The effect of h_1 calponin expression on gallstone formation in pregnancy

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ABSTRACT

Objectives: To explore the effect of h_1 calponin mRNA expression on the biliary tract dynamics, and investigate the molecular mechanisms of gallstone formation in pregnancy.

Methods: This study was carried out in Renmin Hospital of Wuhan University, and in the Department of Molecular Biology and Biochemistry, Wuhan University School of Medicine, Wuhan, China from July to December 2004. Thirty female guinea pigs were divided randomly into 3 groups, the nonpregnant group (n=10) (group A), the 30 days of pregnancy group (n=10) (group B), and the 60 days of pregnancy group (n=10) (group C). Animal models of pregnancy were established on pregnant group guinea pigs through feeding animals with one cage according to female versus male as 4:1. The total cholesterol (TC), total bilirubin (TBiL), total bile acid (TBA) in the bile and the serum estradiol (E_2), progesterone (Pg) levels were determined respectively. Expression levels of h_1 calponin mRNA in gallbladder smooth muscles and Oddi's Sphincter (OS) were evaluated using semiquantitative reverse transcription polymerase chain reaction (RT-PCR).

Results: The concentration of TC, TBiL and the serum E_2 and Pg were more significantly increasing in group C than that in the other 2 groups. However, the concentration of TBA decreased gradually from group A to group C. Up-regulation of h_1 calponin gene expression was observed in the gallbladder smooth muscles in group C, but converse in OS.

Conclusion: The h_1 calponin might play an important role in inducing dysfunction of extrahepatic biliary tract, bile stasis in gallbladder and gallstone formation in pregnancy.

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Epidemiology studies have demonstrated that all the world population, regardless of overall gallstone prevalence, women are twice as likely as men to experience cholelithiasis.[1] The risk is further increased by pregnancy.[2,3] There are 2-4% of pregnant women found to have gallstones during obstetric ultrasound.[4] Pregnancy has a marked effect on the motility of the entire gastrointestinal tract, including gallbladder and Oddi's Sphincter (OS). Gallbladder muscle strips from guinea pigs pretreated with sex hormones have shown that progesterone, rather than estrogens, is the hormone most likely to be responsible for gallbladder hypomotility observed during pregnancy.[5] There is a higher fasting gallbladder volume and incomplete emptying during pregnancy, which appears to be caused by decreased contraction in response to acetylcholine (ACh), cholecystokinin (CCK)-8, and other gastrointestinal

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hormones.[6] Pregnancy may interfere with CCK-8 and Ach-induced gallbladder contraction by altering a step in the excitation-contraction coupling process common to both agonists.[7] Cholecystokinin receptor is coupled with Gai3 protein.[8] Pregnancy may cause a reduced contraction of Gai3 proteins and result in abnormal signal transduction of agonists that act on receptor-G protein coupling mechanisms.[9] However, the exact mechanisms of impaired gallbladder muscle contraction induced by pregnancy remain to be elucidated.

Three homologous calponin isoforms, named h_1, h_2, and acid calponin, have been found in birds and mammals. Based primarily on studies of chicken gizzard smooth muscle (h_1) calponin, calponin has been identified as a family of actin-associated protein that inhibits actomyosin ATPase activity. Evolutionary divergence of the calponin isoforms suggests differentiated function.[10-15] While the role of h_1 calponin in smooth muscle contraction is under investigation. The h_1 calponin, as an important modulator of smooth muscle contraction, has been proved and thought to be specifically expressed in smooth muscle cells in previous studies.[10,16-19] The expression levels of calponin and its messenger RNA (mRNA) were decreasing in OS in guinea pig with gallstone formation. It could increase the pressure of OS, and lead to the stasis of bile and promote the gallstone formation.[20]

In pregnancy, higher serum estrogens and progesterones induce gallbladder hypomotility and OS dysfunction. However, no report on h_1 calponin expression and its effect on gallbladder and OS in pregnant animals with gallstone were available so far.

