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• BASIC RESEARCH •

Screening and identification of proteins mediating senna induced gastrointestinal motility enhancement in mouse colon

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Abstract

AIM: **To isolate the proteins involved in pharmacologic action of senna extract (SE) from mouse gastrointestinal tract and to explore the molecular mechanism of gastrointestinal motility change induced by SE.**

METHODS: **SE was administrated to mice by different routes. Gastrointestinal motility of mice was observed using cathartic, gastrointestinal propellant movement experiments and X-ray analysis. Mouse model for gastrointestinal motility enhancement was established through continuous gastric administration of SE at progressively increased dose. At 3 h and week 3, 4, 6 and 10, morphological changes of gastrointestinal tissues were found under light microscope. Ultrastructural changes of intestinal and colonic tissues at week 6 were observed under transmission electron microscope. The colonic proteomic changes in model mice were examined by two-dimension polyacrylamide gel electrophoresis with immobilized pH gradient isoelectric focusing to screen the differentially expressed proteins, and their molecular masses and isoelectric points were determined. Two N-terminal sequences of the samples were also determined by mass spectrometry.**

RESULTS: **SE (0.3g) caused diarrhea after gastric administration in 1-6h and enhanced gastrointestinal propellant (65.1±7.5%; 45.8±14.6%, $P<0.01$) in mice, but intramuscular and hypodermic injection had no cathartic effect. X-ray analysis of gastrointestinal motility demonstrated that gastric administration of SE enhanced gastric evacuation and gastrointestinal transferring function. At 3 h and week 3 and 4 after gastric administration of SE, light microscopic examination revealed no apparent change in gastrointestinal mucosal tissues, but transmission electron microscopic examination revealed inflammatory changes in whole layer of intestinal and colonic wall. Twenty differential proteins were detected in the colonic tissues of the model mice by two-dimensional electrophoresis, and the N-terminal amino acid sequences of two proteins were determined.**

CONCLUSION: **SE causes diarrhea and enhances gastrointestinal motility through digestive tract administration. Long-term gastric administration of SE induces inflammatory changes and cell damage in the whole gastrointestinal tract. The differential proteins screened from the colonic tissues of the model mice might mediate the enhancing effect of SE on gastrointestinal motility.**

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INTRODUCTION

Senna, a traditional Chinese medicine, has potent cathartic effect[1-4]. Its extract(SE), composed of a few dozens of chemical substances, possesses multiple pharmacological activities. Especially, it can promote the motility and secretion of gastrointestinal tract. However, its application is greatly restricted due to its toxicity[5-17]. Much attention is being paid to the effect of traditional Chinese medicine on the regulation of gastrointestinal motility[18-37]. We analyzed the role of senna in gastrointestinal motility and in diarrhea of mice and observed its action pattern, the ultrastructural changes in the active sites, and the changes in protein expression. In doing so we intended to screen from the gastrointestinal tissues the biological molecules mediating diarrhea and the enhancement of gastrointestinal movement, to elucidate the mechanism of catharsis induced by senna at the molecular level, and to lay a foundation for the development of pharmaceutical agents enhancing gastrointestinal motility.

MATERIALS AND METHODS

Materials

Imported senna (Shaanxi Medicine Corporation) was used, and its quality was confirmed by the Institute of Pharmaceutics, Fourth Military Medical University. The extract was obtained through solvent recovery and stored at -20℃. Acrylamide, bisacrylamide, SDS, Tris base,PMSF (all from Sino-America Biotechnology Co); ultrapure urea (Shanghai Biotechnology Co); ampholyte pH 3-10L, IPG dry strips pH 3-10L, IPGphorTM Isoelectric Focusing System (Pharmacia Co); ultradispersor GF-1 (Jiangsu Qilin Medical Equipment Factory); two-dimensional electrophoresis bath DYY-III 26, gradient mixer (Beijing 61 Factory); electrophoresis apparatus (Bio-Rad Co) were used.

