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Original Article

Distribution changes of interstitial cells of Cajal during cholesterol gallstone formation in guinea pigs fed a high cholesterol diet

Zhen-Peng Huang[1*], Hu Qiu[2,3*], Bao-Ping Yu[2,3]

[1]Department of Internal Medicine, College of Clinical Medicine, Xi'an Medical University, Xi'an, Shannxi Province, P. R. China; [2]Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan, Hubei Province, P. R. China; [3]Key Laboratory of Hubei Province for Digestive System Diseases, Wuhan, Hubei Province, P. R. China. [*]Equal contributors.

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Abstract: Background: Cholesterol gallstone is commonly observed in patients with gallbladder disorders. Interstitial cells of Cajal (ICCs) in the gallbladder are important for regulating gallbladder motility and have a close relationship with cholelithiasis. Aim: The aim of this study was to explore changes in the distribution of gallbladder ICCs during cholesterol gallstone formation. Materials and methods: Thirty guinea pigs were randomly divided into three groups: the control group and study groups. Animals in study groups were fed on high cholesterol diet for 4 weeks or 8 weeks. Animals in the control groups were fed on a standard diet for 8 weeks. Immunohistochemistry was performed to observe the shape, size, morphology, and numbers of ICCs from the neck of the gallbladder to the fundus of the gallbladder, and terminal deoxynucleotidyl transferase dUTP nick-end labeling was performed to detect apoptosis in ICCs from the upper part of the gallbladder to the lower part of the gallbladder. Results: There were no differences in the shape, size, and morphology of the gallbladder ICCs in all groups. Cholesterol gallstones formed in guinea pigs fed on high cholesterol diet. The numbers of gallbladder ICCs were significantly decreased from the neck of the gallbladder to the fundus of the gallbladder, and gallbladder ICC apoptosis was significantly increased from the upper part of the gallbladder to the lower part of the gallbladder in both guinea pigs fed on high cholesterol diet (all $P<0.05$). Conclusion: Cholesterol gallstone formation reduced the density of gallbladder ICCs and increased the frequency of apoptotic gallbladder ICCs from the neck of the gallbladder to the fundus of the gallbladder, and these alterations may affect gallbladder ICC function.

Keywords: Interstitial cells of Cajal, cholelithiasis, c-Kit, cell distribution

Introduction

Cholelithiasis is formation of gallstone, and most of that are cholesterol gallstones. Many factors are involved in the pathogenesis of cholelithiasis, such as hypersaturation of biliary cholesterol, changes of bile salts pool in bile juice, and motility disorders and so on [1, 2]. Most cases of cholelithiasis are accompanied by gallbladder motility disorder and biliary dyskinesia, however, the mechanism is still unknown [2-4]. A recent study suggested that interstitial cells of Cajal (ICCs) play an important role in this disease [5].

ICCs function in the digestive tract smooth muscle by promoting gastrointestinal electrical activity and regulating gastrointestinal tract neurotransmitters [6-11]. In recent studies, ICCs have also been shown to be distributed in the biliary system [12, 13]. Gallbladder motility involves various regulatory mechanisms. Indeed, recent studies have confirmed that ICCs in the biliary system are closely related to the production and spread of the gallbladder spontaneous rhythm and to adjusting gallbladder contraction and gallbladder movement [14]. Additionally, gallbladder ICCs may also have dramatic effects on various biliary system diseases, such as cholelithiasis and acute cholecystitis [15-17]. However, no studies have evaluated changes in the distributions of gallbladder ICCs during the process of cholesterol

Table 1. Serum Lipid Test during cholesterol gallstone formation

	TC*	LDL*	HDL*	TG*
Control Group	1.0250±0.38891	0.7200±0.31113	0.1600±0.04243	0.6767±0.6658
HCD 4 week Group	1.3650±0.12021	0.7950±0.10607	0.1300±0.02828	0.8533±0.01528
HCD 8 week Group	2.0400±0.32249	1.6625±0.10813	0.0670±0.02082	0.9700±0.04000

**F*-values respectively were 8.079, 28.710 and 45.735, *P*-values were 0.027, 0.002, 0.000 and 0.001, respectively.

gallstone formation. Accordingly, in this study, we characterized gallbladder ICCs in order to evaluate changes in the distribution of these cells during cholesterol gallstone formation.

