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DOES BILE PROTECT OR DAMAGE INTERSTITIAL CAJAL-LIKE CELLS IN THE HUMAN GALLBLADDER?

Abstract: The etiology of gallstone disease is considered to be multifactorial, including biliary cholesterol hypersecretion, supersaturation and crystallization, stone formation, bile stasis and mucus hypersecretion and gel formation. Gallbladder hypomotility seems to be a key process that triggers the precipitation of cholesterol microcrystals from supersaturated lithogenic bile. The purpose of the current study was to determine whether ICLCs in the gallbladder were influenced by lithogenic bile. Gallbladder specimens were collected from 30 patients (8 males and 22 females) who underwent elective laparoscopic cholecystectomy for symptomatic gallstone disease. The control group consisted of 25 consecutive patients (11 males and 14 females) who received elective treatment for pancreatic head tumors. ICLCs were visualized in paraffin sections of gallbladders using double immunofluorescence protocol with monoclonal c-kit antibodies and mast cell tryptase. Cholesterol, phospholipid and bile acid concentrations were measured in bile samples obtained by needle aspiration from the gallbladder at the time of surgery. The number of ICLCs in the gallbladder wall was significantly lower in the study group than in the control group (3.35 ± 1.23 vs. 7.06 ± 1.82 cell/FOV in the *muscularis propria*; $P < 0.001$) and correlated with a significant increase in the cholesterol saturation index. The glycocholic and taurocholic acid levels were significantly elevated in the control subjects compared with the study group. The results suggest that bile composition may play an important role in the reduction of ICLC density in the gallbladder.

Key words: interstitial Cajal-like cells, gallstones, cholesterol saturation index, bile acids, bile lithogenicity.

INTRODUCTION

In Europe, 10–20% of the population carry gallbladder stones [1–3]. Many gallstones are silent, but symptoms and severe complications ensue in more than 40% of patients above the age of 40 years, necessitating laparoscopic cholecystectomy [3]. In developed countries, cholesterol gallstones account for 80–90% of the gallstones found at cholecystectomy [4]. The etiology of gallstone disease is considered to be multifactorial, including biliary cholesterol hypersecretion, supersaturation and crystallization, stone formation and growth, bile stasis and mucus hypersecretion and gel formation within the gallbladder. Gallbladder hy-

pomotility may be a key factor in the pathogenesis of cholelithiasis because it allows time for cholesterol microcrystals to precipitate from lithogenic bile that is supersaturated with cholesterol [5–8].

In the light of recent data our knowledge of the physiology of alimentary tract has expanded by the study of a population of specialized cells, the so-called interstitial cells of Cajal (ICCs), which were first described by the Spanish neuropathologist, Ramon Santiago y Cajal in 1889 [9]. These cells have been successfully identified using modern techniques, including immunohistochemistry and electron microscopy [10–12]. ICCs are found along the entire gastrointestinal tract, mostly in close proximity to smooth muscle. ICCs are considered to play an important role in smooth muscle motility. ICCs generate slow waves in phasic gastrointestinal muscles, actively propagate slow waves, and mediate or transduce neural inputs from enteric motor neurons to smooth muscles [13]. One important advance in ICC identification was the recognition that ICCs express the proto-oncogene c-kit, which encodes the transmembrane tyrosine kinase receptor protein c-Kit (CD117) [14–16]. Although the shapes of ICCs vary among species, tissues and tissue layers, all ICCs have the same immunophenotypic characteristics. Therefore, the c-Kit receptor is a reliable marker of ICCs.

The interstitial cells of Cajal were also found in multiple non-enteric organs and referred as to interstitial Cajal-like cells (ICLC), however their morphology and function were best understood in the gastrointestinal system. In the gut, they are believed to act as pacemaker cells, and their loss or dysfunction has been linked to a variety of intestinal motility disorders [17–19]. Therefore, a decrease in the density of ICLCs in the muscle layer of the gallbladder could induce bile stasis and lead to gallstone formation.

The aim of this study was to determine whether ICLC loss was related either to the degree of bile lithogenicity, which was expressed as a lithogenic index (cholesterol saturation index, CSI), or to the bile salt concentrations in patients who suffer from cholecystolithiasis and gallstone-free controls.