Induction of diarrhea by SE in different Administration routes

Thirty Balb/c mice (both sexes, 8 week, 18g-23g) were used. Before experimental manipulation, the mice was fasted without water deprivation for 24h, and each was kept in a single cage re-based with filter paper. After being divided into different groups, the mice were administered 0.3g SE in different routes, and the diarrhea status was examined.

Effect of SE on gastrointestinal motility in mice

Effect of SE on gastrointestinal motility for carbon Thirty Balb/c mice (both sexes, 6-8weeks, 18-23 g) were divided randomly into 3 groups: Group I, II, and Control Group. SE of 0.3g was delivered to each mouse in Group I through gastric administration; 0.3g SE was im injected into each mouse in Group II; and the same volume of 9g·L^{-1} NaCl was delivered through gastric administration to the mice in control group. After 20 min, 0.1 mL of 100 g·L^{-1} mixture of Arabic gum and charcoal powder was administrated to all mice. Then, all mice were killed after 20 min. Whole gastrointestinal tract was taken out and pulled straight. The length of gastrointestinal tract from pylorus to anus and that from pylorus to the anterior extremity of carbon powder were measured. Percentage of migration distance of carbon powder out of whole length was calculated.

Effect of SE on gastrointestinal motility for barium Eight Balb/c mice (both sexes, 8weeks, 18~20 g), fasting without water deprivation within 24 h prior to the experimental manipulation, were divided randomly into 2 groups. SE of 0.3g was given through gastric administration to each mouse in Group I, and the same volume of 9 g·L^{-1} NaCl to each mouse in the control group. After 20 min, 0.8 mL of 1.6kg·L^{-1} barium sulfate suspension was given to each mouse. The mice were put into cloth bags that could restrict their movement, and the bags were placed on the flat of the X-ray digital machine. The migration of barium in the gastrointestinal tract was visualized and X-ray photos were taken at 5 and 40 min, 1, 1.5, 2, and 3h after barium administration[38].

Effect of SE on morphology of gastrointestinal tissue

Thirty Balb/c mice (both sexes, 8weeks, 18-23 g) were fed separately according to their sexes. Ten were classified into the control group, and 20 into the experimental group. SE was given through gastric administration to the mice in the experimental group once a day. The frequency of drug delivery increased progressively from 1·d^{-1} to 4·d^{-1} and the dosage increased progressively from 0.1g·d^{-1} to 0.8g·d^{-1} in 6 weeks. The same volume of 9 g·L^{-1} NaCl was given to the control group. Four mice in the experimental group and 2 in the control group were killed at 3 h, 3, 4 and 6week after reagent administration. Gastric, intestinal and colonic tissues were obtained and fixed in 40 g·L^{-1} paraformaldehyde and embedded routinely with paraffin. Sections were made. Morphological changes were examined under light microscope after HE staining. Intestinal and colonic tissues at week 6 were collected and fixed with 30 g·L^{-1} glutaraldehyde for 6h at 4℃. Ultrathin sections were routinely made. Cellular ultrastructural changes in the whole layer of the intestinal wall were examined under transmission electron microscope.

Two-dimensional polyacrylamide gel electrophoresis of proteins[39]

Establishment of animal model for chronic gastrointestinal motility enhancement Fourty Balb/c mice (both sexes, 8 weeks, 18-20 g) were selected. Twenty five mice in the experimental group received SE through gastric administration once a day, with the frequency and dosage increasing progressively from 0.1g, 1·d^{-1} to 0.8g, 4·d^{-1}. Fifteen mice in the control group received the same volume of 9 g·L^{-1} NaCl.

