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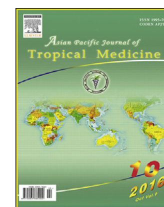

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Effect of emodin on mobility signal transduction system of gallbladder smooth muscle in Guinea pig with cholelithiasis

Bang-Jiang Fang[1], Jun-Yi Shen[1], Hua Zhang[3], Shuang Zhou[2]✉, Chuan-Zhu Lyu[3], Yi-Qiang Xie[4]

[1] *Emergency Department, Long Hua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China*

[2] *Changhai Hospital of Traditional Chinese Medicine, Second Military University, Shanghai 200032, China*

[3] *Traumatology Department, Affiliated Hospital of Hainan Medical University, Haikou 571199, Hainan, China*

[4] *College of Traditional Chinese Medicine, Hainan Medical University, Haikou 571199, Hainan, China*

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ABSTRACT

Objective: To study the effect of emodin on protein and gene expressions of the massagers in mobility signal transduction system of cholecyst smooth muscle cells in guinea pig with cholesterol calculus.

Methods: The guinea pigs were randomly divided into 4 groups, such as control group, gall-stone (GS) group, emodin group and ursodeoxycholic acid (UA) group. Cholesterol calculus models were induced in guinea pigs of GS, emodin and UA groups by lithogenic diet, while emodin or UA were given to the corresponding group for 7 weeks. The histomorphological and ultrastructure change of gallbladder were detected by microscope and electron microscope, the content of plasma cholecystokinin (CCK) and $[Ca^{2+}]_i$ were analyzed successively by radioimmunoassay and flow cytometry. The protein and mRNA of Gsα, Giα and Cap in cholecyst cells were determined by western blotting and real time polymerase chain reaction (RT-PCR).

Results: Emodin or UA can relieve pathogenic changes in epithelial cells and muscle cells in gallbladder of guinea pig with cholesterol calculus by microscope and transmission electron microscope. In the cholecyst cells of GS group, CCK levels in plasma and $[Ca^{2+}]_i$ decreased, the protein and mRNA of GS were down-regulated, the protein and mRNA of Gi and Cap were up-regulated. Emodin significantly decreased the formative rate of gallstone, improved the pathogenic change in epithelial cells and muscle cells, increased CCK levels in plasma and $[Ca^{2+}]_i$ in cholecyst cells, enhanced the protein and mRNA of Gs in cholecyst cells, reduced the protein and mRNA of Gi and Cap in cholecyst cells in guinea pig with cholesterol calculus.

Conclusion: The dysfunction of gallbladder contraction gives rise to the disorders of mobility signal transduction system in cholecyst smooth muscle cells, including low content of plasma CCK and $[Ca^{2+}]_i$ in cholecyst cells, abnormal protein and mRNA of Gs, Gi and Cap. Emodin can enhance the contractibility of gallbladder and alleviate cholestasis by regulating plasma CCK levels, $[Ca^{2+}]_i$ in cholecyst cells and the protein and mRNA of Gs, Gi and Cap.

1. Introduction

Cholelithiasis represents a significant burden on healthcare systems throughout the world, affecting approximately 22.87% of the Han Chinese [1]. Cholelithiasis is also an important public health problem in some Asian countries including China, where cholecystectomy was executed for 11.5% of all hospitalizations between 1985 and 1995 [2–4]. Several mechanisms contribute to gallbladder stone formation, including gallbladder hypo motility, cholesterol super-saturation of bile, destabilization of bile by kinetic protein factors, and abnormal mucin products [2,5,6]. Among them gallbladder motility disorders is more important for

First author: Saul Levy-Blitchtein, Universidad Peruana de Ciencias Aplicadas-UPC, Av. Primavera 2390, Monterrico, Lima, Peru.
Tel: +51 13133333x2704.
E-mail: levysaul45@hotmail.com
✉Corresponding author: Shuang Zhou, Changhai Hospital of Traditional Chinese Medicine, Second Military University, Shanghai 200433, China.
Tel: +86 13918088023
E-mail: zhoushuang8008@163.com