MATERIALS AND METHODS

SUBJECTS

Thirty consecutive patients with symptomatic gallstone disease were scheduled for elective surgery (laparoscopic cholecystectomy) and enrolled to the study group (8 males, mean age 51.9 ± 10.7 y; 22 females, mean age 52.9 ± 15.1 y) (Table 1). The presence of gallstones in the gallbladder was confirmed on ultrasound examination before the operation. They presented with mild, recurrent episodes of biliary colic. None of these patients had associated choledocholithiasis or acute cholecystitis. The control group consisted of 25 consecutive patients (11 males, mean age 62.0 ± 7.6 y; 14 females, mean age 61.0 ± 9.8 y), who were electively

treated for pancreatic head tumors and had no pre- or intraoperative signs of cholelithiasis or jaundice (Table 1). Pancreaticoduodenectomy was performed according to the standard Whipple procedure or the pylorus-sparing Traverso-Longmire technique in patients with resectable lesions. For patients with non-resectable lesions, retrocolic gastroenterostomy with Braun anastomosis was carried out for palliative treatment. Gallbladders that were not affected by primary tumors and did not contain any gallstones were routinely removed. Serum bilirubin levels were measured preoperatively and were normal in both groups. All patients were surgically treated in the First Chair of General, Oncological and Gastrointestinal Surgery at the Jagiellonian University Medical College in 2010.

Table 1

Epidemiological data of patients enrolled in the study.

		Male	Female
Study group	n	8	22
	mean age ± SD	51.9 ± 10.7	52.9 ± 15.1
Control group	n	11	14
	mean age ± SD	62.0 ± 7.6	61.0 ± 9.8
Total	n	19	36
	mean age ± SD	56.9 ± 10.94	54.8 ± 13.6

n — number of patients

ETHICAL APPROVAL AND CONSENT

All surgical samples were retrieved with ethical approval of the Jagiellonian University Bioethical Committee (KBET/30/B/2010), and after full written informed patient consent.

SPECIMENS

Small tissue samples from fresh gallbladder specimens were rinsed with PBS (phosphate-buffered saline, 0.01 M, pH = 7.4), fixed in 4% phosphate-buffered paraformaldehyde, processed and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides and stained with hematoxylin and eosin (H&E) for routine histopathology.

DOUBLE IMMUNOFLUORESCENCE PROTOCOL

Indirect double immunofluorescence after heat-induced epitope retrieval was used to allow the simultaneous visualization of two antigens. The sections were in-

Table 2

Primary antibodies used for immunohistochemical reactions.

Antibody	Species/clonality	Dilution	Manufacturer, Catalog number
Human C-kit/CD117	rabbit/policlonal	1:150	Dako, Glostrup, Dania, A4502
Human mast cell tryptase	mouse/monoclonal	1:800	Dako, M7052