Materials and methods

Animals and animal experiments

Thirty guinea pigs (male and female, weighing 100-120 g) were obtained from Wuhan Institute of Biological Products Co., Ltd. (China). Animals were maintained under standard laboratory conditions (22±2°C with a 12-h light/12-h dark cycle and a relative humidity of 40-60%). All the guinea pigs were allowed free access to food and water. Animal experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University.

Animals were randomly divided into 3 groups, there were 10 guinea pigs in each group. High cholesterol diet (HCD) 4 weeks group and HCD 8 weeks group were the study groups, and each was given the HCD (2% cholesterol) for 4 weeks or 8 weeks before experiments, and the control group was given a standard diet for 8 weeks.

Gallbladder specimen preparation and serum lipid test

Guinea pigs underwent laparotomy and cholecystectomy. Blood was aspirated from the heart and spun at 12,000 rpm for 5 minutes to separate serum, serum total cholesterol (TC), low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL), and triglyceride (TG) concentrations were assessed.

Immunohistochemistry (IHC)

IHC was performed on paraffin-embedded sections using a microwave-based antigen retrieval technique. Gallbladder mucosa and submucosa were peeled away, and the muscular layer was reserved. Gallbladder specimens were fixed in 4% paraformaldehyde solution and embedded in paraffin. Sections were then cut to 5-μm thickness and mounted on positively charged slides. The sections were then identified using rat monoclonal antibodies against CD117/c-Kit (eBioscience, San Diego, CA, USA), and the specimens were incubated at room temperature for 24 h, followed by appropriate incubation with secondary antibodies. Sections were then stained with diaminobenzidine and counterstained with hematoxylin. The stained samples were observed under a light microscope (Olympus BX53, Tokyo, Japan).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays for detection of apoptotic ICCs

TUNEL assays were performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Mannheim, Germany) to assess gallbladder ICC death in both the control group and study groups according to the manufacturer's instructions. Images were obtained using a laser confocal microscope (Olympus FV1000).

Measurement of the numbers of total and apoptotic ICCs

Images of CD117/c-Kit-positive cells and CD117/c-Kit apoptosis-positive cells were taken in three randomly chosen fields (400× magnification) in each section from the neck of the gallbladder (upper part) to the fundus of the gallbladder (lower part) in all groups. Numbers of CD117/c-Kit-positive cells and CD117/c-Kit apoptosis-positive cells were assessed using Image-Pro plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Statistical analysis

All statistical analyses were carried out using SPSS for Windows version 17.0 (SPSS, Chicago, IL, USA). Continuous variables were presented as the mean ± standard deviation (SD).