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cholelithiasis that is triggered by the abnormality of gallbladders smooth muscle movement [7,8]. Impaired muscle contraction and relaxation in gallbladders may be due to an abnormal cholecystokinin (CCK) receptor-binding capacity and a difficult signal-transduction cascade distal to the activation of G proteins [9,10]. The mechanism for abnormal CCK receptor-binding capacity gives rise to decreased membrane fluidity, an excessive cholesterol content and high cholesterol-to-phospholipid ratio, which may affect the functions of G protein signal-transduction cascade, including inhibitory adenylate cyclase G protein (Gi), stimulating adenylate cyclase G protein (Gs), second messengers inositol 1,4,5-trisphosphate (IP_3) and the enzyme Calponin (Cap) [11].

Emodin (1, 3, 8 – t r i h y d r o x y – 6 – m e t h y L -anthraquinone) is a biologically active natural anthraquinone extracted from the roots and rhizomes of *Rheum palmatum* (Chinese name DaHuang), which is one of the most effective traditional Chinese medicines for constipation and has now been officially listed in the Chinese Pharmacopoeia [12]. Some possible mechanisms could be involved in the emodin-induced smooth muscle contraction in the gastrointestinal tract. Firstly, emodin enhances the function of small intestinal peristalsis through inhibiting the secretion of somatostatin and triggering the release of motilin [13]. Secondly, emodin directly contracts the colon smooth muscle by elevating the intracellular Ca^{2+} concentration [14]. It has been reported that emodin has been applied in cholesterol stones and cholecystitis for enhancing the contraction of gallbladder smooth muscle [15]. However, the mechanisms have not been fully elucidated.

This study intends to explore the mechanism of emodin regulating the motility of gallbladder signal transduction function and improving the gallbladder contraction ability, including histological analysis and of cholesterol gallstone in guinea pig, plasma CCK levels and $[Ca^{2+}]_i$ concentration detection, Gs, Gi, Cap protein and mRNA determination in the gallbladder smooth muscle cells.

2. Materials and methods

The antibodies of Gs, Gi, Cap, β-actin were purchased from Abcam. Ursodeoxycholic acid (UDCA') was purchased from Sanwei Pharmaceuticals (Shanghai, China). Benzyl sulphonic acid fluorine (PMSF) was from Shanghai Shengneng gaming biological technology co., Ltd; Acrylamide was from Gibco; Tris-base was from Boehringer; Emodin, sodium dodecyl sulfate (SDS), protease inhibitor cocktail, glycine, ammonium persulfate, DEPC and TEMED were from Sigma; Chemiluminescence chromogenic reagent kit and protein assay kit were from (Thermo Scientific). Hematoxylin and eosin were purchased from Sinopharm Chemical Reagent Co., Ltd. Cholecystokinin (CCK) radiation immunity test kit was provided by the second military medical university neurobiology teaching and research section.

2.1. Animals and grouping

White guinea pig with red eye [200–240 g/body weight, half were male and half were female], were obtained from the Experimental Animals Center of the Shanghai University of Traditional Chinese Medicine (Shanghai, China). Guinea pigs maintained under pathogen-free conditions at a room temperature of (23 ± 3) °C and air humidity of (55 ± 15)% in a 12 h light/12 h dark cycle.

A total of 60 guinea pigs were provided free access to water and were divided into four groups (15 guinea pigs for each group) according to their body weight: control group, gall-stone (GS) group, emodin group and ursodeoxycholic acid (UA) group. Guinea pigs in GS group were fed by lithogenous diet containing 1% cholesterol, 0.5% cholic acid and 15% butter fat for 8 weeks. For gallstone prevention studies, guinea pigs in emodin group were given a lithogenic diet supplemented with emodin (Sigma, USA) at 100 mg/kg every 12 h for 8 weeks. Those in UA group were administered with a lithogenic diet supplemented with UA (80 mg/kg/d) (Shanghai Sanwei Pharmaceuticals, China, No.H31021950) for 8 weeks [16]. The gallbladder samples were taken for detection at the eighth weekend. The experimental protocols were approved by the Committee of Animal Experimentation of the Shanghai University of Traditional Chinese Medicine.

