

C-Kit expression in the gallbladder of guinea pig with chronic calculous cholecystitis and the effect of *Artemisia capillaris* Thunb on interstitial cells of Cajal

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ABSTRACT

Objective(s): To study the c-Kit expression in the gallbladder of cholesterol lithogenic guinea pig model and the effect of *Artemisia capillaris* Thunb on interstitial cells of Cajal (ICCs).

Materials and Methods: A total of 45 guinea pigs were randomly assigned into three groups: the control group (guinea pigs fed a standard diet, normal group); the model group (guinea pigs fed a cholesterol gallstone-inducing diet); and the Chinese medicine group (guinea pigs fed the cholesterol gallstone-inducing diet and treated with *A. capillaris* through intragastric administration, therapy group). Each group had 15 guinea pigs. The gallbladders of the guinea pigs were harvested after 8 weeks. C-Kit expression was detected using an immunohistochemistry staining, real-time PCR, and Western blot analyses. The effect of *A. capillaris* on ICCs was evaluated by muscle strip contraction experiments.

Results: C-Kit expression significantly decreased in the gallbladder of model group, but increased in the Chinese medicine group. The Contractility of guinea pig gallbladder muscle strip significantly improved in the Chinese medicine group.

Conclusion: Our results indicated that *A. capillaris* improves gallbladder impairment by up-regulating c-Kit expression, and it also can improve the contractile response of *in vitro* guinea pig gallbladder muscle strips.

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Introduction

Gallbladder is an important component of biliary tract and plays roles in inspissating, storing, and discharging bile through its rhythmic contraction and relaxation. Cholecystitis and cholelithiasis are mainly caused by insufficient dynamic of gallbladder that might make pathological changes (1-2). Thus, exploring the motor function and mechanism of gallbladder to prevent biliary diseases will be an important area for further study.

Gallbladder wall is similar to gastrointestinal wall in structure. Moreover, gallbladder contraction mainly depends on smooth muscle; it has been demonstrated that interstitial cells of Cajal (ICCs) are widely distributed in the gastrointestinal tract of mammals and play an important role in regulating gastrointestinal motility due to close relation to neuron and smooth muscle cells (3). Studies have shown that biliary tracts of human and guinea pigs

contain ICCs (4-5). The resting muscular tension of gallbladder smooth muscle is significantly reduced after removal of gallbladder ICCs with methylene blue (6). Meanwhile, the expression of c-kit significantly decreased in the gallbladder of guinea pigs on a high cholesterol diet (7).

We speculated that gallbladder ICCs possibly serve the same function as in the gastrointestinal tract through occurrence of spontaneous rhythmic activity of smooth muscle contraction in gallbladder motility disorders (8-10). The expression of proto-oncogene c-Kit in ICCs has been studied before (11). C-Kit is a receptor tyrosine kinase type III which is specifically expressed in ICCs, thus, we could identify ICCs by detecting the expression of c-Kit. For the past few years, many studies found ICCs in different animals by detecting the expression of c-Kit (12-15).

Artemisia capillaris Thunb is from the chapter of Yang Ming disease in Zhang Zhongjing's "Treatise on

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Febrile Diseases", this medication is composed of *A. capillaris*, *Fructus gardeniae*, and *Rheum palmatum L*; which have functions of clearing away heat, removing dampness by diuresis, and eliminating jaundice (16). The *A. capillaries* and *F. gardeniae* of this medicine can remove damp-heat, soothe the liver, and relieve bladder; *Rheum palmatum L* can decrease blood stasis. *A. capillaris* can usually treat yang jaundice, and has shown significant efficacy in treating patients without jaundice but having hepatocholic hydropyrexia(16). *A. capillaris*, *F. gardenia*, and *Rheum palmatum L* and the combined drug (*A. capillaris*) can significantly enhance the function of gallbladder and improve bile stasis(18); however, the exact mechanism remains unclear. 6,7dimethoxycoumarin is the main extract and transition component of *A. capillaris*. Emodin is the active ingredient in *Rheum palmatum L*. while geniposide is the active ingredient of *Fructus gardenia* (17-18). In our experiment, we firstly made guinea pigs model of chronic calculous cholecystitis, and then detected the expression of c-Kit in gallbladder, meanwhile, we tested the expression of c-Kit after the treatment with *A. capillaris* in guinea pigs model group.