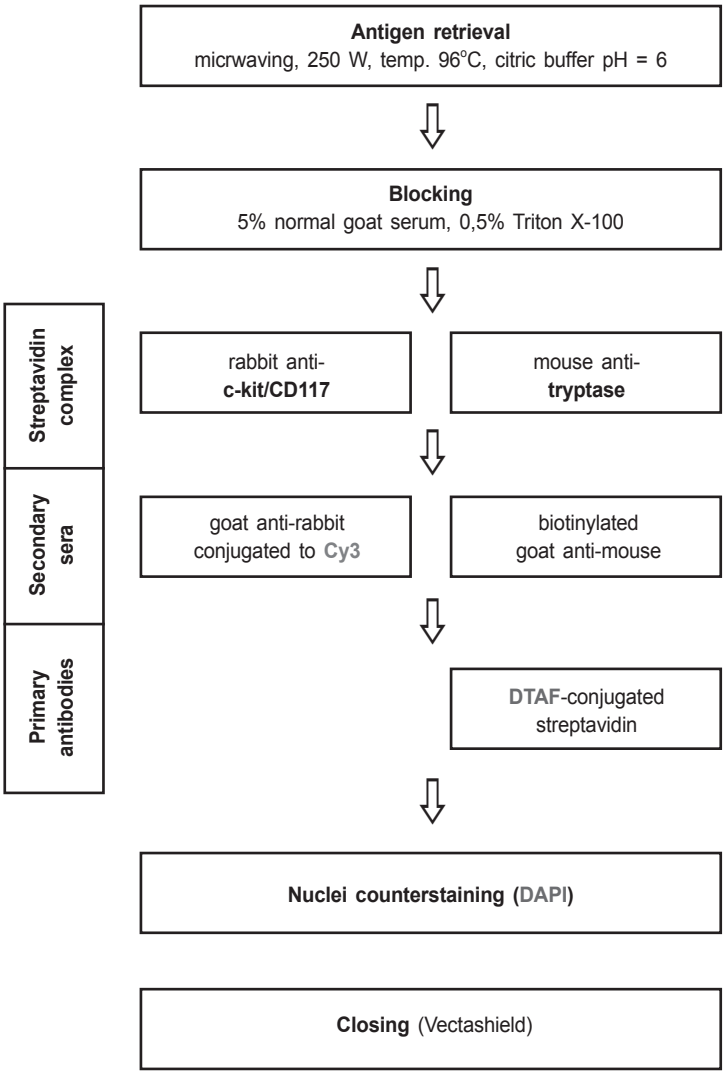

Fig. 1. Block diagram illustrating the steps of double immunostaining protocol applied in the study.

cubated overnight with a mixture of rabbit polyclonal anti-c-Kit antibody (anti -CD117; A4502; Dako, Glostrup, Denmark; diluted 1:150) and mouse monoclonal anti- mast cell tryptase antibody (M7052; Dako; 1:800) (Table 2). The sections were rinsed in PBS and incubated for 1 h at room temperature with a mixture of a Cy3-conjugated goat anti-rabbit antibody (111-165-144; Jackson ImmunoResearch, West Grove, PA, USA; 165-144; 1:600) and a biotinylated goat anti-mouse antibody (115-065-146; Jackson IR; 1:600). After washing in PBS, the slides were incubated with DTAF-conjugated streptavidin (016-010-084; Jackson IR; 1:500 in PBS) for 1 h. After a final rinse in PBS, the nuclei were counterstained with DAPI (D9542; Sigma, St. Louis, MO, USA; 1:30,000) for 30 s. The sections were mounted in Vectashield medium (H-1000; Vector Laboratories, Burlingame, CA, USA) to minimize fluorophore photobleaching (Fig. 1).

MICROSCOPIC EXAMINATION AND QUANTIFICATION OF ICLCS

Microscopy images were captured using Olympus AnalySIS FIVE software on an Olympus BX50 epifluorescence microscope (Olympus, Tokyo, Japan) with an Olympus DP71 digital CCD camera and stored as .TIFF files. Concurrent mast cell tryptase staining enabled c-Kit-positive mast cells to be distinguished from ICLCs. The distribution of ICLCs in the gallbladder corpus was quantitatively assessed by applying the criteria for ICCs (c-Kit positive and tryptase negative). These cells were counted in 10 consecutive high-power fields (400×). The data were expressed as a mean number of cells per 1 field of view (FOV) of gallbladder *muscularis propria*.

GALLBLADDER BILE COLLECTION, SAMPLE ANALYSIS AND LITHOGENECITY INDEX CALCULATION

Bile specimens were obtained from all subjects by needle aspiration of the gallbladder during surgery. Aliquots of bile samples were stored at –70°C prior to biliary lipid composition analysis. The cholesterol and phospholipid concentrations and bile acid levels in the bile samples were determined.

The bile samples were extracted on SEP-PACK-NH_2 columns (500 mg, Waters, USA). Each column was first activated using 6 ml of n-hexane; then, 0.1 ml of centrifuged bile sample was applied to the column, and the flow-through was discarded. The samples were eluted in three 1-ml volumes of a chloroform-isopropanol mixture (3:1, v/v), followed by three 1-ml volumes of methanol. The eluted fractions from each column were collected and dried at 50°C under nitrogen.

The dry residues were reconstituted in 0.5 ml of isopropanol and mixed vigorously. Cholesterol (Randox Laboratories Ltd., UK) and phospholipid (Wako Chemicals, Neuss, Germany) concentrations were measured by enzymatic

methods. The intra- and interassay coefficient of variations were 3% and 4.8% for cholesterol and 5% and 6% for phospholipids, respectively. All determinations were performed with a Cobas-Bio analyzer (Roche).