2.2. Histological analysis

Gallbladder specimens were fixed in 10% buffered formalin, processed by standard techniques and embedded in paraffin. Cross-sectional cuts 3 μm thick were taken from the middle zones of the gallbladder. The sections were stained with hematoxylin and eosin for histopathology, and examined with a light microscope (Zeiss Axioscop 40, Zeiss) by a pathologist who was blinded to the experimental groups. Ten high power fields were observed (×200), and digital images were obtained with a digital camera (Nikon 4500, Japan) and archived.

2.3. Electron microscopy

Obtain the gallbladder specimen fast. The gallbladder slices were prepared for electron microscopy by fixation, dehydration, and embedment in araldite in situ. Portions of the embedment containing suitable cells selected by light microscopy were cut out and affixed to a small metal dowel, sectioned in a Porter-Blum microtome with glass knives, mounted on bare grids, stained with 1.0% $KMnO_4$, and studied in a Siemens Elmiskop I.

2.4. Radioimmunoassay

2 mL blood was obtained from guinea pig heart accurately and injected into precooling, 20 μL 1% heparin anticoagulant. Serum (100 μL was diluted with 400 μL of saline and extracted twice with 2 mL of diethyl ether). The water phase was left frozen in solid carbon dioxide bath and ether extracts were combined and evaporated to dryness. The buffer or standard solution, radio ligand and antiserum, 100 μL, each were added. The tubes for unspecific binding determination contained radio ligand and buffer final volume in each tube was 300 μL The content of tubes was mixed in vortex, and after overnight incubation at 4 °C, 500 μL of dextran coated charcoal (0.25 and 0.025 g/100 mL) was added to each tube. The content was vortexed briefly and after 10 min staying at 4 °C, the tubes were centrifuged at 4 °C and 3 000 rpm for 10 min. The supernatants were decanted into another set of tubes in which the radioactivity of ^{125}I was measured on Berthold gamma counter. The concentration of CCK was calculated from log–logit plot.

2.5. RNA extraction and real-time reverse transcription (RT)-PCR analysis

Total RNA in gallbladder was isolated by using Trizol reagent (Invitrogen, USA) and reverse transcribed into cDNAs by using a First-strand cDNA Synthesis kit (Roche Applied

Science). Real-time PCR was performed for quantitative estimation of Gi, Gs and CAP mRNA. The primers for Gi were sense 5′-CAGAGGATGCATTTTGAGCA-3′ and antisense 5′-CAAAGCAGTTCTGACCACCA-3′. The primers for Gs were sense 5′-AGAAGCAGCTGCAGAAGGAC-3′ and antisense 5′-CCCTCTCCGTTAAACCCATT-3′. The primers for CAP were sense 5′- ACTTCATGGATGGCCTCAAG-3′ and antisense 5′-GTGCCAGTTCTGGGTTGACT-3′. Real-time-PCR was performed in a total volume of 10 μL per reaction. We placed 1 μL of cDNA into a 9-μL reaction mixture that contained 0.1 μL of Taq DNA polymerase, 1 μL of the supplied 10× PCR buffer, 0.5 μL of $MgCl_2$ (50 mmol/L), 0.2 μL of dNTPs (10 mmol/L), 0.15 μL of bovine serum albumin (10 g/L), 0.5 μL of the appropriate sense primer, 0.5 μL of the corresponding antisense primer (3 μmol/L) and 1 μL of the TaqMan probe (3 μmol/L). DEPC–H_2O was added to a final volume of 10 μL. The cycling protocol consisted of an initial 5-min denaturation step at 88 °C, followed by 50 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 20 s, and extension at 72 °C for 20 s. The PCR products were subjected to electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized under UV light. The expression of â-actin mRNA was amplified in parallel as control for RNA loading and RT-PCR efficiency.

