Oral colon-specific drug delivery system reduces the nephrotoxicity of rhubarb anthraquinones when they produce purgative efficacy

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Abstract. Rhubarb is commonly used to treat constipation in China and anthraquinones (AQs) are the active components present in rhubarb. However, an increasing number of studies have reported that AQs induce nephrotoxicity. In the present study, rhubarb total free anthraquinones (RTFA) oral colon-specific drug delivery granules (RTFA-OCDD-GN) were prepared to determine whether RTFA-OCDD-GN could reduce the nephrotoxicity that occurs when AQs produce purgative efficacy. RTFA-OCDD-GN were prepared using pH-enzyme double-layer coating technology and the cumulative release rate of RTFA in RTFA-OCDD-GN was assessed. The first black stool time, the number and state of feces over 8 h were observed to measure the purgative efficacy. In the nephrotoxicity test, biochemical and histopathological examinations were performed following 20 and 40 days administration, and 20 days convalescence. The cumulative release rate of RTFA in RTFA-OCDD-GN was >80% in simulated colonic fluid. RTFA-OCDD-GN produced considerable purgative efficacy compared with rhubarb medical material samples (RMMS). Following 40 days RMMS administration, blood urea nitrogen, creatinine and urine β_2-microglobulin levels in the high-dosage group were significantly increased compared with the control and RTFA-OCDD-GN groups ($P<0.05$). All specimens from the high-dosage RMMS group exhibited swelling/degeneration of renal proximal convoluted tubule epithelial cells. No difference in pathological conditions and biochemical indicators was detected between the RTFA-OCDD-GN groups and the control group. The nephrotoxicity of AQs was significantly reduced following RTFA-OCDD-GN administration, which produced considerable purgative efficacy compared with RMMS.

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Abbreviations: AQs, anthraquinones; OCDDS, oral colon-specific drug delivery system; RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules; RMMS, rhubarb medical material samples; RTFA, rhubarb total free anthraquinones

Key words: anthraquinones, double-layer coating granules, nephrotoxicity, oral colon-specific drug delivery system, purgative efficacy, rhubarb

Introduction

Rhubarb (*Radix et Rhizoma Rhei*; Dahuang in Chinese) is a commonly used traditional Chinese medicine that has been used for cathartic, febrifugal and antidotal purposes for a long time (1). It is most commonly used to produce purgative efficacy. Approximately 10% of traditional Chinese medicine preparations in Chinese Pharmacopoeia (2010 print part I) contain rhubarb. Moreover, >90% of rhubarb-containing preparations utilize its effects on purgative efficacy (2). Anthraquinones (AQs), including emodin, physcion, chrysophanol, aloe-emodin, rhein and their glycosides, and the structure of free AQs (Fig. 1), are the active ingredients of rhubarb and are responsible for its effects on purgative efficacy (3). As Fig. 2 shows, AQs exist both in free (aglycones) and combined (glycosides) forms in rhubarb. Combined AQs are formed by free AQs combining with sugar via β-glycosidic bonding and they are not hydrolyzed by α-glycosidic bond enzymes in the upper gastrointestinal tract (GIT) following oral administration. When delivered to the colon, combined AQs are hydrolyzed by β-glucosidases in the colon, thus releasing free AQs (1,4). Free AQs trigger purgative efficacy by stimulating the colon wall and promoting intestinal peristalsis, resulting in the inhibition of water absorption (1,5). In addition, a small proportion of combined AQs are absorbed by the small intestine and converted to free AQs by the liver. Free AQs then stimulate the colon plexus and inhibit Na^+-K^+-ATPase, thus stimulating purgative efficacy (6,7). Combined AQs can only produce purgative efficacy when taken orally. Free AQs are the ultimate substance of combined

AQs *in vivo* when they exert such purgative action. However, if free AQs are directly taken orally, the vast majority of them are absorbed or destroyed prior to reaching the colon, meaning that they have weak purgative efficacy (8).

At present, preparations containing rhubarb in Chinese Pharmacopoeia only achieve purgative efficacy if rhubarb is all or partly used in original powder. The reason is that rhubarb medicinal materials contain combined and free AQs. Combined AQs readily lose sugar to become free AQs and therefore lose their purgative efficacy during the process of decoction. This is also why the clinical doctors of traditional Chinese medicine require that the rhubarb should be decocted later (8). It is difficult to prepare the original powder using modern methods of preparation. Furthermore, the proportion of combined and free AQs found in rhubarb varies widely among rhubarb grown in different regions or in different batches of rhubarb from the same region, meaning that the purgative efficacy of rhubarb is variable. In view of the aforementioned problems, rhubarb total free anthraquinones (RTFA) containing >50% free AQs have been extracted and it has been demonstrated that they can stimulate purgative efficacy when administered using an oral colon-specific drug delivery system (OCDDS) (9).

At the same time, previous studies have reported that AQ compounds can increase the incidence of renal tubule hyaline droplets and pigmentation, cause renal tubular transparent droplet generation, renal mineralization and bladder cystatin cytoplasm degeneration, as well as induce apoptosis in human proximal tubular epithelial cell line HK-2 cells (10-18). Therefore, careful attention has been given to the safety of rhubarb and its preparations. Such concerns also affect the application of other traditional Chinese medicines containing AQs, including *Radix Polygoni Multiflori*, Aloe and Semen Cassia (19,20).

Therefore, according to the mechanism of purgative efficacy, if free AQs can be released in the colon by OCDDS, it may be possible to reduce the nephrotoxicity that occurs following the stimulation of purgative action. In the present study, rhubarb total free anthraquinones oral colon-specific drug delivery granules (RTFA-OCDD-GN) were prepared using pH-enzyme double controlled colon delivery technology. The vast majority of RTFA in these granules are released in the colon. The nephrotoxicity of RTFA-OCDD-GN and rhubarb medical material samples (RMMS) were also investigated. Rhubarb was extracted and combined AQs and free AQs were preserved as much as possible. The composition of the extract could thus reflect the nature of the original materials. The experimental results suggested that compared with administration of RMMS, the nephrotoxicity of AQs in Sprague Dawley (SD) rats was significantly reduced following administration of RTFA-OCDD-GN, which also stimulated considerable purgative efficacy. The present study provided useful information concerning the safety of long-term rhubarb use in the stimulation of purgative efficacy.

Materials and methods

Materials. Dried root and rhizoma of *Rheum officinale Baill*. Of the Polygonaceae family were purchased from the Anguo Qiao Chinese Herbal Sliced Medicine Co., Ltd. (Hebei, China) and identified by Professor Chunying Zhao, a botanist at Chengde Medical College (Hebei, China). Eudragit S100 was purchased from Shanghai Chineway Pharmaceutical Technology Co., Ltd. (Shanghai, China) and polyethylene glycol-6000 (PEG-6000) was purchased from the Suzhou Zhengxing Chemical Research Institute (Suzhou, China). Chitosan (viscosity of 100 cps) was purchased from the Tianjin Fuchen Chemical Factory (Tianjin, China). Hydroxypropyl methyl cellulose (HPMC), microcrystalline cellulose (MCC) and sodium carboxymethyl cellulose (CMC-Na) were all purchased from Huzhou Zhanwang Pharmaceutical Co., Ltd. (Huzhou, China). Sodium dodecyl sulfate (SDS) and Tween-80 were obtained from Jiaxing Hexin Chemical Industry Co., Ltd. (Tianjin, China). Methyl alcohol of chromatogram grade was purchased from Tianjin Association for Haopeng Chromatography Technology Co., Ltd. (Tianjin, China). Other chemicals and solvents including hydrochloric acid, perchloric acid, ether and chloral hydrate were of analytical grade and obtained from Tianjin Chemical Reagent Company (Tianjin, China). Double-distilled water was used throughout the present study.