In our study, the expression levels of h_1 calponin mRNA in gallbladder smooth muscles and OS, which was evaluated by using semiquantitative RT-PCR, were examined respectively in nonpregnant and pregnant guinea pigs. The objective was to find its connection with gallstone formation and to investigate the cell-signal transduction pathway of biliary duct dysfunction in pregnancy, and to provide a new clinical treatment and prevention for gallstone during pregnancy.

Methods.

Animal model establishment. Thirty clear, female adult, weight of 400 gram to 450 gram guinea pigs, which were purchased from Hubei Provincial Center for Disease Control and Prevention, were divided randomly into 3 groups, the nonpregnant group (n=10) (group A), the 30 days of pregnancy (n=10) (group B) and the 60 days of pregnancy group (n=10) (group C). Animal models of pregnancy were established on pregnant group guinea pigs through feeding animals with one cage according to female versus male as 4:1. The animal, which was observed to have formed the vagina embolus was labeled and recorded as the first day of pregnancy. All animals were housed in thermo-regulated rooms with free access to food and water.

Sample preparation and observation. After a 12 hours fast, the experimental animals were anesthetized with an intraperitoneal injection of 10% chloral hydrate (300 $mg \cdot kg^{-1}$). After being paunched, the fasting volume of gallbladder and the common bile duct were observed and a photograph was taken. Then to take suction all of the gallbladder bile and 3 ml of the portal vein blood. After being centrifuged, the serum was drawn off and conserved at -20°C. The gallbladder, common bile duct, and papilla duodeni were removed altogether, then to be opened to observe for the presence or absence of gallstone. Their mucosa and serosa were carefully peeled off in ice-cold normal physiological saline solution under a dissecting microscope. At last, the gallbladder smooth muscles and OS were immediately stored in liquid nitrogen.

Bile composition analysis. The concentration of total cholesterol (TC), total bilirubin (TBiL) and total bile acid (TBA) of bile were assayed respectively by using methods of oxidase-enzyme circulation and vanadate oxidization. It was performed in the Clinical Laboratory of Renmin Hospital of Wuhan University.

Concentration of serum E_2 and Pg examination. The levels of serum estradiol (E_2) and progesterone (Pg) were assayed using radioimmunoassay (RIA). It was performed in the Department of Nuclear Medicine, Renmin Hospital of Wuhan University.

The isolation of total RNA and RT-PCR. Total RNA was isolated from gallbladder smooth muscles and OS with the TRIzol reagent (Invitrogen, USA) according to the manufacture's instructions. The total RNA (5 μg) was incubated with M-MLV (Moloney Murine Leukemia Virus, Promega, USA) reverse transcriptase to synthesize first-strand cDNA. The composition of a mixture (total volume =25 μl) for reverse transcription was as follows: 200 units of M-MLV reverse transcriptase, 1 × RT buffer, 10 mM DTT, 20 mM each dNTP (dATP, dCTP, dTTP, dGTP), 1.5 μl Oligo (dT) primer, 20 units of RNase inhibitor (Promega, USA).The RT conditions were: 37°C for 90 minutes, 95°C for 5 minutes. For the PCR amplification, 2 μl of RT products was incubated with Taq DNA polymerase (Promega, USA). The composition of the mixture for the PCR amplification (total volume = 50 μl) was as follows:

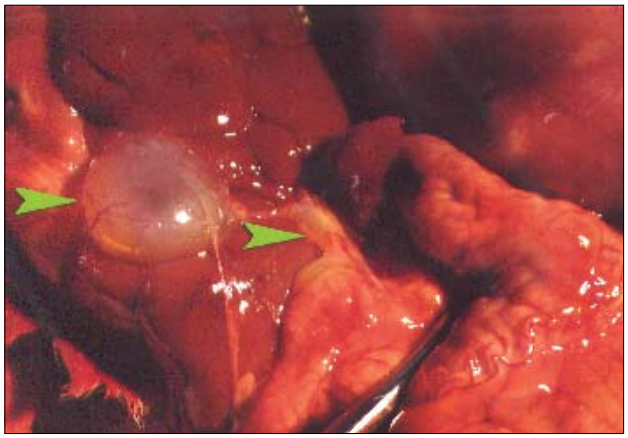

Figure 1 – The gallbladder augmentation and common bile duct distention is evident, and the end of choledoch oncoide, bile stasis in a 60-day pregnant guinea pig.