2.6. Western blot analysis

The gallbladder proteins were homogenized in PBS with protease inhibitor cocktail. The homogenates were centrifuged for 15 min at 14 000 rpm in 4 °C. Supernatants of the tissues were collected and protein concentration was measured with a bicinchoninic acid assay kit. An equal amount of protein from each sample (150 μg) was resolved in 10% Tris-glycine SDS polyacrylamide gel. Protein bands were blotted to nitrocellulose membranes. After incubation for 1 h in blocking solution at room temperature, the membrane was incubated for 24 h with anti-Giá antibodies, anti-Gsá antibodies or anti-CAP antibody at 4 °C, respectively. The secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin) was added and incubated at room temperature for 1 h. Peroxidase labeling was detected with the Western blotting detection system and analyzed by a densitometry system. The relative protein levels of Giá, anti-Gsá or CAP were normalized to â-actin.

2.7. Flow cytometry

The gallbladder of guinea pigs were cut into pieces and washed by PBS repeatedly. The cell suspension was obtained with 40 μm nylon mesh filtration, washed by PBS twice and centrifuged at 1 500 rpm for 5 min. After the supernatant was discarded, each tube was regulated to possess 10^6 cells and mixed fluo-3 markers for [Ca^{2+}] detection on a FACS calibur flow cytometer. Green fluorescence emission of Fluo-3 was detected at 530 nm and the percentages of fluo-3^- cells were quantified with Cell Quest software.

2.8. Statistical analysis

Data are presented as mean ± SE. The significance of the difference in mean values within and among multiple groups was examined with an ANOVA for repeated measures followed by a Duncan's post hoc test. Student's *t*-test was used to evaluate the significance of differences between two groups of experiments (SigmaStat, SPSS). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Emodin can relieve the inflammation of the gallbladder

There were crystal deposits attached with the gallbladder epithelial cells and full of gallbladder by microscope in GS group compared with control group. In the GS group lots of gallbladder epithelial cells disappeared, gallbladder cavity was

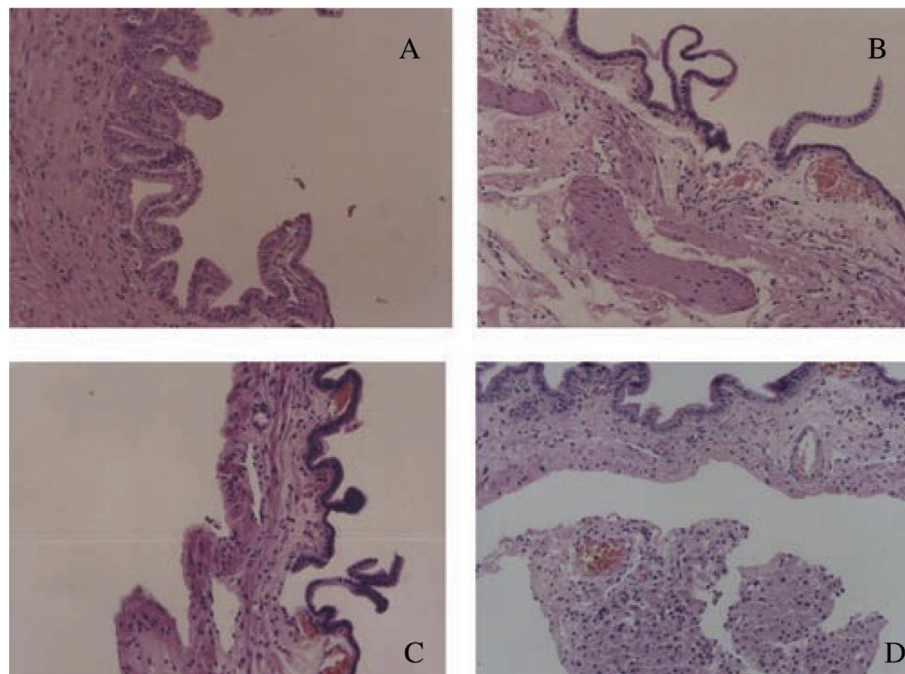

Figure 1. Histological change of gallbladder in different groups (HE × 200). A. Control group. B. GS group. C. Emodin group. D. UA group.