Materials and Methods

Animals

A total of 45 common guinea pigs were provided by the Laboratory Animal Centre of Dalian Medical University. Their weights ranged from 200-250 g. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of First Affiliated Hospital, Dalian Medical University.

The control (normal) group was supplied with normal feed by the Laboratory Animal Centre of Dalian Medical University. The model group and Chinese group was given common feed supplemented with 2% cholesterol, 5% sucrose, and 5% cod liver oil to induce cholesterol gallstone formation.

Groups

The 45 guinea pigs were randomly assigned into three groups with 15 guinea pigs each. The guinea pigs in the control (normal) group were fed a standard diet, the model group was fed the cholesterol gallstone-inducing diet, and the Chinese Medicine (therapy) group was fed the cholesterol gallstone-inducing diet and treated with *A. capillaris* via intragastric administration. All guinea pigs were fed for 8 weeks.

Tissue selection

After 8 weeks, we anaesthetised the guinea pigs via an intraperitoneally injection of chloral hydrate,

and harvested their gallbladders, which were immediately stored in liquid nitrogen after washing. The guinea pigs were all euthanized.

Immunohistochemistry analysis

The guinea pig gallbladders were embedded in optimum cutting temperature, prepared for frozen sections using a freezing microtome (LEICA CM 1850), and fixed on glass slides treated with 3-Aminopropyltriethoxysilane (APES). After drying, the sections were fixed for 10 min in acetone at 4 °C. After washing 3 times with phosphate-buffered saline (PBS), slides were incubated with 10% normal goat serum for 30 min to block nonspecific background staining, and then incubated overnight with primary rabbit anti-mouse c-Kit antibodies (1:50, sc-168, Santa Cruz company) in a humid chamber at 4 °C. Following 3 times washing with PBS, the sections were incubated for 1 hr with species-specific secondary antibodies (sp-9001, Beijing ZSGB-Bio Company) at room temperature, washed 3 times with PBS, stained with DAB, and washed with water. The sections were then observed under an inverted microscope (Nikon ECLIPSE Ti-U) and photographed. The results were analyzed to calculate the mean optical density (OD) using the Image Pro Plus software.

Western blot analysis

Total protein was extracted and quantified via the Bradford method. After calculating protein concentration, the lysates were separated by gel electrophoresis (for 4 hr), before blotting onto membranes. The nonspecific sites on the membranes were blocked with 5% non-fat milk. The blots were incubated overnight with primary rabbit anti-mouse antibodies c-Kit (1:200, sc-168, Santa Cruz Company) and β -actin (1:1000, bs-0061R, Beijing Bosin) at 4 °C, washed 3 times with TBST, and incubated for 2 hr with goat anti-rabbit secondary antibodies (1:5000, ZB-2301, ZSGB-Bio). After washing the membranes were stained with an ECL kit (Thermo, USA), and the gel images were acquired by UVP Bio Spectrum. Semiquantitative analysis was performed via UVP-gel densitometry using Quantity One software.

Real-time PCR

RT-PCR was performed using the following primer sets via Gene Bank: C-Kit (sense) 5'-CCAATTATCCCTCATCGA-3', C-Kit (antisense) 5'-GGGTTTCATCTTTAGCCAC-3', GAPDH (sense) 5'-ACCACAGTCCATGCCATCAC-3', GAPDH (antisense) 5'-TCCACCACCCTGTTGCTGTA-3'. Total RNA was extracted from the gallbladders using PCR kit for reverse cDNA. The PCR conditions were as follows: 40 cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C for 30 sec, and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 5 min. Amplification was visualized using UVP Bio Spectrum via 1.5% agarose gel electrophoresis. We