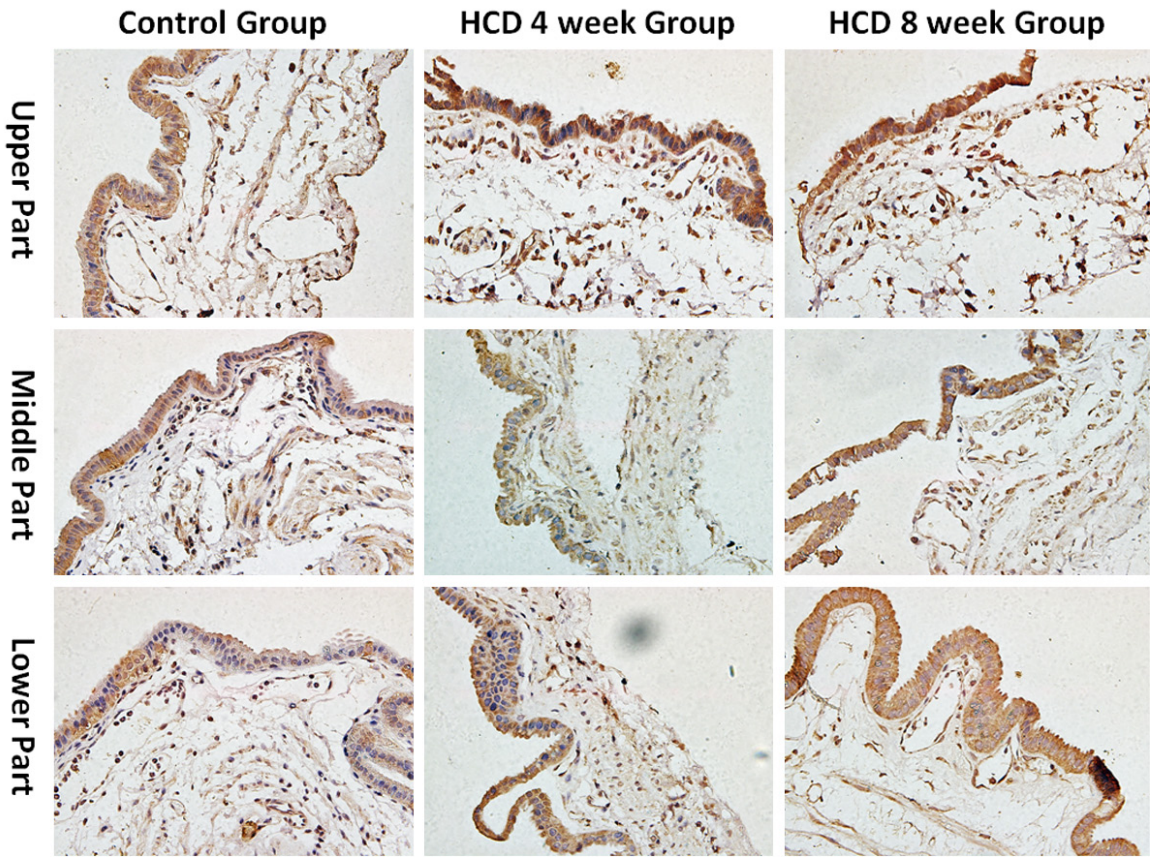


Figure 1. Density of ICCs during cholesterol gallstone formation (400×). The distribution of gallbladder ICCs densities decreased from the neck of the gallbladder to the fundus of the gallbladder and the densities of gallbladder ICCs decreased gradually during cholesterol gallstone formation (all $P<0.01$).

Table 2. Density of ICCs during cholesterol gallstone formation

Group	Part of Gallbladder		
	Upper Part	Middle Part	Lower Part
Control Group*	76.3333±5.85947	54.6667±1.52753	46.3333±3.78594
HCD 4 week Group*	59.3333±3.51188	48.0000±2.00000	43.0000±1.73205
HCD 8 week Group*	37.6667±4.93288	29.3333±5.13160	24.3333±3.21455

*F-values respectively were 52.005, 47.510 and 45.735, P-values were 0.000, 0.000 and 0.000, respectively.

Comparison of continuous variables was carried out using t tests. Comparison of categorical variables was carried out using analysis of variance. A two-sided P value of less than 0.05 was regarded as statistically significant.