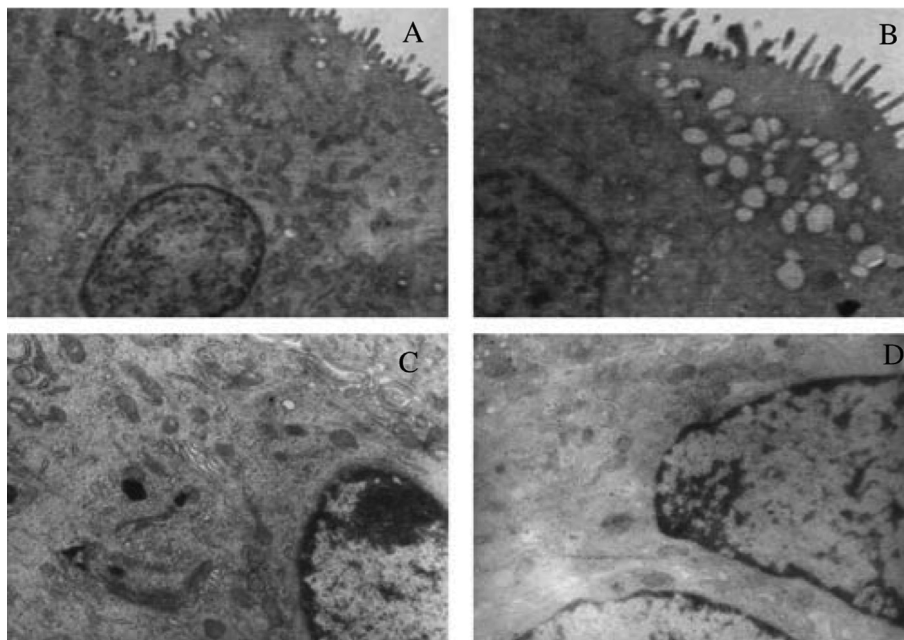

Figure 2. Change of gallbladder epithelial cells in different groups under TEM.
A. Control group (TEM × 7500) B. GS group (TEM × 10000) C. emodin group. (TEM × 13000) D. UA group. (TEM × 13000).

filled with liquid, and the gallbladder wall shrunk. Gallbladder mucosa layer were infiltrated with inflammatory cells and showed edema and hemorrhage (Figure 1A, B). In the emodin group gallbladder epithelial cells showed intact, mild hyperemia and edema appeared in the submucosa. In the ursodeoxycholic acid (UA) group the epithelial cells of gallbladder tissue bears the basic integrity, mild dilatation and hyperemia appeared in the sub-mucosal vessels (Figure 1C, D).

Under transmission electron microscope, the dense deposits, few microvilli, swelling endoplasmic reticulum, expanded Golgi, irregular mitochondria, nuclear membrane shrinkage and adhesion fusion appeared in the guinea pig gallbladder epithelial cells of GS group (Figure 2A, B). Different from GS group, the gallbladder epithelial cells of emodin group and UA group were similar with those of control group (Figure 2C, D).

3.2. Emodin increase plasma CCK levels in the guinea pig with cholesterol gallstone

Radioimmunoassay showed that the plasma CCK level of GS group (63.63 ± 9.14, pmol/L) was lower than the control group (138.91 ± 14.64, pmol/L) ($P < 0.01$). The plasma CCK level of emodin group (102.37 ± 10.19, pmol/L) or UA group (104.65 ± 11.13, pmol/L) was higher than the control group (138.91 ± 14.64, pmol/L) ($P < 0.01$), but still was lower than the control group ($P < 0.01$) (Figure 3).

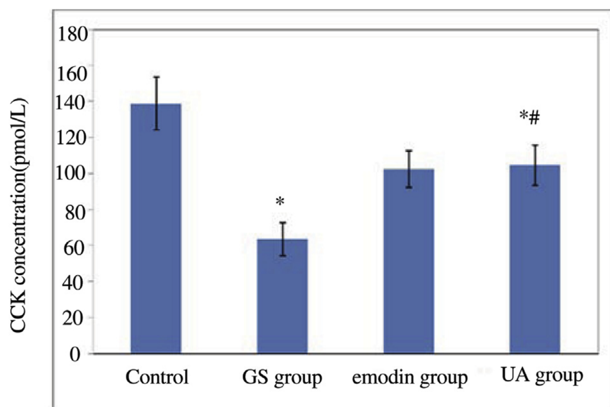

Figure 3. Plasma CCK levels in the guinea pig of different groups.
$^*P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with GS group.