Preparation of protein sample from intestinal tissue of mice Tweenty mice were killed at 6 weeks after receiving gastric administration of SE (10 mice) and 9g·L^{-1} NaCl (10 mice). The colons were collected and put immediately into ice-cold 9g·L^{-1} NaCl containing 0.1mmol·L^{-1}PMSF. The colonic tracts were dissected and colonic contents were washed out. The moisture on the tissues was absorbed with filter paper, and the tissues were put into liquid nitrogen immediately. The whole procedure must be finished in 5 min. Then, the tissues were either stored at -70℃ until used again, or taken for immediate use. In the latter case, the tissues were weighed on electric scale, and lysed by adding tissue lytic solution (9.5mol·L^{-1} urea, 20g·L^{-1} NP, 402g·L^{-1} ampholyte pH 3-10, 20g·L^{-1} 2-ME, 1.5 mmol·L^{-1} EDTA, 40 mmol·L^{-1}Tris and ion-free water) by a mass volume ratio of 1 ∶ 5. Tissue homogenate was prepared with high-speed disperser in ice-bath (3500 r ·min^{-1} ×5s×5), and DNase and RNase (both 0.4 g·L^{-1}) were added into the homogenate. After the incubation for 20 min in ice-bath and addition of PMSF (0.1 mmol·L^{-1}), the homogenate was centrifuged at 10,000×g for 10 min. The supernatant was harvested and stored in designated volume at -70℃ until used again. The total protein concentration in the supernatant was measured by Bradford method[40-42].

Solid-phase pH gradient isoelectric focusing For each group, 100 μg protein sample was solved in 250μL mixture of deuteroxide solution and IPG buffer, placed at room temperature for 1h, and then applied to sample tank which was placed with IPG dry gel strip (13 cm in length), and covered with mineral oil. The isoelectric focusing program was: deuteroxidization for 12 h; isoelectric focusing 0-300 V, 1 h; 300-500 V, 1 h; 500-1000 V, 1 h; 1000-2000 V, 1 h; 2000-4000 V, 1 h; 4000-8000 V, 4 h. All operations above were performed at 20℃.

Equilibration and transfer of IPG slab gels The slab gels were collected and equilibrated with SDS-balanced buffer (50mmol·L^{-1} Tris, 6 mol·L^{-1} urea, 300 g·L^{-1} glycerin, 10 g·L^{-1} DTT, ion-free water) for 15 min followed by a second equilibration with balanced buffer (50 mmol·L^{-1} Tris, 6 mol·L^{-1} urea, 300 g·L^{-1}glycerin, 25 g·L^{-1} idocetamide, ion-free water) for another 15 min. Two pieces of 125 g·L^{-1} separation gel (17cm×17cm×0.15cm) was prepared in absence of sticking gels. IPG slab gels were fixed on the top of the seperation gel using 5 g·L^{-1} agarose. Protein marker was applied at the other terminus.

Second dimension SDS-PAGE Two pieces of 125 g·L^{-1} SDS-PAGE (17cm×17cm×0.15cm) were prepared and underwent polymerization for 2 h in absence of sticking gels; IPG slab gels were fixed onto the top of SDS-PAGE gels; electrophoresis was performedfor 11 h at room temperature (15℃-20℃) with constant electric current (20 mA/gel, 40mA in total) and terminated when the marker arrived at the bottom.

Silver staining of gels The gels were collected and stained with silvery salt based on a modified protocol. The gels were fixed with the fixing solution (300 g·L^{-1} ethanol, 0.5 mol·L^{-1} sodium acetate, 5 g·L^{-1} glutaraldehyde, 2 g·L^{-1} sodium thiosulfate), rinsed with ion-free water for 15 min×3, stained with 1 g·L^{-1} $AgNO_3$ and 0.1 g·L^{-1}formaldehyde for 20 min, washed with ion-free water for 30sec, and colored with 25 g·L^{-1} sodium carbonate, 0.5 g·L^{-1} sodium thiosulfate and 0.1 g·L^{-1} formaldehyde. The color development was terminated with 10 g·L^{-1} acetic acid.