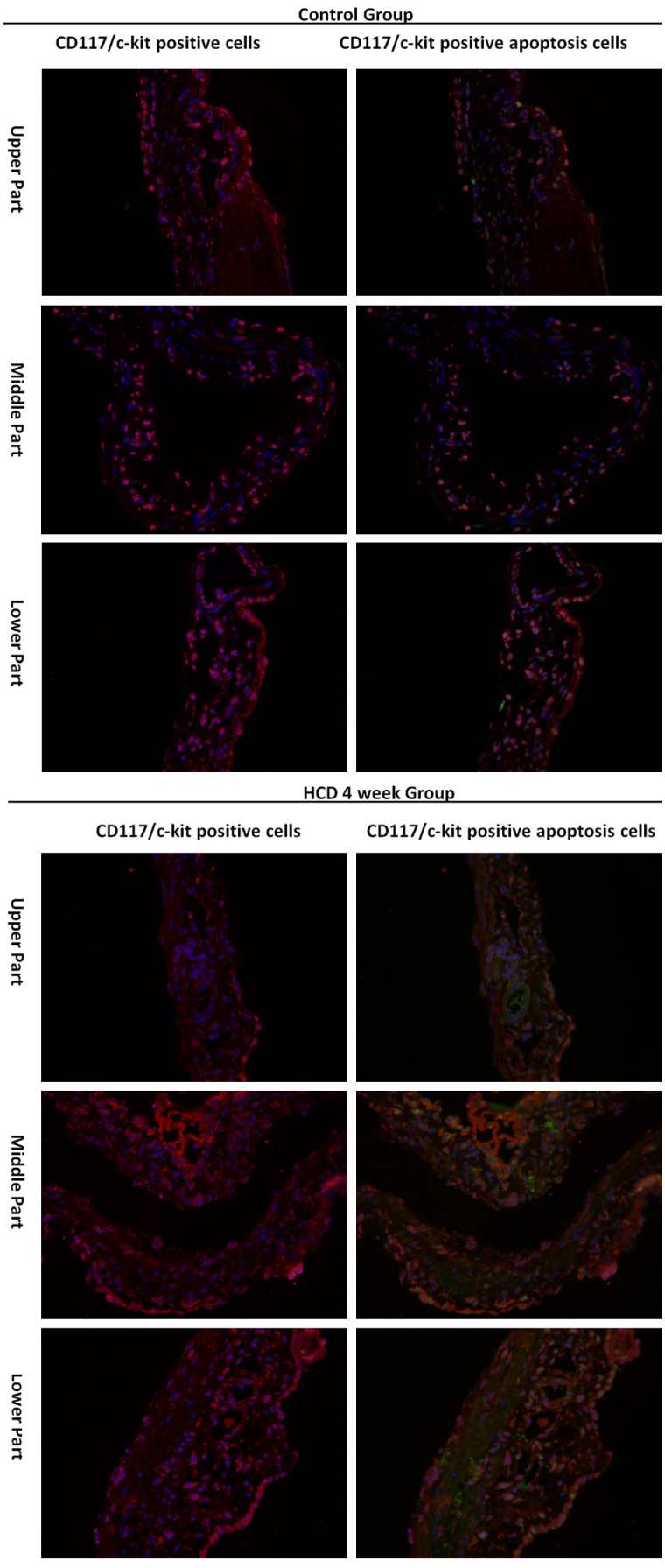
Results

Evaluation of animals

No guinea pigs died both in the control groups and HCD groups. In the control group, gallbladders were normal, with clear, brightly colored bile juice. Gallbladders of guinea pigs in HCD 4 week group were swollen, with granular stones. Gallbladders of HCD 8 week group were very swollen, with thick bile juice and obvious granular, yellow, single or multiple stones.

In the control group, TC, LDL, HDL and TG were significantly lower than the HCD groups, and the HCD 4 week group was much lower than the HCD 8 week group, (all $P<0.01$, respectively). These data are presented in **Table 1**.

These findings suggest that a high cholesterol diet could induce cholesterol gallstone formation.



IHC analysis

We used anti-CD117/c-Kit antibodies to detect CD117/c-Kit protein for identification of ICCs by IHC. After staining, the surface of ICCs was positive for c-Kit. Moreover, when observed under a light microscope, ICCs were fusiform, astrocyte-like, or fusiformis in shape, with 2-5 slender cynapses, and their nuclei were much larger, orbicular or ovate, with little cytoplasm in all groups. We also found that ICCs were mainly located in the muscular layer, with most within the muscularis propria, located primarily parallel to the smooth muscle cells. The ICCs typically appeared individually or in small clusters of 2-3 cells that were connected with each other to form a net-like structure. Mast cells were also immunolabeled positively for c-Kit, had round shapes, and were present in the mucosa of the gallbladders (**Figure 1**).

Measurement of the density of ICCs

The densities of gallbladder ICCs were decreased from the neck of the gallbladder (upper part) to the fundus of the gallbladder (lower part) in all groups. Additionally, the densities of gallbladder ICCs in the neck (upper part), body (middle part), and fundus (lower part) of the gallbladder were all significantly lower in the HCD groups than that in the control group. Additionally, the density of ICCs in different parts of the gallbladder in the HCD 8 week group was lower than that in the HCD 4 week group (all P <0.05, respectively). These data are presented in **Figure 1** and **Table 2**.