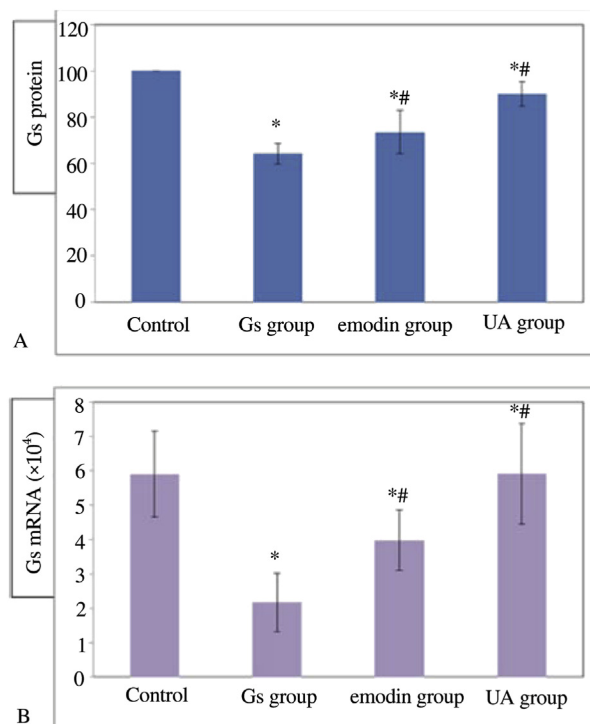

Figure 4. Effect of emodin on Gsá mRNA and protein in gallstone gallbladder.
A. Change of Gs protein in gallstone gallbladder in different groups by Western blot analysis. B. Change of Gs mRNA in gallstone gallbladder in different groups by RT-PCR analysis vs. $^*P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with GS group.

3.3. Effect of emodin on Gsá mRNA and protein in gallstone gallbladder

Western blot analysis demonstrated that Gs alpha protein of the gallbladder in the GS group was lower than that of control group ($P < 0.01$). Compared with GS group, Gs alpha protein production of the gallbladder from emodin group or UA group obviously exceeded. Especially, Gs alpha protein of the gallbladder from emodin group had a little less production than that of UA group (Figure 4A). Gs alpha mRNA of the gallbladder had similar change with Gs alpha protein by RT-PCR analysis (Figure 4B).

3.4. Effect of emodin on Giá mRNA and protein in gallstone gallbladder

Western blot analysis demonstrated that Gi alpha protein of the gallbladder in the GS group was lower than that of control group ($P < 0.01$). Compared with GS group, Gi alpha protein production of the gallbladder from emodin group or UA group obviously elevated. Especially, Gi alpha protein of the gallbladder from emodin group had lesser production than that of UA group (Figure 5A). RT-PCR analysis demonstrated that Gi alpha mRNA changed similarly with Gi alpha protein (Figure 5B).