Analysis of protein map on two-dimensional gels After silvery salt staining, the gels were scanned with transmission laser scanner at 500 bpi resolution and analyzed with image pattern analyzer under identical conditions. The position, shape and density information for each

detected spot was compared to screen obviously differentially expressed proteins and to determine their molecular masses and isoelectric points.

Identification of the screened proteins by sequencing[43] One mg colonic proteins from the animal model was sampled and underwent solid-phase pH gradient isoelectric focusing and second dimension SDS-PAGE and transferred from gel slabs to polyvinylidene difluoride (PVDF) membranes (transferring buffer: CAPS, DTT, methanol and ion-free water, pH 11.0; transferring condition: 350 mA,4.5 h, 10℃). The proteins on the PVDF membranes were stained with 1 g·L^{-1} Coomassie brilliant blue. PDVF membrane was air-dried at room temperature. The protein spots stained with Coomassie brilliant blue were excised from the membrane, and their amino acid sequences at the N-termini were determined by mass spectrometry.

Statistical analysis

Student's *t* test and χ^2 test were used for data measurement and enumeration, respectively.

RESULTS

Cathartic Effect of SE in different Administration routes

One to 1.5 h after gastric administration of SE, mice started to suffer from diarrhea, defecating water-thin feces, which lasted 4-5h. When the mice received im and sc injection of extract of doubled dosage, they did not develop diarrhea within 6 h (Table 1).

Table 1 Carthartic effect of SE in different administration routes

	Groups	Dose / g	*n*	Administration routes	Diarrhea (in 6h)
I	Normal saline	0.3	6	ig	-
II	SE	0.3	6	ig	+(6)[b]
III	Normal saline	0.3	6	im	-
IV	SE	0.3	6	im	-
VC	SE	0.3	6	sc	-

[b]$P<0.01$, *vs* all other groups.

Effect of SE on gastrointestinal motility in mice

Effect of SE on gastrointestinal propellant movement While ig administration of SE could enhance gastric motility, im delivery did not show such effect (Table 2).

Table 2 Effect of SE on gastrointestinal propellant rate in mice ($\bar{x}\pm s$)

	Group	Dose / g	Administration routes	*n*	Propellant rate / %
I	Normal saline	0.3	ig	10	45.8±14.6
II	SE	0.3	ig	10	65.1± 7.5[b]
III	SE	0.3	im	10	48.3±12.4

[b]$P<0.01$, *vs* I, III groups.

X-ray analysis of SE effect on gastrointestinal motility In mice with gastric administration of SE (0.3 g), barium sulfate was excreted at 40 min, and more at 2 h. The migration speed was significantly higher than that shown in the control group (Figure 1).