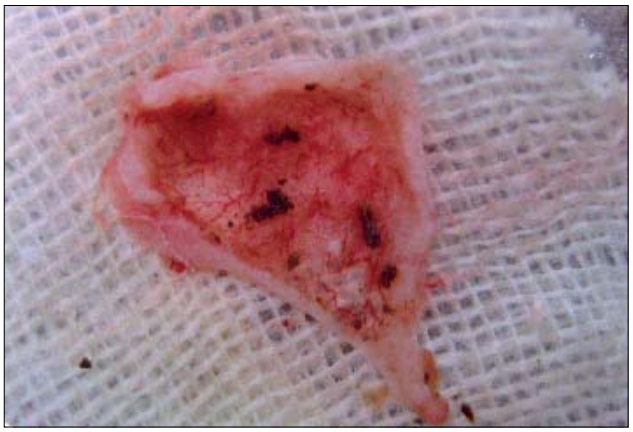

Figure 2 – Cholecystolithiasis formed in the 60 days of pregnancy.

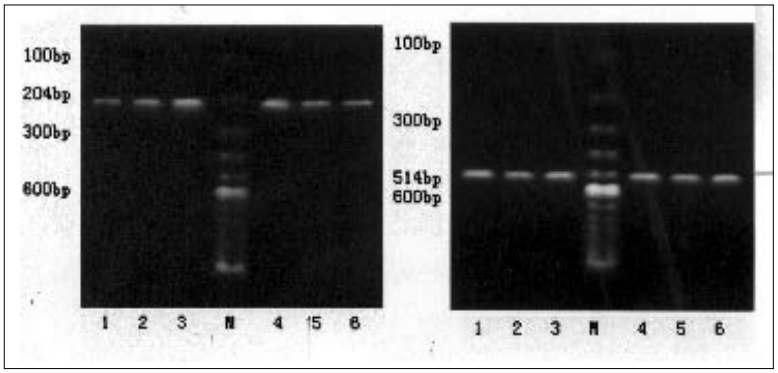

Figure 3 – Detection of h1 calponin mRNA (panel left) and b-actin (panel right) expression by RT-PCR. The PCR amplifications were done for h1 calponin mRNA and b-actin from gallbladder muscles (lane 1-3) and OS (lanes 4-6). Lanes 1 and 4 represent group A, and lanes 2, 5 group B, lanes 3, 6 group C. M represents the DNA size marker.

2 unit of Taq DNA polymerase, 5 μl 10 × buffer, 2 mM $Mgcl_2$, 200 nM each of dNTP, 50 pM each of sense and antisense primers. The primers and thermal cycle profiles used for h_1 calponin were described in the previously published literature.[20] β-actin had the following sequence: sense 5'-TGT GAT GGT GGG AAT GGG TCG G-3', antisense 5'-TTT GAT GTC ACG CAC GAT TTC C-3', which yielded a 514bp product. The cycling conditions used for β-actin were as follows: 5 minutes at 95°C, followed by 30 cycles of 30 seconds 95°C, 60 seconds 50°C, and 30 seconds 72°C. Polymerase chain reaction was completed by a final extension step of 10 minutes at 72°C. The 2 primer pairs were synthesized by biology and technology col. ltd, Augct, Beijing. A 10 μl of PCR product was electrophoresed in 2% agarose gel. The gel was stained with ethidium bromide (EB) and photographed. The absorbency of electrophoresis bands was scanned with computer image disposal system. Expression levels of h_1 calponin mRNA were presented by ratio of h_1 calponin mRNA absorbency and β-actin absorbency.

