found that ICC, SMC, and nerve cells were significantly decreased in the model group, but were enhanced in the three ATL-III groups. The protein and mRNA expressions of C-kit, SCF, PKC, and PI-3K were increased in the ATL-III groups, when compared with model group, albeit at below-normal levels. Therefore, it may be suggested that ATL-III regulates the smooth muscle activity by elevating the number of ICCs, and SCF/c-kit exerts its effect through PKC/PI-3K pathway.

CONCLUSION

The results obtained in this study indicate that ATL-III exerts a significant enhancing effect on the colonic transit function of loperamide-induced STC rats, via a laxative mechanism involving enhancement of the expressions of SCF/c-kit and PKC/PI-3K, and promoting the repair and regeneration of ICC. These effects result in recovery of intestinal motility and defecation ability. Thus, ATL-III has promising potential in the management and prevention of constipation.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this

work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Wang Hao and Gu Yunfei conceived and designed the study. Wang Hao, Gong Yuxia, Li Youran and Xu Minmin performed the experiments. Gu Yunfei carried out analysis of data. Gu Yunfei wrote the manuscript. All authors read and approved the manuscript for publication.

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