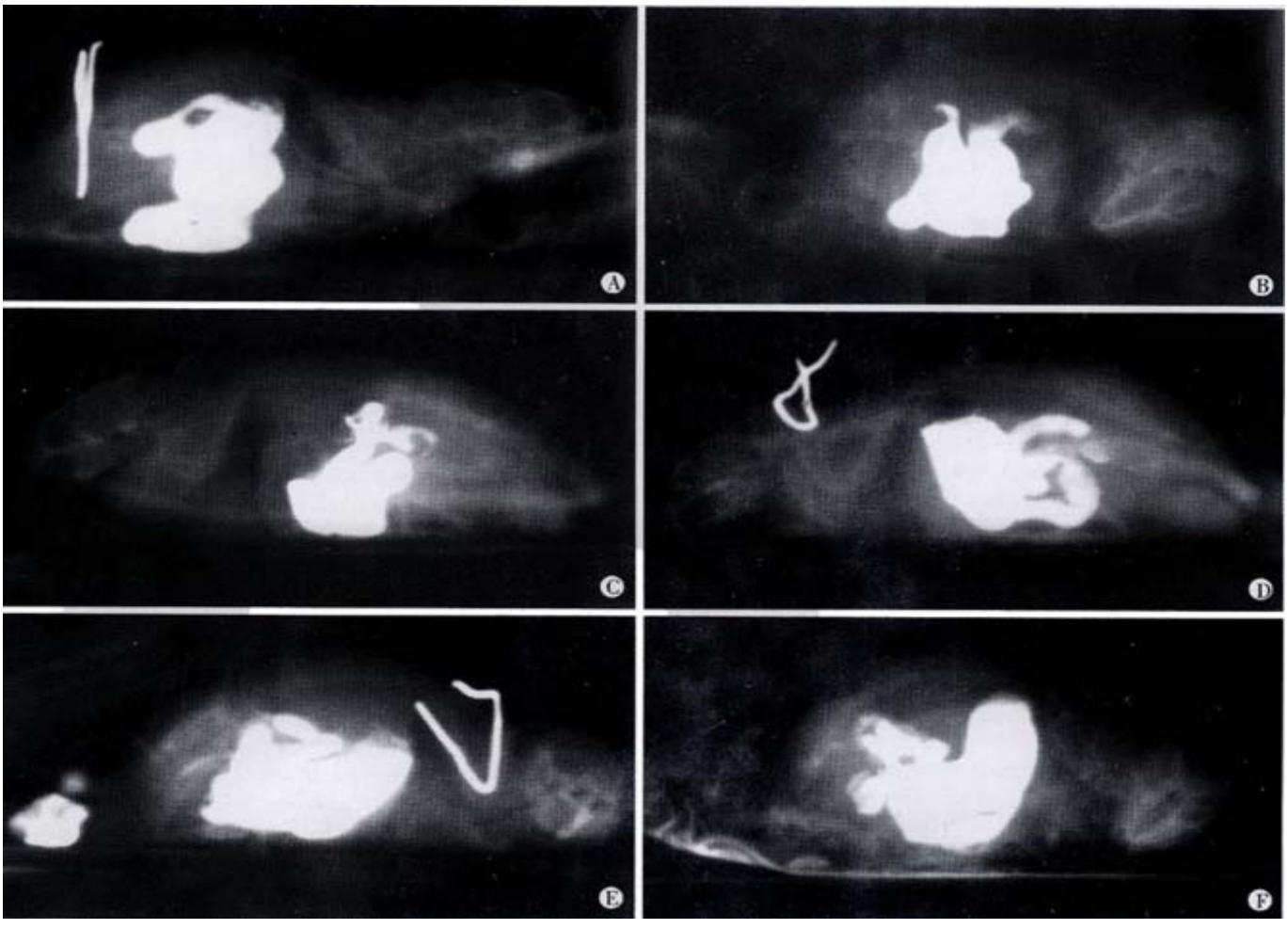

Figure 1 X- rays of gastrointestinal peristalsis in mice administered with SE
A, B, C: 5min, 2h, 3h after 9 g·L-1 NaCl gastric administration, respectively D, E, F: 5 min, 2h, 3h after 0.3 g SE gastric administration, respectively

Morphological effects of SE on gastrointestinal tissue No pathological changes were observed in gastrointerestinal tissues under light microscopy examination at 3 h, 3, 4 and 6weeks after gastric administration of SE. However, at week 6, it was observed under transmission electron microscope that some small intestine epithelial cells underwent degeneration and necrosis. Although the microvilli were normal, the mitochondria in the cells were slightly swollen, with part of the cristae broken. In the mucosa, macrophages containing phagocytic particles were found to increase, and so were plasmacytes. Cell degeneration and necrosis occurred more frequently in the mucosal epithelial cells in colon than those in the small intestine (Figure 2). The mitochondrial abnormalities mentioned above were observed in both small intestine and colon smooth muscle cells, along with the occurrence of vacuolation.