Statistical analysis. All data were expressed as mean ± standard deviation (SD). The Statistical Package for Social Sciences version 11.5 software was used for one-way analysis of variance (Student-Neuman-Keuls, SNK). A p value <0.05 was considered statistical significant.

Results. ***Changes of observation.*** The body weight of pregnant guinea pigs increased significantly. Group B was 560 gram to 740 gram, and group C was 760 gram to 830 gram. The increasing fasting volume of gallbladder-expanding end of choledochus and cholestasis were observed in all animals of group C. After opening the gallbladder, 3 animals of group

C and one of group B demonstrated a gallstone formation. **Figure 1,2**.

Biochemechal analysis of bile. The composition of bile obviously changed in pregnant guinea pigs. Concentrations of both TC and TBiL increased in group B and C, while that of TBA decreased. The concentration of TC (0.03 ± 0.01) mmol/L in group A was increased to that (0.08 ± 0.05) mmol/L in group C (SNK, F=7.227, $p<0.05$), and similarly that of TBiL (27.02 ± 4.56) μmol/L was increased to that (38.53 ± 14.49) μmol/L (SNK, F=4.170, $p<0.05$). While the concentration of TBA (607.65 ± 88.84) μmol/L in group A was decreased to that (341.27 ± 22.00) μmol/L in group C (SNK, F=60.252, p<0.01). These data indicated gallstones were forming in pregnant guinea pigs (**Table 1**).

The changes of serum E_2 and Pg levels. The concentration of E_2 and Pg increased significantly as pregnancy progressed. In comparison with the group A, concentration of E_2 (40.33 ± 5.84) pg/ml and Pg (102.37 ± 40.24) ng/ml in group C increased obviously (F (E_2)=31.359, F (Pg)=32.643, $p<0.001$) (**Table 2**).

The expression levels of h_1 calponin mRNA. In the gallbladder smooth muscles, the expression levels of h_1 calponin mRNA (0.92 ± 0.07) in group C were significantly higher than that (0.57 ± 0.05) in group A (F=115.020, $p<0.001$). On the contrary, in OS, h_1 calponin mRNA expression levels (0.54 ± 0.04) in group C were lower than that (0.97 ± 0.09) in group A (F=99.405, $p<0.001$) (**Table 3**). As shown in **Figure 3**, calponin h_1 mRNA in group C was expressed more abundantly than that in group A in gallbladder smooth muscles. While it was conversely in OS.

Table 1 - Changes of bile biochemical composition in guinea pig.

Groups	TC (mmol/L)	TBiL (μmol/L)	TBA (μmol/L)
A (n=10)	0.03 ± 0.01	27.02 ± 4.56	607.65 ± 88.84
B (n=10)	0.06 ± 0.03	29.67 ± 5.54	505.04 ± 25.25†
C (n=10)	0.08 ± 0.05†	38.53 ± 14.49*	341.27 ± 22.00†‡

*$p<0.05$ versus group A, †$p<0.01$ versus group A, ‡$p<0.01$ versus group B.
TC - total cholesterol, TBiL - total bilirubin, TBA - total bile acid

Table 2 - Changes of serum E_2 and Pg concentration in guinea pig.

Groups	E_2 (pg/ml)	Pg (ng/ml)
A (n=10)	18.21 ± 4.07	6.23 ± 4.57
B (n=10)	28.33 ± 8.16*	51.32 ± 22.03*
C (n=10)	40.33 ± 5.84‡†	102.37 ± 40.24‡†

*$p<0.01$ versus group A, ‡$p<0.001$ versus group A, †$p<0.001$ versus group B.
E_2 - serum estradiol, Pg - progesterone.

Table 3 - Changes of h_1 calponin mRNA expression level in guinea pig.

Groups	Gallbladder smooth muscle	OS
A (n=10)	0.57 ± 0.05	0.97 ± 0.09
B (n=10)	0.68 ± 0.05*	0.75 ± 0.07*
C (n=10)	0.92 ± 0.07*†	0.54 ± 0.04*†

*$p<0.001$ versus group A, †$p<0.001$ versus group B.
OS - Oddi's sphincter.