Animals. A total of 280 male and female SD rats (age, 5-7 weeks; weight, 180-240 g; gender ration, 1:1) were obtained from Tianjin Shanchuanhong Laboratory Animal Science & Technology Co., Ltd. (Tianjin, China; License No. SCXK 2009-0001). Animals were given unlimited access to water and supplied with quantified standard pellet feed (50 g/kg/day) in an environmentally controlled breeding room with a temperature of 22±2˚C and humidity of 40-60%. The breeding room was illuminated by artificial light and rats experienced a 12-h light/dark cycle. Furthermore, the room was regularly disinfected. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The use of the rats was reviewed and approved by the Animal Care Committee of Chengde Medical College (Chengde, China).

RMMS preparation. Briefly, 3 kg rhubarb medicinal materials were extracted with 40% ethanol at 60˚C, three times. The ethanol was recovered and the extract was concentrated and spray-dried. Finally, 330 g extracted product was obtained and the content of total AQs (consisting of ~50% combined AQs and ~50% free AQs) in it was 9.8%, as determined by high performance liquid chromatography analysis using an Agilent 1260 Infinity liquid chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA), equipped with a quaternary solvent delivery system, an online degasser, an autosampler, a column temperature controller and a diode-array detection system that recorded UV spectra in the range of 190-400 nm. Chromatography was carried out on a Diamonsil C18 reversed phase column (250x4.6 mm; 5 μm). The mobile phase consisted of methanol-0.1% perchloric acid aqueous (85:15, v/v) at a flow rate of 1 ml/min. The detection wavelength was set at 254 nm. The final injection volume was 10 μl. The column oven temperature was maintained at 30˚C.

RTFA preparation. Briefly, 3 kg rhubarb medicinal materials were extracted three times using 30% ethanol three times using a previously described method (9). As a result, 28.2 g extracted product was obtained and the content of the total free AQs in

it was 54.5%. The extract product was then used to prepare RTFA-OCDD-GN.

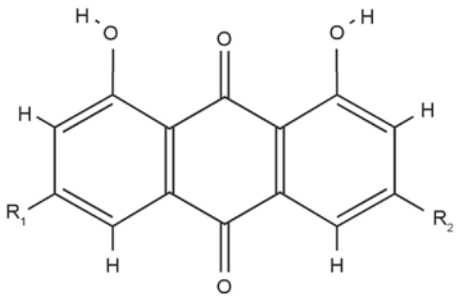
RTFA-OCDD-GN preparation. Sieved RTFA weighing 28 g was mixed with 112 g MCC and 16.8 g PEG-6000. Then, 84 ml aqueous solvent containing 2% (*w*/*v*) HPMC and 2% (*w*/*v*) PEG-6000 was added as an adhesive, and the mixture was granulated using a high-speed stirring machine (Tianjin City Taisite Instrument Co., Ltd., Tianjin, China). The granules were oven-dried at 50˚C and screened through a 50-mesh sieve.

Chitosan was dissolved in 1% HCl aqueous solution (21) at a concentration of 1.25% (*w*/*v*) and stirred overnight with a magnetic stirrer (Zhengzhou Greatwall Scientific Industrial and Trade Co., Ltd, Zhengzhou, China) prior to coating. Glycerin, corresponding to 20% weight of the dry polymer, was used as the plasticizer in the coating formula. The chitosan solution was blended with plasticizer for 1 h. Every 100 g, granules were coated with 420 ml chitosan coating solution in a fluid bed coating apparatus equipped with a bottom sprayer (Chongqing RongKai Machinery Manufacturing Co., Ltd, Chongqing, China). Sub-coated granules were further fluidized for 20 min and oven-dried at 40˚C for 2 h.

Eudragit S100 was dissolved in 95% ethanol aqueous solution. Diethyl phthalate, corresponding to 20% weight of the dry polymer, was used as the plasticizer. Subsequently 3% (*w*/*v*) talcum powder was added as the anti-sticking agent. In order to sufficiently plasticize the polymer and obtain the homogeneous solution, the mixture was then stirred for 24 h. Every 100 g, chitosan-coated granules were coated with 340 ml polymeric solution. Double-layer coated granules were further fluidized for 20 min, oven-dried at 40˚C for 2 h and sieved using a 40-60 mesh. Finally, 300 g RTFA-OCDD-GN was obtained and the content of total free AQs in it was determined to be 8.49% (Fig. 3).

RMMS and RTFA-OCDD-GN were matched into a corresponding concentration mixed suspension with 0.5% CMC-Na and geometrically diluted to the desired concentration of solution according to the dosage regimen (RMMS, 0.66, 0.33 or 0.165 g/kg; RTFA-OCDD-GN, 0.36, 0.18 or 0.09 g/kg). Test drugs were freshly prepared every week and stored at 4˚C prior to further analysis (22). The rats in the control group were given a corresponding volume of 0.5% CMC-Na.

In vitro release test of RTFA-OCDD-GN. An *in vitro* release study of RTFA-OCDD-GN was performed and repeated three times using an RC806 dissolution tester (Tianda Tianfa Science & Technology Co., Ltd., Tianjin, China) using the method in Chinese Pharmacopoeia (2010 print part II) (23). A release test was performed in three different media containing SDS (0.4%, *w*/*v*) for different durations as follows: Medium A for 2 h (0-2 h), 0.1M HCl aqueous solution (pH 1.2); medium B for 4 h (2-6 h), phosphate-buffered saline (PBS, pH 6.8); medium C for 18 h (6-24 h), PBS containing rat cecal contents (pH 7.4) (9,24). Fig. 4 shows the release curve of RTFA in three batches of RTFA-OCDD-GN in these three media. The cumulative release rate of RTFA in RTFA-OCDD-GN was >80% in medium C, while it was only ~6.8% in medium B. RTFA was not released in medium A.



	R_1	R_2
Chrysophanol:	H	CH_3
Aloe-emodin:	H	CH_2OH
Rhein:	H	COOH
Emodin:	OH	CH_3
Physcin:	OCH_3	CH_3

Figure 1. Structure of free anthraquinones.

Purgative efficacy test. 70 rats were randomly and evenly divided into seven groups (each, n=10), including a control group. The groups presented in Table I were differentiated based on the type of drug and dosage. The rats in each group were orally administered drugs once. Each group was administered a mixed suspension with 2% activated carbon and with a volume of 0.5 ml/100 g.