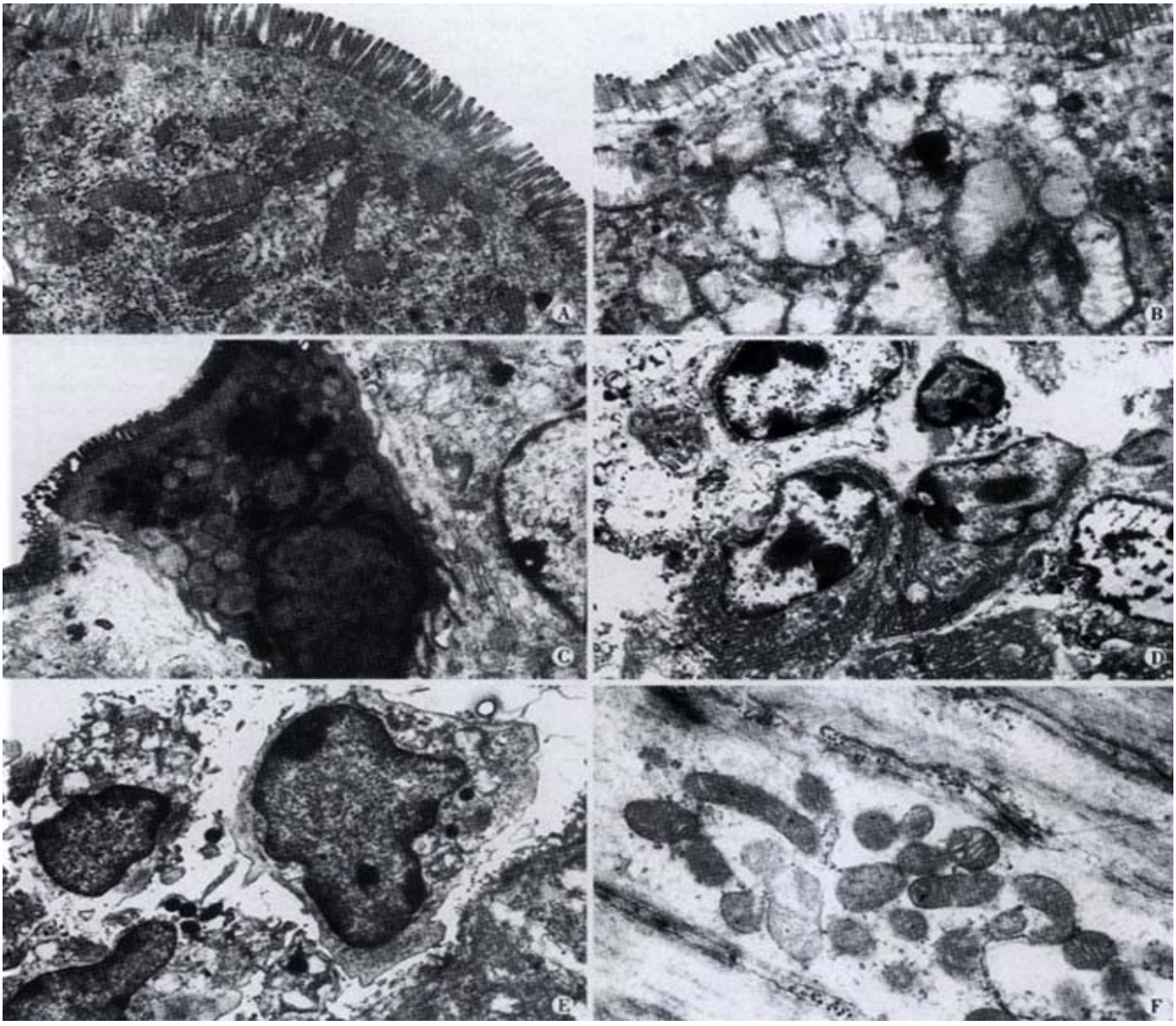

Figure 2 Ultrastructure of intestine and colon cells in mice receiving SE at week 10.
A: Normal epithelial cell; B: Intestinal cell after treatment with SE; C: Colon cell after treatment with SE; D: Increase in submucosal plasmacytes; E: Increase in submucosal macrophages; F: Minor degeneration of smooth muscle cells in intestine