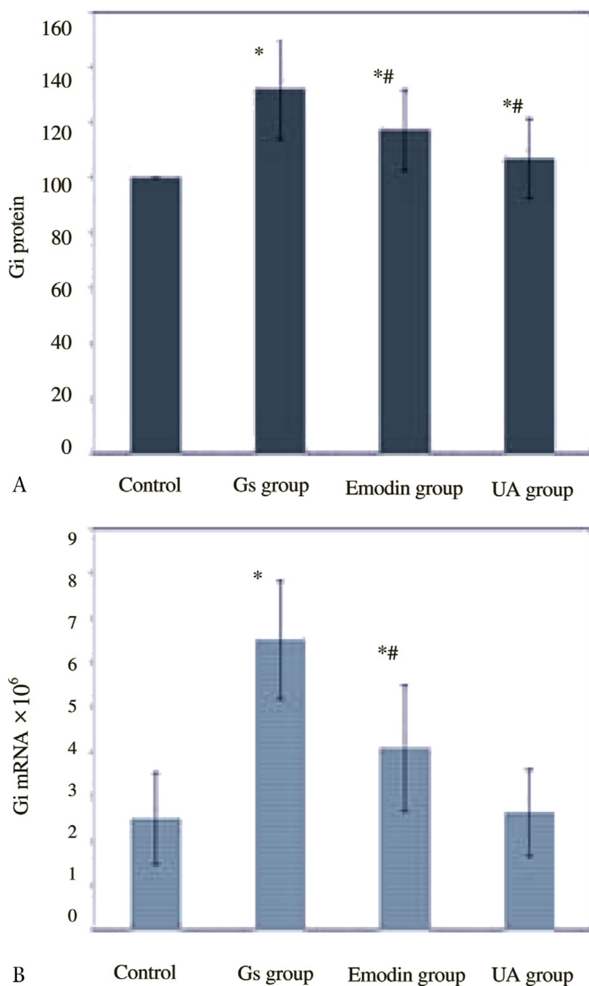

Figure 5. Effect of emodin on Gi mRNA and protein in gallstone gallbladder.

A. Change of Gi protein in gallstone gallbladder in different groups by Western blot analysis. B. Change of Gi mRNA in gallstone gallbladder in different groups by RT-PCR analysis. $^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with GS group.

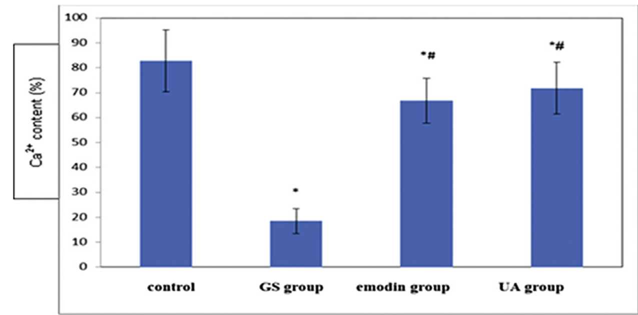

Figure 6. Change of emodin on $[Ca^{2+}]_i$ concentration in different groups.

3.5. Effect of emodin on Ca^{2+} concentration and cap in gallbladder cells of the guinea pig with cholesterol calculus

Flow cytometry experiments showed that the $[Ca^{2+}]_i$ concentration in gallbladder cells of GC group was lower ($P < 0.01$) compared with control group. Both emodin and UA can increase $[Ca^{2+}]_i$ concentration in gallbladder cells compared with GC group ($P < 0.01$) (Figure 6). As calcium binding protein, Cap protein of the gallbladder in the GS group was higher than that of control group according to Western blot analysis ($P < 0.01$). Compared with GS group, Cap protein production of the gallbladder from emodin group or UA group obviously descended. Especially, Cap protein of the gallbladder from emodin group had lesser production than that of UA group ($P > 0.05$) (Figure 7A). RT-PCR analysis demonstrated that Cap mRNA of the gallbladder had similar change with Cap protein (Figure 7B).