Rats in all groups fasted for 12 h and were individually placed in metabolic cages (with filter paper spread beneath) following their respective treatments. The first black stool time, the number and state of feces in 8 h were observed. According to the state of the feces, stools were divided into five types: Normal, soft stools, loose stools, semi-liquid stools and watery stools. The non-normal stools were considered to be positive reaction of purgation.

The rats were performed with formal management until the drug *in vivo* metabolized completely. And then they were repeated used for other experiments.

Experimental animals and administration. A total of 210 rats were randomized into seven groups (all n=30) according to the results of the efficacy test. The administration groups received appropriate drugs and the control group received physiological saline. The rats were perfused with a 0.5 ml/100 g of the previously described RMMS and RTFA-OCDD-GN solutions once a day for 40 days. Animals were weighed once a week and drug dosage was adjusted based on body weight changes. In each group, one third of the rats were sacrificed via exsanguination following anesthesia with chloral hydrate (350 mg/kg) after 20 days of administration (n=10), one third after 40 days of administration (n=10) and one third after 20 days of convalescence (n=10) (25).

Observation of toxicity signs. Body weight, general behavior, urine biochemistry and routine urine [urine β2-microglobulin (β2-MG), urobilinogen (URO), urine bilirubin (BIL), leucine (LEU), ketobodies (KET), protein (PRO), glucose (GLU),

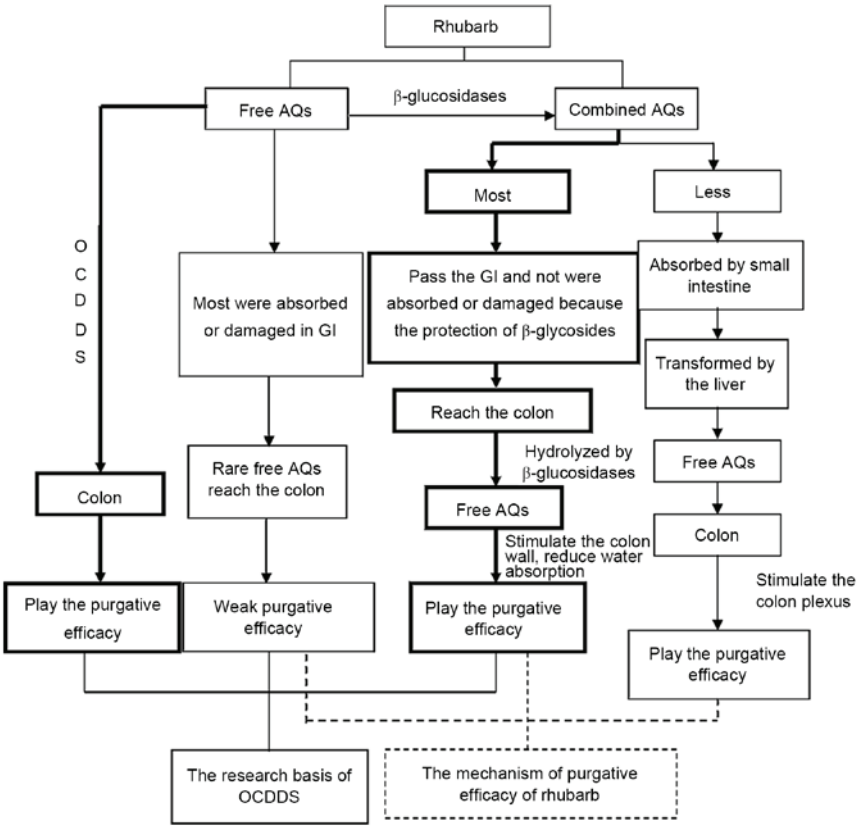


Figure 2. Mechanism of the purgative efficacy of rhubarb and the research basis of OCDDS. AQ, anthraquinones; OCDDS, oral colon-specific drug delivery systems.

nitrite (NIT), urine occult blood (BLD)] and blood biochemistry [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea nitrogen (BUN) and creatinine (CREA)] were measured using an automatic biochemical analyzer (Type 7180, Hitachi, Ltd., Tokyo, Japan) after 20 days administration, a special protein instrument (IMMAGE® 800; Beckman Coulter, Inc., Brea, CA, USA) after 40 days administration and a urine analyzer (H-500; DIRUI Industrial Co., Ltd., Changchun, China), after 20 days of convalescence. Rats from each group were individually housed in metabolic cages for 24 h and their urine was collected. Animals were subsequently anesthetized with chloral hydrate (350 mg/kg; intraperitoneal injection) and blood samples were collected. Rats were sacrificed via exsanguination for necropsy examination. Internal organs including heart, liver, spleen, lung, kidney, adrenal gland, uterus, ovary and testes were dissected and weighed and gross pathological observations were performed by histopathological evaluation (26).

Histopathological evaluation. Organs were fixed in 10% neutral formalin at room temperature for at least 24 h (27). Fixed organs were dehydrated in 70% alcohol, embedded in paraffin, cut into sections 4-5 μm thick and stained with hematoxylin & eosin. The histological sections were evaluated under light microscopy (Nikon Eclipse CI; Nikon Corporation, Tokyo, Japan) for pathological changes of nephrotoxicity.

Statistical analysis. Experimental data were processed using the statistical software SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean ± standard deviation. Significant differences between groups were analyzed by one-way analysis of variance and Wilcoxon and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Purgative efficacy test. The first black stool time of the groups receiving the drug was earlier than that of the control group ($P<0.05$). There were no significant differences between the RTFA-OCDD-GN and RMMS groups at equal dosage (Table II). Rats treated with high-dosage RMMS primarily defecated semi-liquid and watery stools. The rats treated with high-dosage RTFA-OCDD-GN primarily defecated loose and soft stools. The rats treated with middle-dosage RMMS and RTFA-OCDD-GN primarily defecated loose and soft stools. The rats treated with low-dosage RMMS and RTFA-OCDD-GN primarily defecated soft and normal stools. The control group primarily defecated normal stools. The number of total feces excreted in the RMMS and RTFA-OCDD-GN groups was increased compared with the control group ($P<0.05$), however, no difference between the RMMS and RTFA-OCDD-GN groups was detected (Table III).

According to the purgative efficacy test, the efficacy of the RMMS groups was comparable with the RTFA-OCDD-GN groups administered the same dosages. Therefore, the dosages of drugs given in the nephrotoxicity test were the same as those administered in the purgative efficacy test.