Gastrointestinal proteins in mice

Establishment of mice model for chronic gastrointestinal movement enhancement Gastrointestinal propellant movement experiment and X-ray analysis of gastrointestinal motility demonstrated that gastric administration of SE could significantly enhance the gastrointestinal motility in Balb/c mice. Therefore, continuous gastric administration of SE might keep the gastrointestinal tract in constant enhanced motility. At week 6, the weight of the mice in the model group was significantly lower than that in the control group.

Differential expression of gastrointestinal tissue proteins in mice In the first dimension IPG isoelectric focusing, pH gradient was pH 3-10 L, 100 μg protein sample was added. In the second dimension IPG -PAGE 125 $g \cdot L^{-1}$ uniform gel was used, the gels were stained with silver salt (Figure 3).

Identification of differentially expressed proteins The colonic proteins of one control mouse and one model mouse were screened and compared, and the process repeated for 5 such pairs. Twenty proteins were found different in abundance (Table 3).

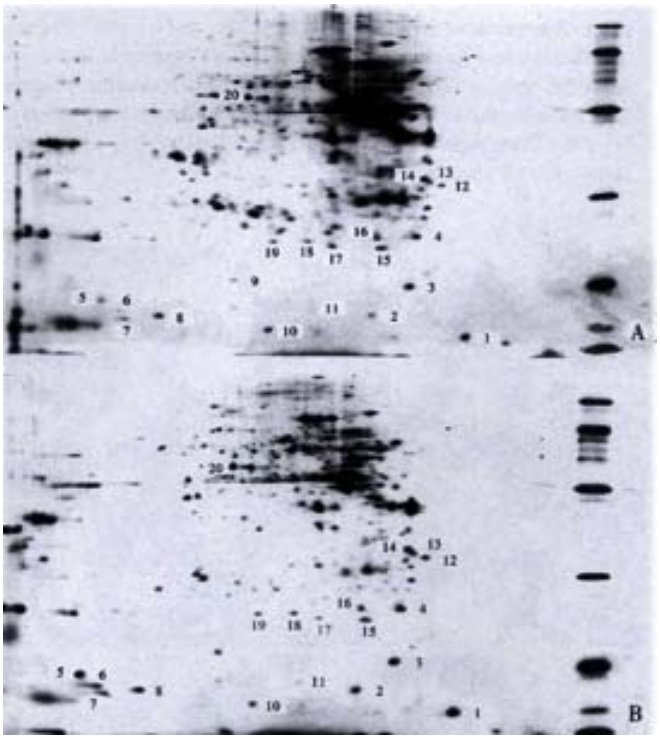

Figure 3 Proteins on two-dimension electropherosis maps
A: Normal mice; B: Model mice

Table 3 Identification of the differential proteins

No.	Molecular mass/ku	Isoelectric point/pI	No.	Molecular mass/ ku	Isoelectric Point/pI
1	15.0	3.9	11	18.0	5.9
2	17.5	5.2	12	33.0	4.3
3	19.0	4.7	13	34.0	4.5
4	26.0	4.6	14	35.0	4.6
5	18.0	8.8	15	24.0	5.1
6	17.5	8.6	16	26.0	5.2
7	16.5	8.5	17	24.5	5.7
8	17.0	8.0	18	25.0	6.1
9	20.0	7.0	19	25.0	6.5
10	16.0	6.6	20	52.0	7.0

Identification of differential proteins by sequencing The N-terminal amino acid sequences of protein No. 4 and No 12 were determined by sequencing (No. 4: N-MIX/IYR-C; No 12: N-GFXDX/L-C). Protein database search showed that these two proteins were unknown proteins.