Individual bile acids were measured by reverse-phase high-performance liquid chromatography with an isocratic solvent system (Waters, USA). Prior to chromatographic separation, bile acids were extracted from bile samples using SEP-PACK C18 columns (Waters, USA). All columns were activated using 5 ml of methanol and 5 ml of water, after which 0.1 ml of bile mixed with phosphate buffer (0.07 mmol/l, pH 7.0) and 0.1 ml of internal standard were applied. Then, the columns were washed with 10 ml of water, 3 ml of 10% acetonitrile and another 10 ml of water. The bile acids were eluted in 3 ml of methanol. The eluates were dried at 37°C under nitrogen, and dry residues were re-dissolved in 1 ml of an acetonitrile-water mixture (1:1, v/v). Each sample was filtered using a Millex GN filter (13 mm) and separated chromatographically using an XTERRA RPC-18 column (18.5 µm × 3.9 mm × 150 mm, Waters, USA) with detection at 200 nm.

The mobile phase (flow rate 2 ml/min) contained 10% acetonitrile in a mixture of methanol and 0.1 M monobasic potassium phosphate (60:40, v/v, pH 4.50). Before use, the solvent was filtered through a 0.45 µm filter (type HV, Millipore, Bedford, MA, USA). An elution profile of conjugated bile acid standards (Sigma, St. Louis, MO, USA) was obtained by injecting 20 µl of a standard bile acid mixture in methanol that contained glycocholic acid (1.640 mmol/l), taurocholic acid (1.488 mmol/l), glycochenodeoxycholic acid (1.696 mmol/l), glycodeoxycholic acid (1.696 mmol/l), taurochenodeoxycholic acid (1.532 mmol/l) and taurodeoxycholic acid (1.532 mmol/l).

Based on cholesterol, phospholipid and total bile acid concentrations, the cholesterol saturation index (CSI) also known as lithogenecity index was obtained from Carey's critical tables [20, 21]. Bile samples with a CSI equal to 1 or more were considered supersaturated.

DATA ANALYSIS AND STATISTICAL EVALUATION

The data were expressed as mean and standard deviation (SD). The results were analyzed using a one-way analysis of variance (ANOVA), followed by a post-hoc LSD test. Pearson's correlation test was used to examine the relationship between continuous variables. *P* values less than 0.05 were considered to indicate statistical significance. All statistical analyses were performed using STATISTICA 9.0 software (StatSoft, Tulsa).

RESULTS

The interstitial Cajal-like cells were identified as c-Kit-positive nucleated cells with no co-expression of mast cell tryptase. ICLCs were located predominantly in the gallbladder corpus, but were also spotted occasionally in its fundus and neck. ICLCs had a centrally located nucleus and were showing characteristic spindle morphology; nevertheless, sparse, round tryptase-negative/c-Kit-positive cells were also present. Numerous ICLCs were detected, mostly in the *muscularis propria*, and some ICLCs were observed in the connective tissue separating smooth muscle bundles (Fig. 2).

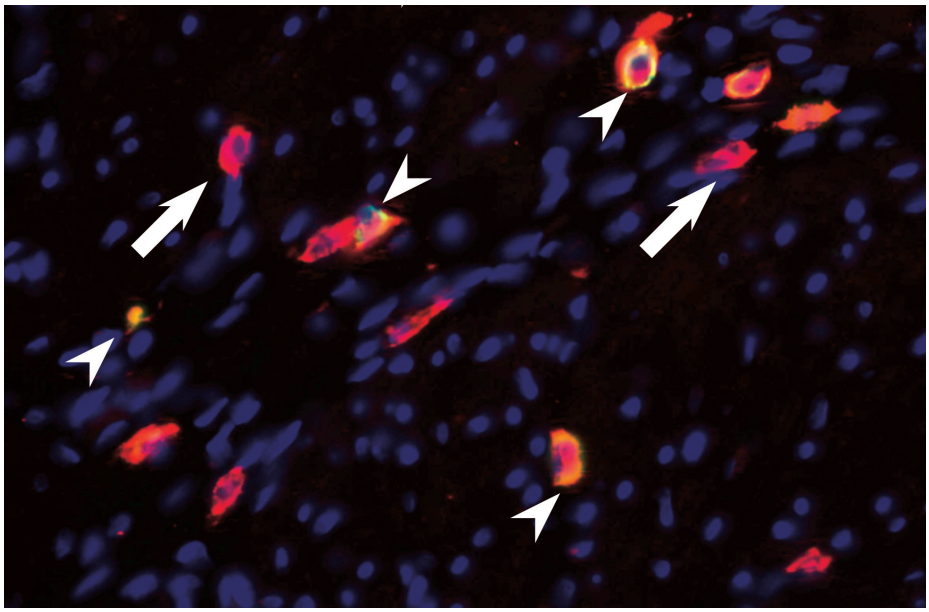

Fig. 2. Cross-section of the gallbladder wall stained for CD117 (red) and tryptase (green). The nuclei are counterstained with DAPI (blue). CD117-positive/tryptase-negative ICLCs (arrows) and CD117-positive/tryptase-positive mast cells (arrowheads) are indicated.