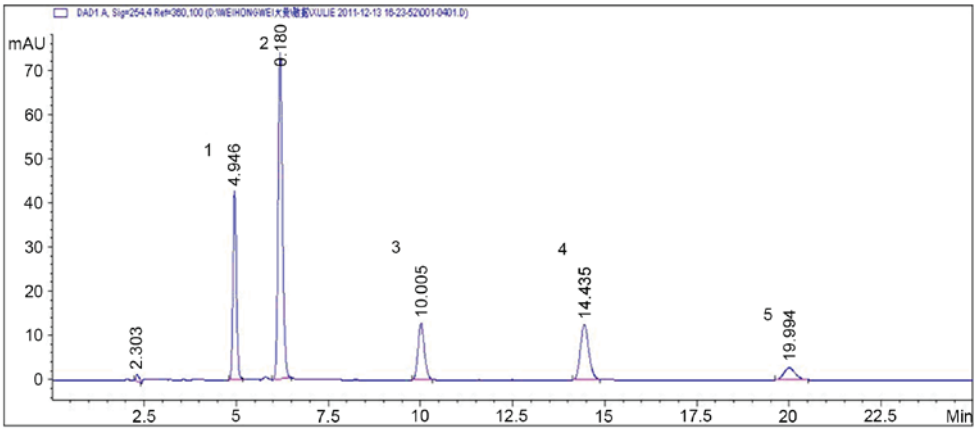


Figure 3. RTFA-OCDD-GN were analyzed by high performance liquid chromatography. The chromatogram of free anthraquinones in RTFA-OCDD-GN (1, aloe-emodin; 2, rhein; 3, emodin; 4, chrysophanol; 5, physcion) is presented here. RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules.

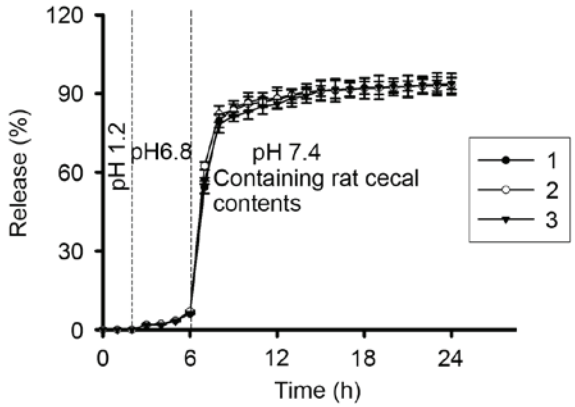


Figure 4. Release curve of RTFA in three batches of RTFA-OCDD-GN in the three media. The cumulative release rate of RTFA in RTFA-OCDD-GN was >80% in the simulated colonic fluid containing rat cecal contents, while it was only ~6.8% in the small-intestinal fluids. RTFA did not release in the simulated gastric fluids. RTFA, rhubarb total free anthraquinones; RTFA-OCDD-GN, rhubarb free anthraquinone oral specific drug delivery granules; 1, first experiment; 2, second experiment; 3, third experiment.

General behavior evaluation of RMMS group. Rats in the high-dosage group defecated semi-liquid and loose stools. Stools were reddish-brown and urine was dark red. Coarse, haggard hair was exhibited by rats following 20 days of drug administration and reddish pigmentation was observed on the back, abdomen, hind leg and testicle. Furthermore, emaciation and inactivity were observed in all rats following 40 days drug administration. These symptoms indicate adverse effects on the survival status of rats (28). These symptoms resumed to normal or improved following convalescence. Similar but less severe symptoms were observed in rats in the middle-dosage group. By contrast, rats in the low-dosage group generally defecated normally, although some soft stools were observed. The fecal quantity of this group was greater than that of the control group.

General behavior evaluation of the RTFA-OCDD-GN groups. There were no differences in terms of general behavior between the treatment groups and control group. The weight gain observed in the high- and middle-dosage groups was lower than that of the control group, while it was greater than that of RMMS groups at the same dosages.

Effect on blood biochemical indicators. Following 20 days RMMS administration, BUN, CREA (Fig. 5) ALT and AST levels (Fig. 6) in the high-, middle- and low-dosage groups were all increased compared with the control and RTFA-OCDD-GN groups, however these differences were not significant. ALP levels in the high- and middle- dosage groups were increased compared with the control group, however this difference was not significant. Following RMMS administration for 40 days, BUN and CREA levels in the high-dosage group, CREA levels in the middle-dosage group and ALT levels in the low-dosage group were increased compared with the control and RTFA-OCDD-GN groups respectively (all $P<0.05$). Following convalescence all returned to normal levels.

No significant differences were observed among BUN, CREA, ALT and AST levels in different RTFA-OCDD-GN groups at different stages compared with the control group (Figs. 5 and 6).

Effect on urine biochemical indicators. Following 20 days RMMS administration, urine β_2-MG levels in the high-dosage RMMS group were increased compared with the control and RTFA-OCDD-GN groups ($P<0.05$). Furthermore, following 40 days RMMS administration, urine β_2-MG levels in the high- and middle-dosage groups were increased compared with the control and RTFA-OCDD-GN groups ($P<0.05$; Fig. 7). In both groups, levels of urine β_2-MG returned to normal following convalescence.

No significant differences in urine β_2-MG levels were observed among any RTFA-OCDD-GN groups at different stages compared with the control group (Fig. 7).

Effect on urine routine indicators. As presented in Table IV following 20 days RMMS administration, BIL and LEU levels in the high-, middle- and low-dosage groups, KET and NIT levels in the high- and middle-dosage groups were increased compared with the control group ($P<0.05$). There were no significant change in URO, BLD, PRO and GLU levels in all groups compared with the control group. Following 40 days

Table I. The groups of purgative efficacy test based on drug and dosage.

Group	Dosage (g/kg)	The quantity equivalent to the original medicinal materials (g/kg)	The content of total AQs (mg/kg)	The content of combined AQs (mg/kg)	The content of free AQs (mg/kg)
RTFA-OCDD-GN	0.36	6	32.18	0	32.18
	0.18	3	16.09	0	16.09
	0.09	1.5	8.05	0	8.05
RMMS	0.66	6	64.68	32.38	32.30
	0.33	3	32.34	16.22	16.12
	0.165	1.5	16.17	8.11	8.06
Control	-	-	-	-	-

n=10 in all groups. AQ, anthraquinones; RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules; RMMS, rhubarb medical material samples.

Table II. The first black stool time in all groups.

Group (contain 2% activated carbon)	Dosage (g/kg)	The first black stool time (min)
RTFA-OCDD-GN	0.36	263.3±5.7[a]
	0.18	291.1±9.3[a]
	0.09	303.6±11.2[a]
RMMS	0.66	257.9±4.7[a]
	0.33	289.0±11.7[a]
	0.165	310.1±13.3[a]
Control	-	349.9±10.3

[a]P<0.05, compared with control group; n=10 for all groups. RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules; RMMS, rhubarb medical material samples.

RMMS administration, BIL, KET, PRO, LEU, GLU and NIT in the high-, middle- and low-dosage groups, URO in the low-dosage group were increased compared with the control group (P<0.05). No significant change in BLD levels in all groups were found compared with the control group. Following the convalescence period, only the increase in PRO and NIT levels in the high-dosage group was still greater than that of the control group (P<0.05), but the difference was smaller than that observed at 40 days. The increase in the levels of all the indicators except for NIT in the middle-dosage group was not significantly greater than that of the control group (P>0.05). The increase of URO, BIL, LEU and NIT levels in the low-dosage group were still greater than that of the control group (P<0.05), however the degree was decreased compared with that at 40 days. Other indicators in the low-dosage group were similar to the control group.

