Morphological and Crystal Chemical Characteristics
of Gallbladder Biomineralization

Roman Moskalenko¹, Sergiy Danilchenko², Artem Piddubnyi¹, Oleksandr Kravets³,
Inna Chorna⁴, Olena Kolomiets¹, Anatolii Romaniuk¹

¹Department of Pathology, Sumy State University, Sumy, Ukraine
²Institute of Applied Physics of National Academy of Science, Sumy, Ukraine
³Department of General Surgery, Sumy State University, Sumy, Ukraine
⁴Department of Biophysics, Biochemistry, Pharmacology and Biomolecular Engineering,
Sumy State University, Sumy, Ukraine

SUMMARY

Pathological biomineralization can be found in some gallbladder (GB) diseases such as chronic
calculous cholecystitis (CCCh), gallbladder cancer (GBC) and porcelain gallbladder (PGB).
The aim of the work was to analyze the morphology of pathological biomineralization in GB
tissue in CCCh, GBC and PGB.

Five cases of PGB, 10 samples of CCCh and 5 cases of GBC with biomineralization were selected
for this study. All samples were examined by histology, histochemistry and scanning electron microscopy
with X-ray diffraction.

The X-ray diffraction of mineral deposits of PGB wall and GB concretions revealed their different
mineral composition. All PGB and GBC samples had the presence of hydroxyapatite. Calcium-containing
GB concretions were composed of calcium carbonate with the presence of trace amounts of other calcium
phosphate phases (vaterite, dolomite).

We did not find cancer in all PGB cases. The different crystal phases of biominerals were found in
the wall (PGB and GBC) and in the GB cavity (CCCh) during pathology development. The difference
between mineral content of biominerals can be caused by various conditions and mechanisms of their
formation.

Key words: cholecystitis, gallbladder cancer, porcelain gallbladder, hydroxyapatite, calcium carbonate

Corresponding author:
Roman Moskalenko
E-mail: r.moskalenko@med.sumdu.edu.ua
INTRODUCTION

Gallbladder (GB) diseases are often accompanied by pathological biomineralization (PBM) development. PBM occurs in chronic calculous cholecystitis (CCCh) (cholelithiasis), gallbladder cancer (GC) and porcelain gallbladder (PGB) (1).

Chronic calculous cholecystitis is extremely common pathology. Thereby, the causes, formation mechanisms and chemical composition of GB stones have been well studied (2, 3). Other pathologies, such as gallbladder cancer (GBC) and PGB, were studied to a lesser degree according to their low incidence. Moreover, the PBM in GBC and PGB are actually "terra incognita" (1).

GBC is more common in women with a ratio 5:1. The age of patients ranges from 50 to 70 years (1, 2). GBC prevalence depends on the geographical and ethnic features and varies from 1.59 cases per 100.000 (Maori women, New Zealand) to 22 cases per 100.000 (women, North India) (4). Calcification of the GB wall is associated with GBC in 12-62% of cases (5-7).

PGB is a rare manifestation of chronic GB disease. It has dense calcification deposits in the GB wall and can be found in 0.06-0.8% of cholecystectomies (8). The causes of PGB are exactly unknown. PGB was first described in 1929 as a GB with fragile consistency, uncolored wall and its widespread calcification (6).

The aim of the work was to analyze the morphology of pathological biomineralization in GB tissue in CCCh, GBC and PGB.

MATERIALS AND METHODS

Ethics statement

A written informed consent was obtained from all patients. This research was approved by the Medical Ethics Committee of The Sumy Regional Clinical Hospital and Medical Institute of Sumy State University (Protocol No.3/6, June 07 2016).

Sample collection

All patients were selected during the period 2012-2014 at the surgical department of the Sumy Regional Clinical Hospital. Patients were hospitalized routinely with the diagnosis: "Chronic calculous cholecystitis." Laparoscopic cholecystectomy and abdominal drainage were performed in patients under endotracheal anesthesia.