The number of ICLCs in the gallbladder wall corpus was significantly lower in the study group than in the control group (3.35 ± 1.23 vs. 7.06 ± 1.82 cell/FOV in the muscularis propria; $P < 0.001$) (Table 3).

Mast cells were present in all layers of the gallbladder wall and predominantly localized to the lamina propria. In immunostained slides, c-Kit and tryptase double-positive mast cells were generally round or oval shaped, with a centrally located nucleus.

Using mast cell tryptase to identify and distinguish c-kit positive mast cells from ICLCs highlighted the morphological variability in mast cells in these spe-

Table 3

Number of ICLCs per field of view and mm^2 of tissue. Study group vs control group.

	Study group			Control group		
	Mean value	SD	95% CI	Mean value	SD	95% CI
ICLC/FOV	3.35	1.24	2.85–3.85	7.06	1.82	6.20–7.40
ICLC/mm^2 of tissue	26.27	11.27	22.06–30.48	56.42	14.62	49.58–63.26

cimens and the potential for misidentification with conventional c-kit immunohistochemistry.

The study revealed a significant decrease in the mean concentrations of glycocholic and taurocholic acids in the bile from patients with cholelithiasis as compared with the controls ($P < 0.02$ and $P < 0.05$, respectively). Furthermore, there was also a positive correlation between the ICLC count and the concentrations of glycocholic ($r = 0.45$, $P = 0.039$) and taurocholic ($r = 0.32$, $P = 0.05$) acids.

No significant differences in the concentrations of the other bile acids examined (glycochenodeoxycholic, glycodeoxycholic, taurochenodeoxycholic and taurodeoxycholic acid) were observed between the 2 groups of patients. There were also no significant differences in the concentrations of cholesterol, bile salts or phospholipids in the bile between the 2 groups (Table 4).

Table 4

Biliary lipid composition of gallbladder bile sampled at the time of surgery (mmol/l). Data are mean ± standard deviation (SD).

	Study group		Control group	
	Mean value	SD	Mean value	SD
BS	92.96	54.78	114.1	65.74
PC	32.38	17.57	31.89	21.27
Ch	8.72	6.44	7.74	6.57
GCA	35.66	16.66	43.44	32.40
TCA	9.00	7.66	15.18	11.09
GCDCA	24.64	14.94	25.71	16.41
GDCA	22.00	13.24	17.23	16.57
TCDCA	8.64	6.55	9.69	7.00
TDCA	3.01	1.46	2.34	1.30

BS — bile salts, PC — phospholipids, Ch — cholesterol, GCA — glycocholic acid, TCA — taurocholic acid, GCDCA — glycochenodeoxycholic acid, GDCA — glycodeoxycholic acid, TCDCA — taurochenodeoxycholic acid, TDCA — taurodeoxycholic acid

The calculated cholesterol saturation index was significantly higher in patients with cholecystolithiasis (1.23 ± 0.84) than in the controls (0.78 ± 0.33) ($P < 0.05$). The results revealed an important negative correlation (r = –0.62, $P = 0.001$) between the CSI and the ICLC count in the gallbladder wall.

DISCUSSION

The discovery that ICC could be labeled with antibodies to the c-kit, a tyrosine kinase receptor coded by the proto-oncogene c-kit, led to the successful characterization of ICC throughout the gut [22], which had only been thought possible previously using TEM.