Discussion. Gallstone disease is a complex disorder where both environmental and genetic factors contribute towards susceptibility to the disease. It is a major health problem worldwide, particularly in adult population. The risk of gallstone formation is associated with gender, obesity, age, family history and ethnic background of individuals in the population. Previous studies have shown that pregnancy is a gallstone conducive state. But the exact mechanisms of gallstone formation during pregnancy remains unclear. We studied the cause of biliary tract disorder during pregnancy through examining the changes of h_1 calponin gene expression in the pathway of muscle contraction, and did some helpful exploration for research of gallstone etiology in pregnancy.

It is well known that the engorging and emptying of gallbladder are result of mutual effects of gallbladder, cystic duct and OS. Gallbladder hypomotility can lead to increase fasting volumes of cholecyst, prolong the cholerrhagia and promote cholestasis. The dysfunction of OS appears to increase contractility and block the normal bile excretion. The disordered motility of gallbladder and OS, and incomplete evacuation of bile from the gallbladder promote stasis of bile in the gallbladder, and static bile further facilitates nucleation, crystallization and agglomeration into gallstone.

Our study firstly investigates the molecular mechanisms of gallbladder hypomotility and OS pressure increasing in pregnancy at gene grade. The molecular mechanisms of smooth muscle contraction involve the 2 pathways: 1. Myosin light chain kinase (MLCK). The kinase phosphorylate MLCK result in activation of ATPase and smooth muscle contraction.[21] 2. Protein kinase C (PKC). Phosphorylation of

calponin by PKC cause loss of its ability to inhibit ATPase, which also promote the smooth muscle contraction.[22-24] The h_1 calponin is a smooth muscle-specific contractile protein.[10,18,19] It was reported previously that the expression levels of myosin in OS had no changes during gallstone formation in guinea pig, whereas that of calponin decreased.[20] It indicated that calponin expressions in animals with gallstone were specific, which maybe take the key regulatory protein for the motility of bile duct. Our experimental results reveal that the levels of h_1 calponin mRNA in pregnant animals are more significantly increasing than that of in nonpregnant group in gallbladder smooth muscles. In contrast, it decreases in OS. In previous studies, it has been demonstrated that calponin inhibits actomyosin ATPase activity and makes smooth muscle contraction subdued.[19,25] So, the up-regulation of h_1 calponin mRNA expression would induce increasing h_1 calponin expression, and it will result in strengthening inhibition of smooth muscle contraction, gallbladder muscles contraction impaired and bile stasis in gallbladder. In contrast, the expression levels of h_1 calponin mRNA decreased in OS in pregnant guinea pigs, which would induce down-regulation of h_1 calponin and weak the inhibition of smooth muscle contraction, and thereby caused OS pressure increasing and bile drainage being handicapped. These changes make the conspiracy of bile duct on bile excretion upside down and cause cholestasis. Furthermore, it aggravates the bile stasis and makes gallbladder bile easier to be nucleating and retention of the precipitated microcrystals, agglomerating into stones. In our experiment, we observed that the fasting gallbladder volumes increasing, cholestasis in cholecyst, and the end of the common bile duct expanding and cholestasis existed in all pregnant animals, and there were 1 animal in group B and 3 in group C developed gallstone. These results indicate that the disorganized bile duct kinetics and changes of bile hydrodynamics promote gallstone formation in pregnant guinea pigs.