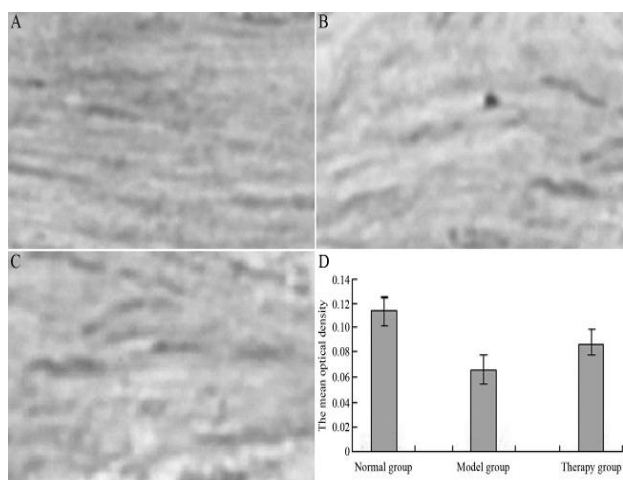


Figure 1. A: ICCs normal group; B: ICCs model group; C: ICCs therapy group; D: Mean optical density (OD) of the three groups. The OD of the model group was lower than those of the normal group ($P < 0.05$) and the therapy group ($P < 0.05$)

used Quantity One software to detect the OD of all strips and relative OD (OD c-Kit/OD β -actin).

Preparation of in vitro muscle strip tissues and contraction experiments

Each guinea pig was intraperitoneally injected with 4-5 ml of 10% chloral hydrate anesthesia. Surgical instruments in the gallbladder were taken off from neck. Mesangial and blood vessels were removed and washed 2-3 times in PBS solution. Along the longitudinal axis of the gallbladder, the gallbladder smooth muscle strips with 8 mm length and 3 mm width were prepared. The mucosal and serosal layers were removed, and each gallbladder muscle strip was divided into two. The prepared strips were attached to the muscle tension transducer hook, the muscle perfusion bath temperature was set to 37 °C, and the strips were infused into the freshly prepared Krebs solution. Then, 95% O₂ and 5% CO₂ gas mixture was supplied, and the initial tension of 1.0 g was given to the hook as preload. After approximately 15-20 min, smooth muscle strips appeared to have more regular amplitude of spontaneous stabilized contractions. Successively, cholecystinin octapeptide (CCK-8) was added to the base tank Krebs to stimulate the smooth muscle strips, which began to shrink significantly. Each group was given 1×10^{-5} , 1×10^{-6} , and 1×10^{-7} concentration gradients of CCK-8. Based on records, after the addition of CCK-8, gallbladder contracted frequency and contractions began to increase significantly after shrinkage. The degree of change in each contraction tension at the same stimuli and concentration in the three groups of smooth muscle strips were compared. CCK-8 was added 1 min prior to tension of the gallbladder

smooth muscle strips as a control value. After each addition of CCK-8, numerical values were used as the effect values, and results were expressed as mean \pm SD. Comparison between the two groups were analyzed by t-test method; and $P < 0.05$ was considered statistically significant.

Statistical analysis

Statistical analysis were performed using SPSS 16.0 software and all results were presented as mean \pm SD. Statistically significant differences between groups were determined using a Student's t-test. Differences with $P < 0.05$ were considered statistically significant.

Results

Immunohistochemistry analysis

The ICCs were dyed brown and irregularly distributed in the muscular layer of the gallbladder, which presented shuttle or satellite shapes, and formed a network. Few positive-staining ICCs were observed in the model group and which were lighter in colour (Figure 1B) compared with the normal group (Figure 1A) and the therapy group (Figure 1C). The mean OD ratios of the normal group, the model group, and the therapy group were 0.1135 ± 0.0118 , 0.0652 ± 0.0110 , and 0.0871 ± 0.0119 , respectively (Figure 1D). This finding indicates that the c-Kit-positive ICCs in the model group were significantly fewer than the normal and the therapy groups ($P < 0.05$).