The distributions of gallbladder ICC densities decreased fr-

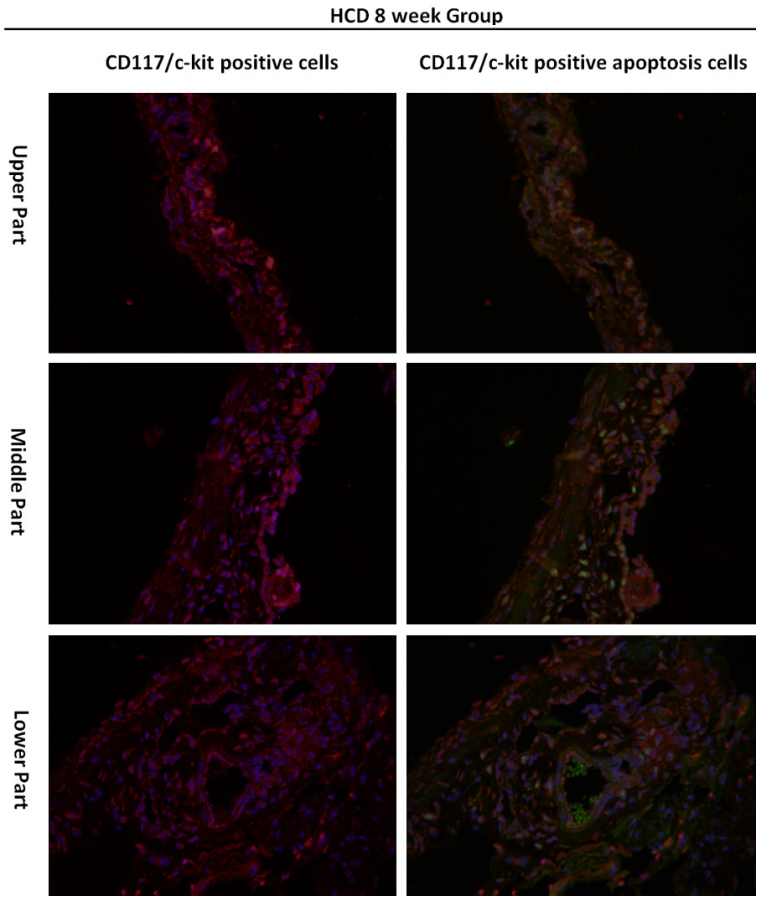


Figure 2. TUNEL assays for analysis of apoptosis ICCs (400×). The distribution of apoptotic gallbladder ICCs increased from the neck of the gallbladder to the fundus of the gallbladder, and the density of apoptotic gallbladder ICCs was increased gradually during cholesterol gallstone formation (all $P<0.01$).

om the neck of the gallbladder to the fundus of the gallbladder. Additionally, the structures of gallbladder ICCs were damaged in cholelithiasis and the densities of gallbladder ICCs decreased gradually during cholesterol gallstone formation. These changes may affect the function of gallbladder ICCs.

TUNEL assays for analysis of apoptosis

The numbers of apoptotic gallbladder ICCs increased from the neck of the gallbladder to the fundus of the gallbladder in all groups. Additionally, the numbers of apoptotic gallbladder ICCs were significantly higher in the HCD groups than in the control group for all portions of the gallbladder, and the numbers of apoptotic ICCs in individual parts of the gallbladder were higher in the HCD 8 week group than in the HCD 4 week group (all $P<0.01$, respectively). These data are summarized in **Figure 2** and **Table 3**.

The distributions of apoptotic gallbladder ICCs increased from the neck of the gallbladder to the fundus of the gallbladder. In cholelithiasis, gallbladder ICCs were lost, and the density of apoptotic gallbladder ICCs was increased gradually during the process of cholesterol gallstone formation.