In addition, previous studies have shown that pregnancy can increase prevalence of cholelithiasis. The high levels of circulating progesterone can weak gallbladder muscle in response to CCK and reduce its contraction.[9] It can interfere with the expression of CCK receptor[26,27] or G protein coupled with CCK receptor, and result in abnormal signal transduction. Also, progesterone can increase OS pressure.[28] While estrogen may increase biliary cholesterol saturation.[29] And it also has the function of inhibiting OS motility.[30] Other experiment had demonstrated that estradiol could markedly degrade the amplitude and frequency of gallbladder muscles contraction. Our experiment indicate that the concentration of serum E_2 and Pg increase as pregnancy progresses and some of the pregnant animals were found with gallstone formation, and the bile composition such as TC, TBiL and TBA altered obviously in pregnant animals, which was in accordance with changes of lithogenic bile. These provide the evidence that pregnancy promotes incidence of cholelithiasis. So, we supposed that it be estrogen and progesterone that induced the changes of h_1 calponin mRNA expression in gallbladder muscles and OS in pregnancy. And its mechanism may be that estrogen and progesterone bind into each corresponding receptor in the gallbladder and OS, following to trigger the changes of G protein or CCK receptor expression and dysfunction of signal synthesis, and lead to abnormality of h_1 calponin mRNA expression through pathway of PKC, which stimulates the muscles contraction or relaxation, in the end. But it remains to be further investigated that the relationship between hormones of pregnancy and expression of h_1 calponin mRNA.

In summary, we suggest that the changes of h_1 calponin mRNA expression in gallbladder smooth muscles and OS in pregnant guinea pigs may be responsible for the disorganization of biliary duct motility and gallstone formation in pregnancy. We can conceive that if we take measures to prevent female steroid hormones from affecting gallbladder smooth muscles and OS contraction in pregnancy, or reverse the intracellular h_1 calponin mRNA expression levels of gallbladder muscles and OS, it will contribute to preventing emergence of gallstone formation in pregnancy. However, during gallstone formation in gestation, the agonists and the mechanisms of signals transduction and regulation induced the changes of h_1 calponin mRNA expression, which remain to be further explored in the future.

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References

1. Graham G, Baxi L, Tharakan T. Laparoscopic cholecystectomy during pregnancy: A case series and review of the literature. ***Obstet Gynecol Surv*** 1998; 53: 566-574.
2. Barbara L, Sama C, Labate AMM, Taroxi F, Rusticali AG, Festi D. A population study on the prevalence of gallstone disease: the Sirmoine study. ***Hepatology*** 1987; 7: 913-917.
3. Scragg RKR, McMichael AJ, Seamark RF. Oral contraceptives, pregnancy, and endogenous oestrogen in gallstone disease: a case-control study. ***BMJ*** 1984; 288: 1795-1799.
4. Carlos A. C, Bahman S, Nicolas J, Steven CS, David G, Dilip P, et al. Surgical management of biliary gallstone disease during pregnancy. ***Am J Surg*** 1999; 178: 545-548.