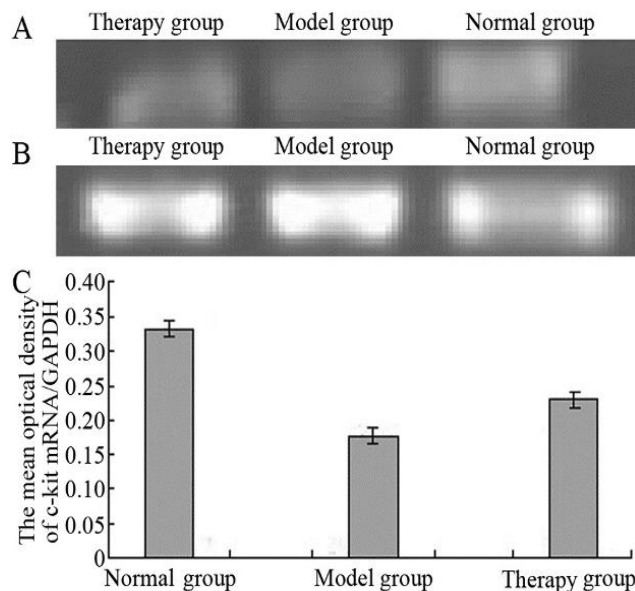


Figure 2. A: C-Kit mRNA expression; B: GAPDH mRNA expression; C: Mean optical density (OD) of the three groups. The OD of the model group was lower than those of the normal group ($P < 0.05$) and the therapy group ($P < 0.05$)

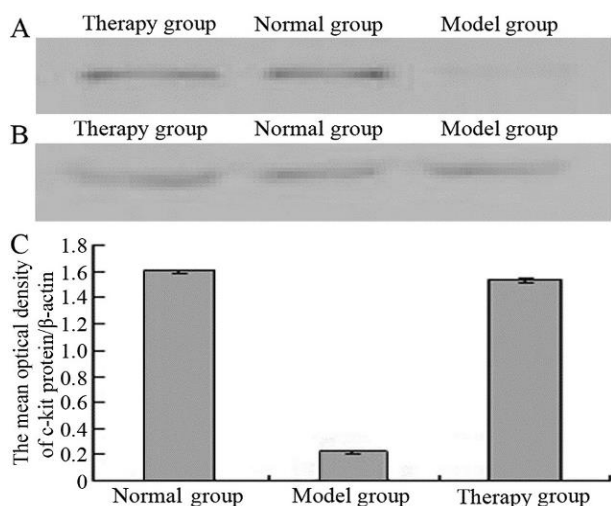


Figure 3. A: C-Kit expression; B: β -actin expression; C: Mean optical density (OD) of c-Kit/ β -actin. The OD of the model group was lower than the normal group ($P < 0.05$) and the therapy group ($P < 0.05$).

RT-PCR

The mRNA expression of c-Kit and GAPDH was examined using PCR (Figures 2A and 2B). The mean OD ratios (C-Kit/GAPDH) of the control group, the model group, and the therapy group were 0.3314 ± 0.0110 , 0.1779 ± 0.0134 , and 0.2300 ± 0.0117 , respectively (Figure 2C). This data indicate that the c-Kit mRNA expression in the model group was significantly lower than those in the control and the therapy groups ($P < 0.05$).

Western blot analysis

The protein expression of c-Kit and β -actin was examined using Western blot analysis (Figures 3A and 3B). The mean OD ratios (C-Kit/ β -actin) of the control group, the model group, and the therapy were 1.6056 ± 0.0157 , 0.2219 ± 0.0154 , and 1.5346 ± 0.124 , respectively. This data indicate that the c-Kit protein expression in the model group was significantly lower than those in the control and the therapy groups ($P < 0.05$; Figure 3C).