DISCUSSION

Senna is a traditional Chinese medicine containing various chemicals, the effective components of which are sennoside A, B, C and D. It has been shown that sennosides are decomposed into Rhein anthrone by bacteria in colon, and then take effects on the colonic smooth muscle, significantly promoting colonic motility in animals and humans. The mechanism is that sennoside and its active forms affect on the intestinal mucosal epithelium and submucosal nerve bundles, stimulating prostaglandin (PG) synthesis and endogenous acetylcholine release, and subsequently enhancing colonic smooth muscle contraction.[44-46] Meanwhile, sennosides may affect directly the colonic smooth muscle, evoking its spontaneous spike potential and promoting its contraction[47]. In addition, sennosides can stimulate the colonic mucosa to release PG, NO and 5-HT, efficiently inducing the excretion of water and electrolytes by epithelial cells[48-50]. Our study showed that 1-1.5 h after gastric administration of SE, mice started to suffer from diarrhea, defecating water-thin feces, which lasted 4-5h. When the mice received im injection of extract of doubled dosage, they did not develop diarrhea within 6 h, but after 6 h, 3 mice had light diarrhea. It suggested that the possible pathway might be that the drug delivered through im injection entered blood circulation and was excreted into the intestinal tract with bile. With hypodermic injection of larger dosage of extract, no mice developed diarrhea. However, no enhanced gastrointestinal motility was observed after im injection of SE. These results indicate that the cathartic effect of SE can only be exerted through the digestive tract rather than other pathways.

Our study also indicated that SE could promote the propellant movement in mice, a result in agreement with the reports of other researchers. Although most researchers believe that the active components of senna mainly work on colon and promote colonic motility. We think that it may also affect small intestine, as our gastrointestinal propellant movement experiment has revealed that it promoted the motility of mice small intestine in mice. In order to locate the effective site of the drug, we continuously observed the movement of marked liquid ($BaSO_4$) in the gastrointestinal tract after administration of SE, and discovered that it in hibited rather than promoted gastric empty; however, it promoted small intestinal motility, and especially colonic motility.

We made histological examinations of mouse gastrointestinal tracts at different stages after gastric administration of SE. No apparent change was found under light microscope, but transmission electron microscopy showed lesions and degenerative changes of small intestinal mucosal epithelial cells, increase in submucosal macrophages and plasmacytes, minor degeneration of smooth muscle cells, and severe extensive degeneration and necrosis of colonic epithelial cells. Mengs *et al*[10] observed the changes in guinea pig colonic epithelial cells after continuous gastric administration of sennoside for 2 weeks, and discovered epithelial cell degeneration. These results show that SE can injure the intestinal cells.

Using improved two-dimensional electrophoresis of proteomic analysis[42,51,52], we compared the protein expression in the colon tissues of promoted gastrointestinal motility model mice and that of normal controls. The conditions in the whole process were identical for the two groups, and the location, shape, size and density of many protein spots were similar on the two-dimensional electrophoresis maps. Hence, the two groups were comparable. Image analysis showed differential proteins in the colon tissues of the model animals; the differences were primarily the increase or decrease in the amount of protein expression. Most of the differential proteins had moderate or low molecular mass, as shown in Table 3, which are probably regulatory proteins induced or affected by SE. Two of the proteins were sequenced with mass spectrometry and were confirmed to be novel ones through protein database search. However, the pharmacological functions they mediate remain to be discovered.

SE administered through the gastrointestinal tract promotes diarrhea and gastrointestinal motility, especially that of the small intestine and the colon; it also injures the digestive tract mucosa and smooth muscles, and promotes differential protein expression in the colon tissues of model animals. We have isolated and identified some of the molecules that may be involved in mediating the motility promotion and secretion effect of senna, and will continue to investigate the molecular mechanism of the aforementioned effects so as to lay the foundation for further research.

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