5. Xu QW, Scott RB, Shaffer EA. Adverse effect of female sex hormones on biliary lipid composition in the ground squirrel: different influences on the liver versus gallblader and intestinal motility. ***Gastroenterology*** 1996; 110: A1362.
6. Ryan JP. Effect of pregnancy on gallbladder contractility in the guinea pig. ***Gastroenterology*** 1984; 87: 674-678.
7. Ryan JP. Calcium and gallbladder smooth muscle contraction in guinea pig: effect of pregnancy. ***Gastroenterology*** 1985; 89: 1279-1285.
8. Chen Q, Chitinavis V, Xiao ZL, Yu P, OH S, Biancani P, Behar J. Impaired G protein function in gallbladder muscle from progesterone-treated guinea pig. ***Am J Physiol*** 1998; 274: G283-G289.
9. Xiao ZL, Chen Q, Piero B, Jose B. Mechanisms of gallbladder hypomotility in pregnant guinea pigs. ***Gastroenterology*** 1999; 116: 411-419.
10. Matthew JD, Khromov AS, McDuffie MJ. Contractile properties and proteins of smooth muscles of a calponin knockout mouse. ***J Physiol*** 2000; 529: 811-824.
11. Sugenoya Y, Yoshimura A,Yamamura H. Smooth muscle calponin in mesangial cells: Regulation of expression and a role in suppressing glomerulonephritis. ***J Am Soc Nephrol*** 2002; 13:322-331.
12. Miettinen MM, Sarlomo RM, Kovatich AJ. Calponin and Yh caldesmon in soft tissue tumors:consistent h-caldesmon immunoreactivity in gastrointestinal stromal tumors indicates traits of smooth muscle differentiation. ***Mod Pathol*** 1999; 12: 756.
13. Horiuchi A, Nikaido T, Ito K. Reduced expression of Calponin h1 in leiomyosarcoma of the uterus. ***Lab Invest*** 1998; 78: 839-846.
14. Mosunjac MB, Lewis MM, Lawson D. Use of a novel marker, calponin, for myoepithelial cells in fine needle aspirates of papillary breast lesions. ***Diagn Cytopathol*** 2000; 23: 151-155.
15. Horiuchi A, Nikaido T, Taniguchi S. Possible role of calponin h1 as a tumor suppressor in human uterine leiomyosarcoma. ***J Natl Cancer Inst*** 1999; 91: 790-796.
16. Gao JM, John MH, Jin JP. Complete nucleotide sequence, structural organization, and an alternatively spliced exon of mouse h1-calponin gene. ***Biochem Biophys Res Commun*** 1996; 218: 292-297.
17. Chie S, Junji N, Sei K, Shoske T, Hideo K. Expression of calponin mRNA in porcine aortic endothelial cells. ***Biochem Biophys Res Commun*** 1996; 222: 195-200.
18. Tang DC, Kang HM, Jin JP, Fraser ED, Walsh MP. Structure-Function relations of smooth muscle calponin: the critical role of serine-175. ***J Bio Chem*** 1996; 271: 8605-8611.
19. Jin JP, Wu D, Gao JM, Rita N, Stephen K. Expression and purification of the h1 and h2 isoforms of calponin. ***Prot Expr Purif*** 2003; 31: 231-239.
20. Lu WT, Tang DC, Cao S, Yu CS. The study on the relationship between the expression of calponin and gallstone formation. ***J Tongji Med Univ*** 1997; 17: 86-89.
21. He J, Stephens NL. Calcium and smooth muscle contraction. ***Mol Cell Biochem*** 1994; 135:1-9.
22. Walsh MP. Calmodulin and the regulation of smooth muscle contraction. ***Mol Cell Biochem*** 1994; 135: 21-24.
23. Tang DC, Xiang JZ, Lu WT. Calponin: a new regulatory protein for smooth muscle contraction. ***Prog Biochem Biophys*** 1996; 23: 325-329.
24. Mohammed EM. Calponin. ***Int J Biochem Cell Biol*** 1996; 28: 1185-1189.
25. Takahashi K,Yoshimoto R, Fuchibe K, Fujishige A, Mitsuisaito M, Hori M, et al. Regulation of shortening velocity by calponin in intact contracting smooth muscles. ***Biochem Biophys Res Commun*** 2000; 279: 150-157.
26. Norikazu S, Kyoko M, Shinji S, Setsuko K, Minoru O, Takako K, et al. Lack of Cholecystokinin-A Receptor Enhanced Gallstone Formation. ***Dig Dis Sci*** 2003; 48: 1944-1947.
27. Miyasaka K, Takata Y, Funakoshi A. Association of cholecystokinin A receptor gene polymorphism with cholelithiasis and the molecular mechanisms of this polymorphism. ***J Gastroenterol*** 2002; 37: 102-106.
28. Sean T, Attila N, Cliver W, Pamela A, Lipsett, Samuel S, et al. Progesterone alters biliary flow dynamics. ***Am Surg*** 1999; 229: 205-209.
29. Everson GT. Gallbladder function in gallstone disease. ***Gastroenterol Clin North America*** 1991; 20: 85-110.
30. Tierney S, Qian Z, Lipsett PA, Pitt HA, Lillemoe KD. Estrogen inhibits sphincter of Oddi motility. ***J Surg Res*** 1994; 57: 69-73.