Following 20 days of RTFA-OCDD-GN administration, BIL, LEU and NIT levels in the high-, middle- and low-dosage groups, as well as the KET level of the high- and middle-dosage groups were increased compared with the control group (P<0.05). There were no significant changes in URO, BLD, PRO and GLU levels in all groups compared with the control group. Following 40 days RTFA-OCDD-GN administration, levels of BIL, KET, LEU and NIT in all groups, PRO and GLU in the high- and middle-dosage group and URO in the low-dosage group were increased compared with the control group (P<0.05). No significant changes of other indicators were observed compared with the control group. Following convalescence, PRO, KET and NIT of the high-dosage group, NIT of the middle-dosage group, URO, BIL and LEU of the low-dosage group were still greater than that of the control group (P<0.05), but the difference was lower compared with that at 40 days (Table IV). All other indicators were at similar levels compared with the control group.

Following 20 days convalescence, certain urine routine indicators of the RMMS and RTFA-OCDD-GN groups did not return to normal levels. This suggests that these rats may suffer from inflammation (Table IV).

System autopsy and histopathological evaluation. No macroscopic pathological changes were observed in the animals in all RMMS groups. Following 40 days RMMS administration, the increase of organ coefficient (organ weight/body weight) in kidney, testicle and adrenal of the high-dosage RMMS group was greater than that observed in the control group (P<0.05). Following the convalescence period, all values returned to normal levels.

Table V and Fig. 8 present the results of the histopathological evaluation. Histological examination indicated that following 20 days of RMMS administration at high-dosage, one (1/10) specimen exhibited swelling and degeneration of RPCTECs, causing narrowing of the lumen. Following 40 days RMMS administration at high-dosage, all 10 specimens showed swelling/degeneration of RPCTECs to different extents, causing the lumen to narrow (marked as '+'), as well as epithelial cell shedding (marked as '++'). In seven cases, '+' was observed and '++' was found in three cases. Four specimens of the middle-dosage RMMS group exhibited the aforementioned pathological changes and the degree of pathological changes occurring in these cases was '+'. No pathological changes were observed in the low-dosage RMMS group. Other tested organs did not exhibit marked

Table III. The number and state of feces over 8 h in all groups.

Group	Dosage (g/kg)	The frequency of different types of feces observed					
		Normal stools	Soft stools	Loose stools	Semi-liquid stools	Watery stools	Total
RTFA-OCDD-GN	0.36	0.6±0.52	7.2±0.64[a]	6.1±0.31[a]	1.4±0.34	0	15.3±1.12[b]
	0.18	1.8±0.42	10.4±0.87[a]	3.0±0.14	0	0	15.2±1.46[b]
	0.09	8.2±0.23	5.2±0.09[a]	0.8±0.42	0	0	14.2±1.35[b]
RMMS	0.66	0.4±0.52	0.3±0.48	2.3±0.16	7.8±0.22[c]	5.8±0.14[c]	16.6±2.31[b]
	0.33	2.8±0.29	3.6±0.24	4.6±0.73	3.1±0.13[c]	1.2±0.15[c]	15.3±1.60[b]
	0.165	8.8±0.97	4.1±0.35	0.7±0.48	0	0	13.6±1.91[b]
Control	-	8.9±1.01	0.2±0.42	0	0	0	9.1±0.58

[a]P<0.05, compared with RMMS group; [b]P<0.05, compared with control group; [c]P<0.05, compared with RTFA-OCDD-GN group. n=10 for all groups. RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules; RMMS, rhubarb medical material samples.

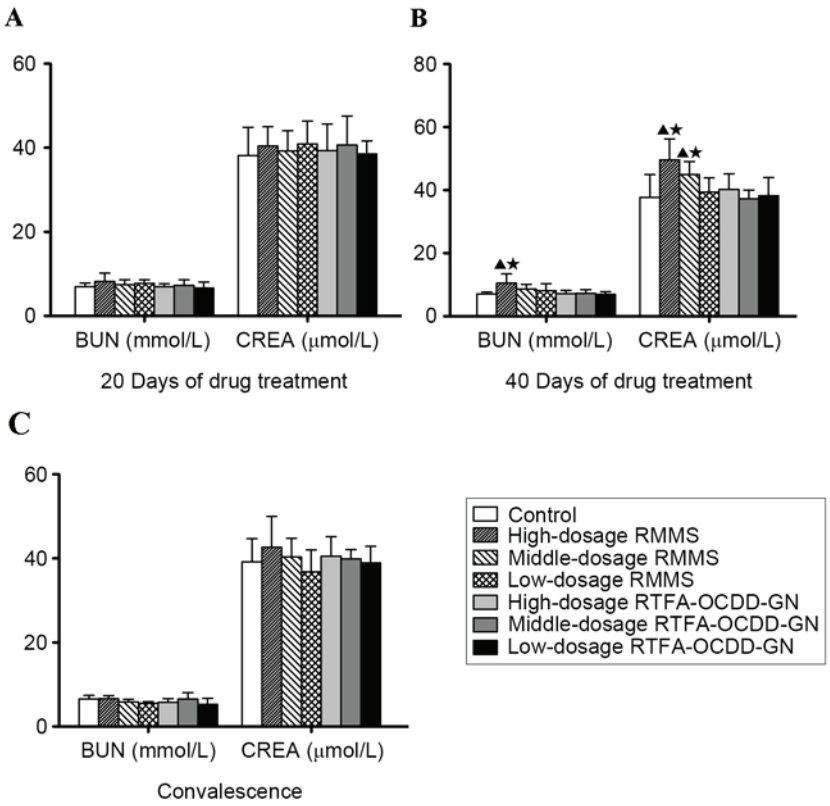


Figure 5. Effect on blood biochemical indicators. Following RMMS administration for: (A) 20 days, no significant differences in BUN and CREA levels were detected between experimental and control groups; (B) 40 days, BUN and CREA levels of the high-dosage group, CREA levels of the middle-dosage and low-dosage groups were increased compared with the control and RTFA-OCDD-GN groups (P<0.05), respectively. (C) The two indices returned to normal levels following convalescence. ▲P<0.05, compared with control group; ★P<0.05, compared with RTFA-OCDD-GN group. BUN, blood urea nitrogen; CREA, creatinine; RMMS, rhubarb medical material samples; RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules.

pathological changes compared with the control group. Following convalescence, visible pathological changes could still be observed in one specimen of the high-dosage RMMS group (marked as '+').

Following RTFA-OCDD-GN administration, no macroscopic pathological, organ coefficient and pathological changes were observed in any of the groups.