Taken together, the results from TUNEL assays support the findings of IHC analysis and demonstrate that during cholesterol gallstone formation, the density of gallbladder ICCs was reduced, and gallbladder ICC death is increased from the neck of the gallbladder to the fundus of the gallbladder.

Discussion

Cholesterol gallstone formation is a complicated process and many factors, such as hyper-saturation of biliary cholesterol, changes of bile salts pool in bile juice, and motility disorders, are involved [1, 2]. Surgery is usually required for the treatment of cholelithiasis and acute cholecystitis [18]. Moreover, most cases of cholelithiasis are complicated by biliary dyskinesia [2-4].

Gallbladder motility is involved in various regulatory mechanisms, including gallbladder smooth muscle and nervous circuit activity, and gallbladder ICCs play an important role in this process [19]. ICCs were first identified in the gastrointestinal tract in 1893 [20-23]. More recent studies have shown that ICCs are also distributed in the gallbladder and extrahepatic biliary duct both guinea pigs and humans [24-26]. Moreover, previous studies have shown that the specific marker CD117/c-Kit is expressed on the surfaces of ICCs [14]. Thus, in this study, we found that after IHC, the surfaces of gallbladder ICCs were positive for c-Kit,

Table 3. Density of apoptotic ICCs during cholesterol gallstone formation

Group	Part of Gallbladder		
	Upper Part	Middle Part	Lower Part
Control Group*	5.6667±2.51661	16.3333±2.08167	19.6667±1.15470
HCD 4 week Group*	9.6667±1.52753	18.6667±3.21455	26.0000±5.29150
HCD 8 week Group*	22.6667±0.57735	25.6667±1.52753	36.3333±0.57735

*F-values respectively were 7.000, 14.811 and 154.778, P-values were 0.027, 0.005 and 0.000, respectively.

and gallbladder ICCs were distributed throughout the muscular layer of the gallbladder, most of which were arranged parallel to smooth muscle cells. Gallbladder ICCs typically appeared individually or in small clusters of 2-3 cells that were connected with each other to form a net-like structure. In contrast, mast cells were also positive for c-Kit and showed round shapes. These cells were located in the mucosa of the gallbladders, but were difficult to identify.

ICCs in the biliary system may have functions as smooth muscle pacemakers to produce and maintain the spontaneous rhythm of the gallbladder, adjusting the contraction and movement of the gallbladder [6-12]. Recent studies have confirmed that ICCs in the biliary system are involved in initiating pacemaker activity to adjust gallbladder movement, and decreases in the number of gallbladder ICCs are related to gallbladder motility disorders [27]. Additionally, gallbladder ICCs may have close relationships with various biliary system diseases, such as acute cholecystitis and cholelithiasis [28]. A previous study confirmed that gallbladder ICC numbers were decreased in cholelithiasis [15]. Additionally, in this study, we found that, during cholesterol gallstone formation, the number of gallbladder ICCs was significant lower, whereas the number of apoptotic gallbladder ICCs was increased.

The physiology of gallbladder motility involves three phases: a slow emptying that occurs immediately after ingestion of food, followed by refilling and then rapid emptying [29]. Gallbladder contraction and emptying occurs from the fundus to the body and neck of the gallbladder [30]. Physiologically, the densities of gallbladder ICCs were decreased from the neck of the gallbladder to the body and fundus [14]. Moreover, during cholesterol gallstone formation, the densities of gallbladder ICCs were also decreased from the upper part of the gallbladder to the body of the gallbladder and finally the lower part of the gallbladder. Thus, these data indicate that changes in the distribution of gallbladder ICCs may be closely related to gallbladder motility disorder.

In summary, our study indicates that cholesterol gallstone formation causes structural damage to gallbladder ICCs, reduces the density of gallbladder ICCs, and increases apoptosis in gallbladder ICCs from the neck to the fundus of the gallbladder. Thus, these changes may affect the function of gallbladder ICCs and contribute to gallbladder motility disorder.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhen-Peng Huang, Teaching and Research Section of Internal Medicine, College of Clinical Medicine, Xi'an Medical University, Xi'an 710021, Shannxi Province, P. R. China. Tel: +86-15827622430; E-mail: huang_zhenpeng@126.com

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