The responses of gallbladder muscle strips to different concentrations of CCK-8

Gallbladder muscle strips were under 1.0 g of initial tension, within 10-20 min; a gradual emergence of spontaneous voluntary contraction of the gallbladder was observed. The amplitude was changed significantly after the addition of CCK-8 concentration gradients of 1×10^{-5} , 1×10^{-6} , and 1×10^{-7} mol/l in the normal, model, and therapy groups, respectively. After the addition of 3 concentration gradients of CCK-8, gallbladder contractions according to descending concentrations of CCK were as follows: in the normal group, 1.731 ± 0.312 g, 1.548 ± 0.281 g, and 1.246 ± 0.250 g (Figure 4A); in model group, 1.420 ± 0.288 g, 1.268 ± 0.270 g, and

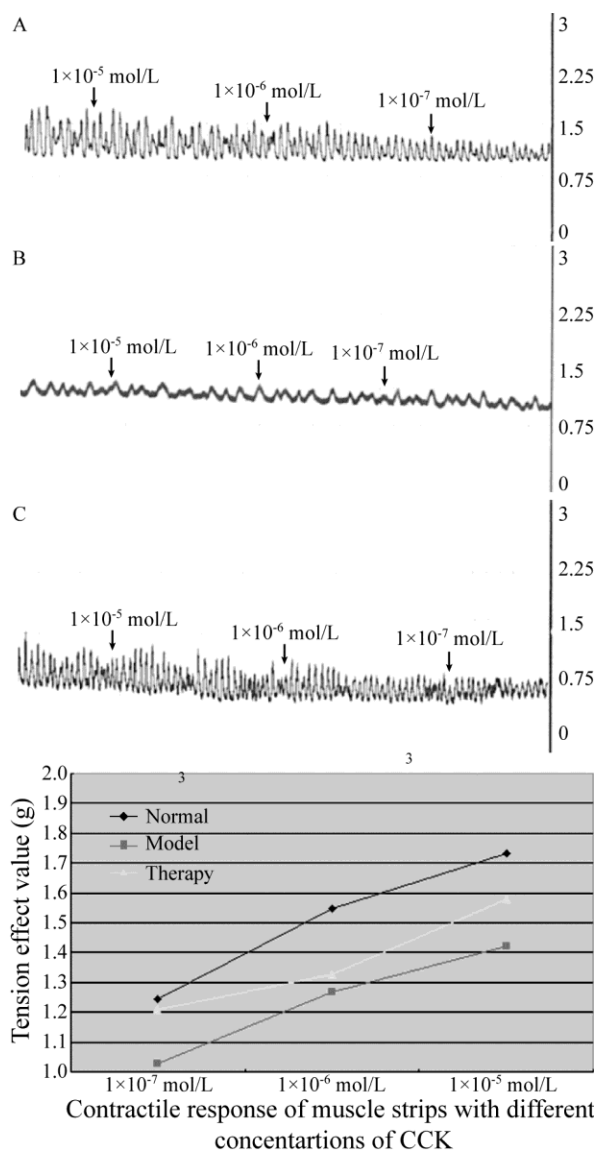


Figure 4. A: The contraction amplitude of gallbladder muscle strips of normal group. B: The contraction amplitude of gallbladder muscle strips of model group. C: The contraction amplitude of gallbladder muscle strips of therapy group. D: Tension effect value. The tension effect value of the model group was lower than the normal group ($P < 0.05$) and the therapy group ($P < 0.05$).

1.029 ± 0.233 g (Figure 4B); in the therapy group, 1.578 ± 0.266 g, 1.326 ± 0.237 g, and 1.207 ± 0.211 g (Figure 4C).

Discussion

Chronic calculous cholecystitis is a common disease. It has been demonstrated that disorders or conditions characterized by altered gallbladder motility predispose patients to gallstone formation (19). Moreover, gallbladder emptying and refilling, and even bile updating were all inhibited in patients with gallbladder gallstone (20). Constriction rate (21) and constriction activities of muscle strips (22)

in gallbladder of cholesterol gallstone were both much lower than those in healthy gallbladder. Therefore, weak gallbladder motility is a main factor that lead to gallstone formation and cause secondary histopathologic alterations of cholecystitis and cholelithiasis (1-2).

ICCs widely distributed in the gastrointestinal tract of mammals and closely related with neuron and smooth muscle cells, play an important role in regulating gastrointestinal motility. Furthermore, regulatory effect of ICCs networks for gastrointestinal motility will be a hot topic in the gastrointestinal dynamic abnormality field.