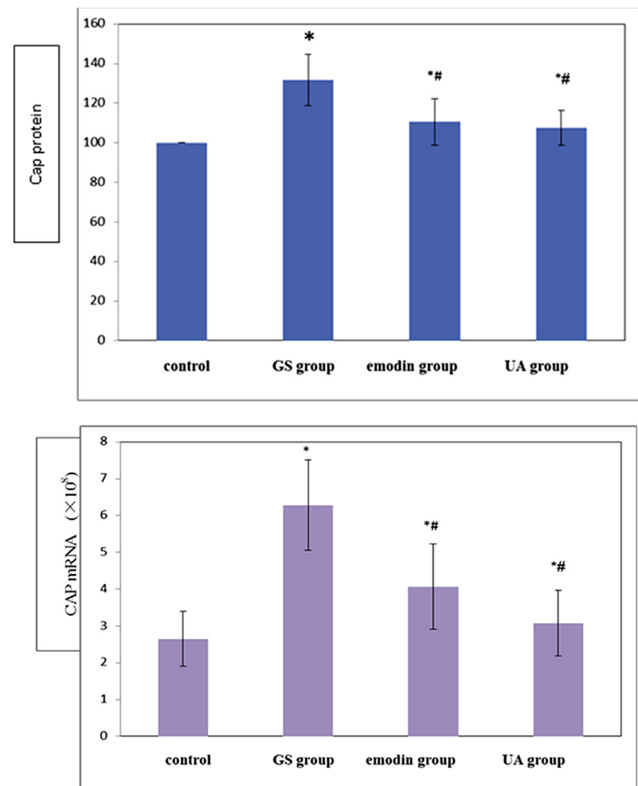

Figure 7. Effect of emodin on Cap mRNA and protein in gallstone gallbladder.

$^{*}P < 0.05$ compared with control group; $^{\#}P < 0.05$ compared with GS group.

4. Discussion

The injury of gallbladder epithelial cells and abnormality of smooth muscle movement initiated gallbladder motility disorders. In China, rhubarb has been applied in the treatment of cholesterol stones for hundreds of years. As main effective ingredient of rhubarb, emodin has been demonstrated that emodin increased the resting tension of gallbladder smooth muscle strips in a dose-dependent manner [17]. In the present study, we found that both emodin and UA relieved injury of epithelial cells in gallbladder of guinea pig with cholesterol calculus.

CCK is a gastrointestinal hormone that is produced and secreted by intestinal cells. It has been found that plasma concentrations of CCK significantly increased after ingestion, which can promote a significant contraction of the gallbladder [18]. Lack of CCK induces gallbladder hypomotility that prolongs the residence time of excess cholesterol in the gallbladder, leading to rapid crystallization and precipitation of solid cholesterol crystals [19]. CCK interacts with CCK receptor-A (CCK-R) in gallbladder, which elicits the contraction of gallbladder by the activation of post-membrane signaling passage in smooth muscle [20]. The results of this study showed that emodin made a significant increase in plasma concentrations of CCK and raised gallbladder motility in guinea pig with cholelithiasis. Combined with Flow cytometry experiments showed that the $[Ca^{2+}]_i$ concentration impaired muscle contraction and relaxation in gallbladders may be due to an abnormal CCK receptor-binding capacity and a difficult signal-transduction cascade distal to the activation of G proteins [9,10]. The mechanism for abnormal CCK receptor-binding capacity gives rise to decreased membrane fluidity, an excessive cholesterol content and high cholesterol-to-phospholipid ratio, which may affect the functions of G protein signal-transduction cascade, including inhibitory adenylate cyclase G protein (Gi), stimulating adenylate cyclase G protein (Gs), second messengers inositol 1,4,5-trisphosphate (IP_3) and the enzyme Calponin (Cap) [11]. Calponin is a calcium binding protein. Calponin tonically inhibits the ATPase activity of myosin in smooth muscle. Phosphorylation of calponin by a protein kinase, which is dependent upon calcium binding to calmodulin, releases the calponin's inhibition of the smooth muscle ATPase. In the cholecyst cells of GS group, CCK levels in plasma and $[Ca^{2+}]_i$ decreased, the protein and mRNA of Gs were down-regulated, the protein and mRNA of Gi and Cap were up-regulated. Emodin significantly decreased the formative rate of gallstone, improved the pathogenic change in epithelial cells and muscle cells, increased CCK levels in plasma and $[Ca^{2+}]_i$ in cholecyst cells, enhanced the protein and mRNA of Gs in cholecyst cells, reduced the protein and mRNA of Gi and Cap in cholecyst cells in guinea pig with cholesterol calculus.

The dysfunction of gallbladder contraction gives rise to the disorders of mobility signal transduction system in cholecyst smooth muscle cells, including low content of plasma CCK and $[Ca^{2+}]_i$ in cholecyst cells, abnormal protein and mRNA of Gs, Gi and Cap. Emodin can enhance the contractibility of gallbladder and alleviate cholestasis by regulating plasma CCK levels, $[Ca^{2+}]_i$ in cholecyst cells and the protein and mRNA of Gs, Gi and Cap.

Conflict of interest statement

All authors have no conflict of interest to disclose.

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