All five cases of PGB were evaluated as clinical findings in female patients at the age of 61.4 ± 1.54 years.

PBM in CCCh was represented as intraluminal GB concretions. The stones with a high inorganic component were used for this study. The organic calculi were destroyed during the sample processing (burning in a muffle furnace). Therefore, all CCCh samples are presented by GB calcium stones. Ten female patients with age range 54.4 ± 1.77 years were examined.

The study also involved five cases of GBC with PBM (1 male and 4 female patients, mean age 67.6 ± 4.32 years) (Table 1).

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, years</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Mineral</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>f</td>
<td>PGB</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>f</td>
<td>PGB</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>f</td>
<td>PGB</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>f</td>
<td>PGB</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>f</td>
<td>PGB</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite, Tricalcium magnesium phosphate</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite, Tricalcium magnesil phosphate</td>
</tr>
<tr>
<td>8</td>
<td>52</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite, vaterite</td>
</tr>
<tr>
<td>9</td>
<td>55</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite, vaterite</td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite, dolomite</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite, dolomite</td>
</tr>
<tr>
<td>12</td>
<td>62</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite</td>
</tr>
<tr>
<td>13</td>
<td>63</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite, dolomite</td>
</tr>
<tr>
<td>14</td>
<td>63</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite, dolomite</td>
</tr>
<tr>
<td>15</td>
<td>51</td>
<td>f</td>
<td>CCCh</td>
<td>Calcite</td>
</tr>
<tr>
<td>16</td>
<td>57</td>
<td>m</td>
<td>GBC</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>17</td>
<td>58</td>
<td>f</td>
<td>GBC</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>18</td>
<td>70</td>
<td>f</td>
<td>GBC</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>19</td>
<td>75</td>
<td>f</td>
<td>GBC</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>20</td>
<td>78</td>
<td>f</td>
<td>GBC</td>
<td>Hydroxyapatite</td>
</tr>
</tbody>
</table>
**Histology and histochemistry**

The presence of significant mineral deposits in some cases required an intensive decalcification with EDTA.

All tissue samples were fixed in 10% formaldehyde in PBS, dehydrated in ethanol and xylene, embedded in paraffin. 4-μm-thick sections were used for the hematoxylin-eosin, Alizarin red and Von Kossa staining.

**X-ray diffraction**

Mineral component was isolated by burning at 200°C for 1 hour. X-ray diffraction studies were performed on diffractometer DRON4-07 ("Burevestnik", Russia). Radiation CuKα (wavelength 0.154 nm) was used under conditions of focusing due to Bragg-Brentano (θ-2θ) (θ-Bragg’s angle) (9). The current and the voltage at the X-ray tube were 20 mA and 30 kV, respectively. The samples were scanned at the continuous recording mode (speed 2°/min) in a range of angles 2θ between 10 and 60°. All procedures of the experimental data processing were performed by the usage of the licensed software support package and results processing (DIFWIN-1, TOO "Etalon TCP"). Identification of crystalline phases was carried out by automatic comparison of the obtained results with the database cards Powder Diffraction File 2 without imposing restrictions on the elemental composition of the samples; the software package Crystallographica Search-Match (Cryosystems, Oxford) was used in the work.

**Scanning electron microscopy**

Scanning electron microscopy (SEM) with X-ray microanalysis was performed on tissue sections of GB by the REMMA 100U (SELMI, Ukraine). Briefly, 10 μm thick tissue sections were placed on the graphite plates. Paraffin sections were submerged to the three sets of xylene for 5 minute each, followed by three sets of 96°C ethanol for 5 min and finally rinsed with distilled water. Slides were placed into thermostat at 56°C for 10 minutes for drying.

**RESULTS**

**Morphological study**

PGBs maintained their shape after their surgical removal. GBs were whitish-gray and dense during palpation. The GB wall was thickened to 1.0 - 1.2 cm, dense, the tissue was crumbled. The mucosa exfoliated easily as thin plates with gray-yellowish color. Cracks were found on a mucosal surface of PGB, which was similar to the surface of old porcelain tableware (Figure 1A).