Discussion

If the stool is not smooth, the waste and toxins produced by digestion and metabolism of gastrointestinal food may cause poisoning. Moreover, they can induce gallstones, hemorrhoids, colon cancer and the onset of other diseases. Rhubarb can maintain the smooth stool, therefore, cholesterol, creatine and other

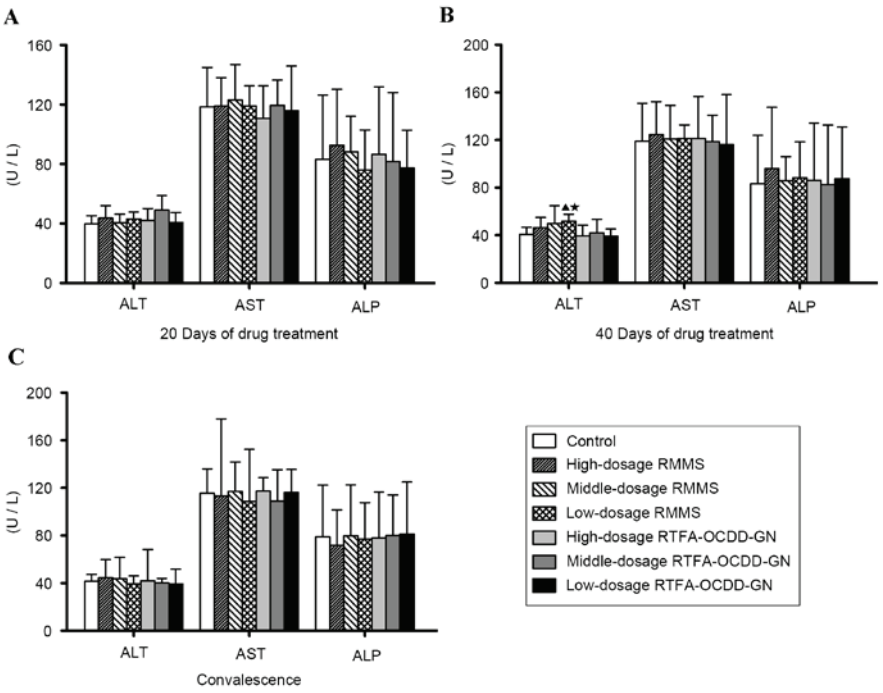


Figure 6. Effect on blood biochemical indicators. Following RMMS administration for (A) 20 days, there were no significant differences between the experimental and control groups; (B) 40 days, the ALT level of the low-dosage group was increased compared with control and RTFA-OCDD-GN groups (P<0.05). (C) ALT returned to normal after convalescence. ▲P<0.05, compared with control group; ★P<0.05, compared with RTFA-OCDD-GN group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; RMMS, rhubarb medical material samples; RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules.

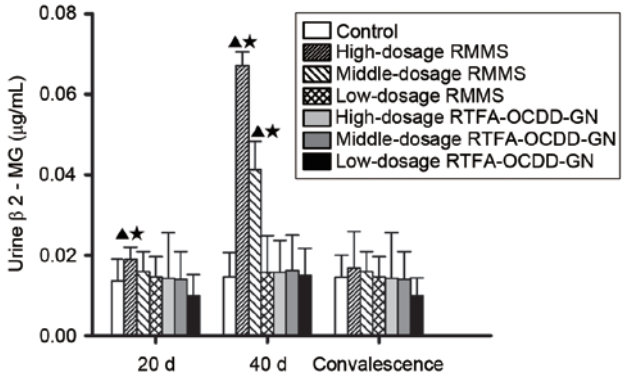


Figure 7. Effect on β_2-MG following drug administration for 20 and 40 days, and 20 days of convalescence. Following 20 days RMMS administration, urine β_2-MG levels of the high-dosage RMMS group were increased compared with the control and RTFA-OCDD-GN groups (P<0.05). Following 40 days RMMS administration, urine β_2-MG levels of the high- and middle-dosage group were increased compared with the control and RTFA-OCDD-GN groups (P<0.05), respectively. Both of them returned to normal levels after the convalescence. ▲P<0.05, compared with control group; ★P<0.05, compared with RTFA-OCDD-GN group. β_2-MG, urine β2-microglobulin; RMMS, rhubarb medical material samples; RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules.

harmful substances in the blood can be quickly eliminated and the blood becomes clean, which aids the recovery of patients. Modern medicine has recognized that rhubarb can promote gastrointestinal peristalsis by stimulating purgative efficacy. This can eliminate dry knot and endotoxin in the intestinal tract, improve blood circulation and reduce intracranial pressure and cerebral edema. Therefore, rhubarb may serve an important role in the treatment of acute cerebrovascular disease and its complications (29). Rhubarb can eliminate intestinal paralysis and reduce stasis by promoting intestinal peristalsis, which may maintain the unobstructed drainage of bile and pancreatic juice, as well as controlling inflammation of the biliary tract more effectively. This eliminates gallstone pancreatitis from the source. An effective curative effect has been observed in patients with severe acute pancreatitis following the clinical application of rhubarb (30). The aforementioned reports prove that rhubarb can also be used to treat other diseases due to its purgative efficacy.

In a previous study, RTFA containing >50% free AQs was extracted in the secondary development of '*San-huang* Tablets'. However, RTFA could not induce purgative efficacy at the prescription dosage. Under this condition, RTFA granules with pH-sensitive materials were prepared as an adhesive, which is able to alter RTFA release in the GIT. RTFA in these granules could be partly released in the colon, therefore the granules were used to prepare '*San-huang* dispersible Tablets', which had the same features as OCDDS. A purgative efficacy test proved that '*San-huang* dispersible Tablets' exerted a stronger cathartic effect compared with '*San-huang* Tablets' (9).

In the current study, film-coating technology was used to prepare RTFA-OCDD-GN based on the pH sensitive-enzyme triggered principle (31). Double layer granules were prepared using chitosan as inner layer and Eudragit as an outer enteric layer. As a type of alkaline polysaccharide, chitosan can be only dissolved in acidic solution and biodegraded by special bacteria. The granules can pass through the stomach under the protection of the outer enteric layer and are not released due to the presence of chitosan in the small intestine. In the colon, chitosan is

Table IV. The effect of urine routine at different stages (n=10).

A, 20 days

Biochemical indicator	RTFA-OCDD-GN (g/kg)			RMMS (g/kg)			Control	Result
	0.36	0.18	0.09	0.66	0.33	0.165		
URO	10	10	10	10	10	10	10	-
URO	0	0	0	0	0	0	0	+
URO	0	0	0	0	0	0	0	++
BIL	6[a]	7[a]	8[a]	6[a]	5[a]	7[a]	10	-
BIL	2[a]	2[a]	2[a]	1[a]	3[a]	2[a]	0	+
BIL	2[a]	1[a]	0[a]	3[a]	2[a]	1[a]	0	++
BIL	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0	+++
KET	6[a]	7[a]	9	6[a]	6[a]	9	9	-
KET	2[a]	2[a]	1	1[a]	2[a]	1	1	+-
KET	2[a]	1[a]	0	3[a]	2[a]	0	0	+
KET	0[a]	0[a]	0	0[a]	0[a]	0	0	++
BLD	9	9	9	9	9	9	9	-
BLD	1	1	0	0	1	0	0	+-
BLD	0	0	1	0	0	1	1	+
BLD	0	0	0	1	0	0	0	++
PRO	9	9	10	9	9	10	9	-
PRO	1	0	0	1	0	0	1	+-
PRO	0	1	0	0	0	0	0	+
PRO	0	0	0	0	1	0	0	++
PRO	0	0	0	0	0	0	0	+++
LEU	5[a]	4[a]	3[a]	3[a]	2[a]	3[a]	10	-
LEU	2[a]	1[a]	0[a]	1[a]	0[a]	0[a]	0	+-
LEU	2[a]	3[a]	7[a]	2[a]	4[a]	7[a]	0	+
LEU	1[a]	2[a]	0[a]	4[a]	3[a]	0[a]	0	++
LEU	0[a]	0[a]	0[a]	0[a]	1[a]	0[a]	0	+++
GLU	10	10	10	10	10	10	10	-
GLU	0	0	0	0	0	0	0	+
GLU	0	0	0	0	0	0	0	++
NIT	5[a]	7[a]	8[a]	5[a]	6[a]	10	10	-
NIT	5[a]	3[a]	2[a]	5[a]	4[a]	0	0	+