ICCs are classified into several different subtypes (1). ICC-MY located between the circular and longitudinal muscle layers, serve as pacemaker cells and generate slow waves (2). ICC-IM are densely distributed throughout the circular and longitudinal cardiac muscle layers. ICC-IM of the stomach and colon show a simple elongated spindle shape to transmit neural signal (3). ICC-DMP/ICC-SEP are located between the inner thin and outer thick sublayers of the circular muscle (4) ICC-SM are distributed under the mucous membrane (23).

ICCs located between gastrointestinal nerve and smooth muscle cells form a special network, called nerve-ICC-smooth muscle network. A gap junction that is consisted of junction proteins has been found between intestinal smooth muscle and ICC-DMP (24).

More and more studies have shown that gastroesophageal reflux disease, gastrointestinal inflammatory diseases (ulcerative colitis and Crohn's disease), and diabetic gastroparesis involve impaired morphological features or functions of ICCs and even impaired nerve-ICC-smooth muscle network. At present study, we considered that ICCs and nerve-ICC-smooth muscle, called enteric-nervous system (ENS), were mediators to control gastrointestinal smooth muscle and were involved in gastrointestinal neurotransmitter signal transduction. Thus, ENS will play very important role in gastrointestinal motility disorders diseases.

We studied whether mechanisms of gallbladder and gastrointestinal contraction function were the same, and ICCs could mediate gallbladder contracted activities or not.

Previous studies have demonstrated the following results:

Spontaneous myoelectrical activity was obviously recorded in gallbladders and no significant differences of myoelectrical activities were seen between smooth muscles of gallbladder and gastrointestinal smooth muscle (6).

Resting muscular tension of gallbladder smooth muscle reduced significantly after removal of gallbladder ICCs with methylene blue (6).

Both gallbladder ICCs and smooth muscles exhibit spontaneous rhythmic Ca^{2+} flashes, however, the gap

junction uncouples greatly reduced Ca^{2+} flashes in gallbladder smooth muscle, but they persisted in gallbladder ICCs (9).

Expression of c-kit was significantly decreased in the gallbladders of guinea pigs of high cholesterol diet (7).

Cholecystokinin (CCK)-A receptors (CCK-AR) on the gallbladder ICCs indicated CCK induced gallbladder muscle strip contractions through the ICCs or CCK-AR (10).

All above implied that gallbladder ICCs might mediate gallbladder spontaneous rhythmic activity and smooth muscle contraction. However, few studies researched how ICCs could regulate gallbladder motility. Some studies found that due to gallbladder wall chronic inflammation, gallbladder motility was decreased, even presented cholestasis (25-26); furthermore, Ca^{2+} concentration was obviously reduced in gallbladder smooth muscles based on laboratory tests. They demonstrated that Chinese medicine could be used to improve cholestasis and enhance gallbladder contraction function in cholesterol calculus model. Another study also found that the expression of c-kit was significantly decreased in the gallbladders of guinea pigs of high cholesterol diet (27), but its possible mechanisms are not clear.

A. capillaris soup is a traditional Chinese medicine used for liver and gallbladder diseases. It consist of *A. capillaries*, *F. gardeniae*, and *Rhubarb*, which could promote gallbladder contraction (16, 28).

Our study showed that the protein and mRNA expression of c-Kit in normal group was much higher than those in cholesterol gallstone model group, but significantly lower than those in therapy group that guinea pigs were treated with *A. capillaris*. This data indicated that ICCs were decreased or impaired, this leads to depressed gallbladder contraction in model group. Thus, we considered that ICCs could mediate regulatory effect of gallbladder contraction activities. Moreover, *A. capillaris* enhanced gallbladder contraction and inhibited gallbladder ICCs to be impaired by high cholesterol diet. *A. capillaris* consists of three different ingredients, so, the responsible ingredient or ways that affect contraction function of ICCs and gallbladder should be further studied.

Conclusion

Our results indicate that *A. capillaris* improves gallbladder impairment by up-regulating c-Kit expression, and it also can improve the contractile response of *in vitro* guinea pig gallbladder muscle strips.

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