Most importantly, the kit receptor is a specific marker for ICC and kit antibodies have been used extensively to identify ICC in gastrointestinal tissues as well as in many extraintestinal organs where these cells are known as interstitial Cajal-like cells (ICLCs). We used paraffin sections labeled with c-kit to provide information about these cells in the human gallbladder. As a result, the ICLCs were found throughout the thickness of the gallbladder wall, from the gallbladder dome to the bladder neck. We reported a significant decrease in the density of ICLCs in the gallbladder wall in patients with gallstones. Twofold reduction of the number of interstitial Cajal-like cells may significantly affect motility of the gallbladder and result in cholelithiasis. Literature reports existence of correlation between reduced number of ICC in gastrointestinal diseases and abnormal gastrointestinal motility [23–25]. Numerous papers indicate presence of abnormalities of the gallbladder emptying in patients with cholecystolithiasis [26–28]. Two recent studies in guinea pigs would support this. Lavoie *et al.* showed that the kit receptor regulates the excitability of gallbladder smooth muscle by modulating the generation and rhythmicity of electrical activity [29]. Xu *et al.* found that gallbladder contraction is mediated by CCK-A receptors and these are expressed by ICC [30]. On this basis one can hypothesize that abnormal gallbladder motility may result from low number of Cajal-like cells in the gallbladder wall and lead to abnormalities in bile outflow, ultimately with formation of concrements in its lumen. From a physiological point of view, Cajal-type of interstitial cells might represent, through analogy to the gastrointestinal tract an essential player in the physiology of a digestive cavitary organ such as gallbladder, imposing the rhythm of bile release (pace-maker cells). The presented results confirm our previous findings that the number of c-Kit immunopositive (concomitantly tryptase-immunonegative) cells that from the phenotypic point of view could correspond to Cajal-like cells, is reduced in patients with cholelithiasis [14]. Some authors claim that impaired gallbladder motility is evoked by absorption of cholesterol from lithogenic bile through the gallbladder wall [31]. Excessive amounts of cholesterol in the smooth muscles of the gallbladder wall may stiffen the sarcolemmal mem-

brane and impair the signal transduction mediated by protein G, resulting from CCK-A binding to its receptor, ultimately paralyzing the gallbladder contractility [32]. Therefore, we evaluated the cholesterol saturation index (CSI), which is an accepted parameter relevant to bile lithogenicity in both groups of patients [33]. We found an increased CSI in the patients with gallstones, which could be related to the loss of ICLCs. However, the exact mechanism underlying the destructive influence of bile on ICLCs remains still unclear. Our findings are supported by Hu *et al.*, who demonstrated that the expression of c-Kit mRNA and c-Kit protein in the gallbladder wall were significantly decreased in the gallbladders of guinea pigs fed a high cholesterol diet [34]. However, the Hu *et al.* study provided insight into the pathogenesis of gallstone formation, similar to the studies by Xu and Shaffer, by showing that gallbladder hypomotility resulted from increased cholesterol levels [35]. The excess cholesterol in the smooth muscle of the gallbladder attenuates the ability of the muscle to contract as a result of alterations in signal transduction, changes in ion channel activity, decoupled membrane receptor–ligand interactions and disturbances in contractile protein activity [36].

It has also been reported that chronic administration of imatinib may be related to increased gallstone formation in patients treated for chronic myelogenous leukemia. Imatinib is a specific tyrosine kinase inhibitor and is widely used in the treatment of GIST and other malignancies. Imatinib can increase the plasma estrogen levels via cytochrome P450-dependent metabolic pathways and possibly lead to increased cholesterol excretion with reduced excretion of biliary salts and the development of gallbladder stones [37]. Because imatinib damages ICLCs, gallstone formation could be directly related to the loss of ICLCs in the gallbladder wall.

The observed decrease in the number of ICCs in the gallbladder wall of patients with cholelithiasis in our study must be taken into consideration as a possible mechanism underlying gallbladder dysmotility resulting from the increased lithogenic properties of bile. The role of ICLCs in the regulation of bile duct motility appears to be an important pathological factor in gallstone disease.

Altered composition of bile in patients with cholelithiasis influences ICLCs within the gallbladder musculature. Considering the pivotal role of interstitial Cajal-like cells in the regulation of gallbladder and extrahepatic bile duct motility, we conclude that a reduction in ICLC number may be a consequence of the toxicity of the supersaturated bile, while some other bile components (glycocholic and taurocholic acids) may exert protective effects on ICLCs.

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CONFLICT OF INTEREST STATEMENT

None declared.

This manuscript was based entirely on PhD thesis of doctor Artur Pasternak.

LIST OF ABBREVIATIONS

ICC — interstitial cells of Cajal
ICLC — interstitial Cajal-like cells
CSI — cholesterol saturation index
H&E — hematoxylin and eosin
FOV — field of view
ANOVA — one-way analysis of variance

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