B, 40 days

Biochemical indicator	RTFA-OCDD-GN (g/kg)			RMMS (g/kg)			Control	Result
	0.36	0.18	0.09	0.66	0.33	0.165		
URO	10	10	6[a]	10	10	5[a]	10	-
URO	0	0	4[a]	0	0	5[a]	0	+
URO	0	0	0[a]	0	0	0[a]	0	++
BIL	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	10	-
BIL	8[a]	7[a]	2[a]	6[a]	7[a]	2[a]	0	+
BIL	1[a]	3[a]	8[a]	2[a]	2[a]	8[a]	0	++
BIL	1[a]	0[a]	0[a]	2[a]	1[a]	0[a]	0	+++
KET	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	9	-
KET	0[a]	4[a]	7[a]	0[a]	3[a]	7[a]	1	+-
KET	8[a]	5[a]	3[a]	6[a]	6[a]	3[a]	0	+
KET	2[a]	1[a]	0[a]	4[a]	1[a]	0[a]	0	++
BLD	9	10	10	9	10	10	9	-

Table IV. Continued.

Biochemical indicator	RTFA-OCDD-GN (g/kg)			RMMS (g/kg)			Control	Result
	0.36	0.18	0.09	0.66	0.33	0.165		
BLD	0	0	0	0	0	0	0	+-
BLD	1	0	0	1	0	0	1	+
BLD	0	0	0	0	0	0	0	++
PRO	0[a]	3[a]	10	0[a]	2[a]	8[a]	10	-
PRO	4[a]	5[a]	0	3[a]	5[a]	1[a]	0	+-
PRO	2[a]	1[a]	0	2[a]	2[a]	1[a]	0	+
PRO	1[a]	1[a]	0	2[a]	1[a]	0[a]	0	++
PRO	3[a]	0[a]	0	3[a]	0[a]	0[a]	0	+++
LEU	0[a]	0[a]	4[a]	0[a]	0[a]	0[a]	10	-
LEU	0[a]	0[a]	0[a]	0[a]	0[a]	1[a]	0	+-
LEU	0[a]	3[a]	1[a]	0[a]	2[a]	1[a]	0	+
LEU	2[a]	4[a]	5[a]	4[a]	4[a]	5[a]	0	++
LEU	8[a]	3[a]	0[a]	6[a]	4[a]	3[a]	0	+++
GLU	7[a]	9[a]	10	6[a]	8[a]	7[a]	10	-
GLU	3[a]	1[a]	0	4[a]	2[a]	2[a]	0	+
GLU	0[a]	0[a]	0	0[a]	0[a]	1[a]	0	++
NIT	3[a]	4[a]	7[a]	3[a]	2[a]	1[a]	10	-
NIT	7[a]	6[a]	3[a]	7[a]	8[a]	9[a]	0	+

C, Convalescence

Biochemical indicator	RTFA-OCDD-GN (g/kg)			RMMS (g/kg)			Control	Result
	0.36	0.18	0.09	0.66	0.33	0.165		
URO	10	10	7[a]	10	10	6[a]	10	-
URO	0	0	3[a]	0	0	4[a]	0	+
URO	0	0	0[a]	0	0	0[a]	0	++
BIL	10	10	6[a]	10	10	4[a]	10	-
BIL	0	0	3[a]	0	0	3[a]	0	+
BIL	0	0	1[a]	0	0	3[a]	0	++
BIL	0	0	0[a]	0	0	0[a]	0	+++
KET	8[a]	9	10	8	9	9	9	-
KET	2[a]	0	0	2	0	1	1	+-
KET	0[a]	1	0	0	1	0	0	+
KET	0[a]	0	0	0	0	0	0	++
BLD	10	10	9	10	10	9	9	-
BLD	0	0	0	0	0	0	1	+-
BLD	0	0	1	0	0	1	0	+
BLD	0	0	0	0	0	0	0	++
PRO	7[a]	9	9	6[a]	9	9	9	-
PRO	2[a]	1	1	3[a]	1	1	1	+-
PRO	1[a]	0	0	1[a]	0	0	0	+
PRO	0[a]	0	0	0[a]	0	0	0	++
PRO	0[a]	0	0	0[a]	0	0	0	+++
LEU	9	10	6[a]	9	10	5[a]	9	-
LEU	0	0	4[a]	0	0	5[a]	1	+ -
LEU	1	0	0[a]	1	0	0[a]	0	+
LEU	0	0	0[a]	0	0	0[a]	0	++
LEU	0	0	0[a]	0	0	0[a]	0	+++
GLU	10	10	10	10	10	10	10	-

Table IV. Continued.

Biochemical indicator	RTFA-OCDD-GN (g/kg)			RMMS (g/kg)			Control	Result
	0.36	0.18	0.09	0.66	0.33	0.165		
GLU	0	0	0	0	0	0	0	+
GLU	0	0	0	0	0	0	0	++
NIT	7[a]	8[a]	9	6[a]	6[a]	8[a]	9	-
NIT	3[a]	2[a]	1	4[a]	4[a]	2[a]	1	+

[a]P<0.05 vs. control. RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules; RMMS, rhubarb medical material samples; URO, urobilinogen; BIL, urine bilirubin; KET, ketobodies; BLD, urine occult blood; PRO, protein; LEU, leucine; GLU, glucose; NIT, nitrite; -, negative; +, positive; +-, +, ++ and +++, increasing positive degree.

Table V. Results of histological examinations.

Group	Dosage, g/kg	20 days		40 days		Convalescence	
		+	++	+	++	+	++
RTFA-OCDD-GN	0.36	0	0	0	0	0	0
	0.18	0	0	0	0	0	0
	0.09	0	0	0	0	0	0
RMMS	0.66	1[a,b]	0	7[a,b]	3[a,b]	1[a,b]	0
	0.33	0	0	4[a,b]	0	0	0
	0.165	0	0	0	0	0	0
Control	-	0	0	0	0	0	0

[a]P<0.05, compared with control group; [b]P<0.05, compared with RTFA-OCDD-GN group. n=10 for all groups. RTFA-OCDD-GN, rhubarb free anthraquinone oral colon-specific drug delivery granules; RMMS, rhubarb medical material samples; +, swelling/degeneration of RPCTECs at different extents, causing the lumen to narrow; ++, epithelial cell shedding.

metabolized by bacteria and RTFA is released from the granules to stimulate purgative efficacy. Therefore, the release rate of RTFA is associated with the number of special bacteria in the colon, but is not under the influence of physiological factors, such as gastrointestinal movement and food (32). Bacteroides metabolizing chitosan are present in the colon and their number may be as large as 10-100 times of *Escherichia coli*, which is favorable in the metabolism of chitosan, present in the colon (33,34). Therefore in the current study, chitosan, which was used as inner coating film, had no influence on the release rate and the purgative efficacy of RTFA. However, rhubarb combined AQs are metabolized to free AQs by bifidobacterium in the colon (35). As a type of beneficial bacteria, bifidobacterium is important for humans, however its presence in the human colon gradually decreases with age and over the course of some diseases. A change in the number of *Bacillus bifidus* distributed in the colon may affect the purgative efficacy of orally administrated rhubarb. The fact that RTFA-OCDD-GN stimulated purgative efficacy with the same efficiency as rhubarb in the current study indicates that the purgative efficacy produced by RTFA-OCDD-GN may be more stable for such individuals than that stimulated by rhubarb.

A number of studies have documented the nephrotoxicity of emodin and other AQ compounds (10-18). Emodin may increase the incidence of renal tubule hyaline droplets and pigmentation (15). The National Toxicology Program of the USA reported that oral administration of emodin for >14 weeks causes renal tubular transparent droplet generation, renal mineralization and bladder cystatin cytoplasm degeneration, and has mutagenic and carcinogenic effects *in vitro* (16). Additionally, it has been demonstrated that emodin and rhein can induce apoptosis in human proximal tubular epithelial cell line HK-2 cells (17). Furthermore, administration of total rhubarb AQs for 13 weeks induces nephrotoxicity in SD rats: Tissue slice examination demonstrated that renal tubule epithelial cells swelled and denatured (18). In the current study, marked nephrotoxicity was observed following the treatment of rats with RMMS for 40 days. However, no nephrotoxicity, apart from some abnormal urine routine indicators, was observed in any of the groups receiving RTA-OCDD-GN. At equal original medicinal dosage and considerable purgative efficacy, the content of total AQs (only free AQs) in RTA-OCDD-GN was nearly half of that (combined and free AQs) in RMMS, whereas the nephrotoxicity of RMMS was significantly greater than RTA-OCDD-GN. Oral colon drug delivery technology may significantly reduce the nephrotoxicity of rhubarb AQs compared with RMMS. It solves the nephrotoxicity problem by using pharmaceutical technology.

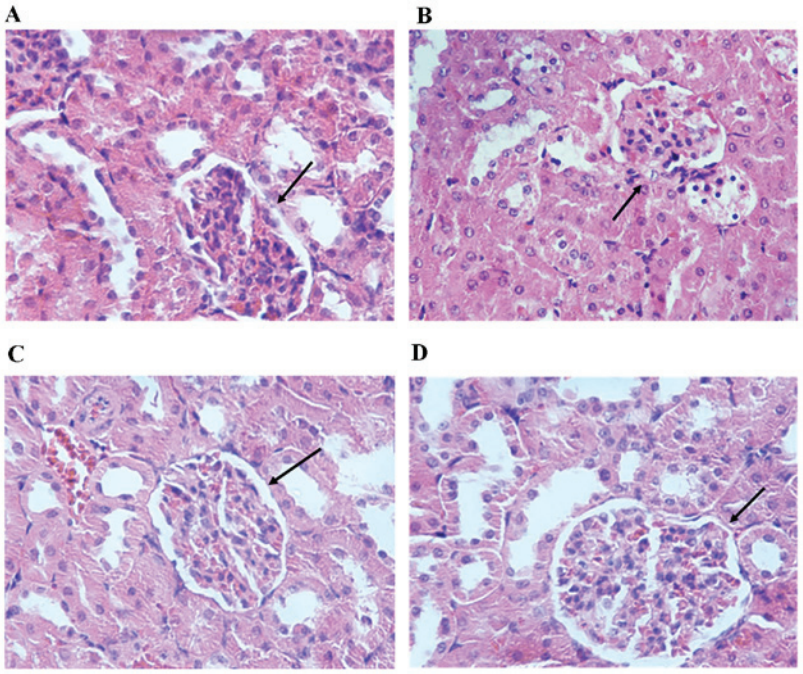


Figure 8. Results of histological examination. (A) Control group, (B) 40 days of administration of high-dosage RMMS, (C) 40 days of administration of high-dosage RTFA-OCDD-GN and (D) convalescence. As the arrows indicate, following 40 days of rhubarb medical material sample administration, the high-dosage group exhibited swelling/degeneration of the renal proximal tubule epithelial cells, causing lumen narrowing, as well as epithelial cell shedding. No pathological changes were observed in the other three groups. Magnification, x400. Hematoxylin and eosin staining.

According to the mechanism of purgative efficacy, after RMMS is taked orally, a proportion of combined AQs may cause nephrotoxicity when they are absorbed. At the same time, free AQs may also cause nephrotoxicity due to their absorption. In a previous study performed by the authors of the current study, rat stomachs and the intestinal absorption of RTFA were studied *in situ*. The results suggested that free AQs are primarily absorbed in the GIT and rarely absorbed in the colon (36). In the current study, RTFA was prepared to RTFA-OCDD-GN. In PBS containing rat cecal contents (pH 7.4), >80% of RTFA in RTFA-OCDD-GN was released. RTFA-OCDD-GN may stimulate considerable purgative efficacy and it was indicated that the nephrotoxicity of AQs was significantly reduced compared with that of RMMS. All of these results infer that RTFA in RTFA-OCDD-GN may not be absorbed into the bloodstream following oral administration and thus does not produce nephrotoxicity. In another study, the pharmacokinetic characteristics of orally administered rhubarb AQs in rats were compared with rhubarb and RTFA-OCDD-GN. The results showed that, compared with rhubarb group, the area under the plasma concentration time curve, the peak concentration, the biological half-life andapparent volume of distribution of aloe-emodin, rhein, emodin and chrysophanol in rats administered with RTFA-OCDD-GN were significantly decreased, and the time to reach peak concentration of the four analytes was prolonged. Simultaneously, AQ prototype excretion rates in urine and feces of aloe-emodin, rhein, emodin, chrysophanol and physcion were all increased. These findings suggested that oral colon-specific drug delivery technology induced free AQs colon-specific release following oral administration. This allowed AQs to not only exhibit the corresponding purgative effect but also to avoid intestinal absorption and promote excretion, thereby greatly reducing the nephrotoxicity of rhubarb (37).

In the present study, RTFA-OCDD-GN was prepared by pH-enzyme double-layer coating technology and the cumulative release rate of RTFA in RTFA-OCDD-GN in the simulated colonic fluid achieved the desired effect of colon-specific drug delivery. Purgative efficacy test results revealed that RTFA-OCDD-GN produced considerable purgative efficacy compared with RMMS. Following 40 days of drug administration, marked nephrotoxicity was observed in the RMMS groups, whereas no significant difference was detected between the RTFA-OCDD-GN groups and the control group. The experimental results suggested that the nephrotoxicity of AQs was significantly reduced when RTFA-OCDD-GN were used, compared with RMMS. The present study provided a novel form of administration of rhubarb and offered useful information about the safety of long-term use of rhubarb with purgative efficacy.

Acknowledgements

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