Macropreparations of PGB had a different spread of PBM and ranged from the total calcification of the organ to calcification of large parts of the GB wall (more than 50% of the GB wall).

![Figure 1. GB with PBM. A. The PGB wall with a massive deposition of biominerals (indicated by arrow), the preparation is fixed in formalin; B – macropreparation of GBC (tumor thickening of the walls is showed by the arrow); C – calculus from the patients with CCCh.](image-url)
GBC specimens were collapsed, had gray-pink color of serous membranes. GB tissues were soft; the walls were thickened to 0.6 - 0.7 cm (Figure 1B). Visible small gray nodules with a diameter of 0.1 - 0.2 cm, hemorrhages, fibrous tissue overgrowth were found in GBC. One GBC patient had polyps with the diameter of 0.1-0.3 cm at the GB mucosa.

GBs with CCCh were of pink-grayish color and they were collapsed. The slight wall thickening to 0.4 - 0.5 cm was defined (normal 0.2 - 0.3 cm). The GB mucosa had velvety surface with pinpoint hemorrhages. The GB wall had fibrous tissue overgrowth and hemorrhages. White-yellowish round shaped calculi with a diameter of 0.3-2.5 cm (Figure 1C) were found inside the GB.

**Histological examination**

Histological examination of the gallbladders shows the typical pathological changes of organ tissue. A moderate mixed cell inflammatory infiltration was revealed in the wall tissues of all PGB cases. Inflammation was accompanied by fibrosis, hemorrhages, muscular hypertrophy, hyalinosis and stagnation of secretion (Figure 2A, C).
Calcium deposits were predominantly located in the muscular layer along the muscle fibers and connective tissue. The size of these deposits varied from 0.1 to 5.0 cm. Muscle fibers were similar to "metallic net" in the places of evident calcification (Figure 2E, 3C). Histological examination of CCCh tissue revealed a diffuse inflammatory infiltration with lymphocytes and histiocytes, connective tissue excrescence, small hemorrhages and edema. Interstitial deposits of calcium compounds were not found.

GBC was found as a spread of atypical glandular tissue in the mucosa and muscle layer of GB. Cancerous tissue formed single atypical glands and tenia of tumor cells. All GBC cases were represented as adenocarcinoma. Focal inflammatory infiltration, connective tissue excrescence, and blood vessels formation were observed around the tumor tissue and calcifications in the GB wall (Figure 2F). Calcifications appeared as small single formations, which where found often in malignant glands or they where associated with the tumor. Desquamation of typical epithelial was found in glands with calcium deposits (Figure 2B, D, F; 3B, D).

**Scanning electron microscopy**

SEM revealed mineralized elements of GBs as white and gray colored objects with the signs of degradation as fragmentation and cracks (artifact material damage during the samples' processing). Electronic scanograms of PGB wall showed the presence of massive biomineral deposits. Calcium compounds were colocalized with fibrous tissue elements (muscles, connective tissue) (Figure 4A). SEM of mineralized GBC tissues revealed single foci of calcification with jagged borders. They were round-shaped and corresponded to the neoplastic glands shape (Figure 4B). X-ray diffraction of PGB and GBC showed a similar chemical composition and ratio of calcium and phosphorus, which corresponds to hydroxyapatite (Figure 4). X-ray diffraction patterns of PGB (Figure 5) corresponded to the structural data of hydroxyapatite (Ca_{10}(PO_4)_6(OH)\_2, JCPDS № 9-0432).

A significant broadening and overlap of diffraction peaks indicated a low level of the material crystallinity, a small size of the region with a regular periodic structure (crystallites or mosaic blocks) and a big amount of defects, which led to the lattice microdeformations. The semiquantitative evaluation of the crystallite size using Scherrer’s formula (9) in a perpendicular direction to the crystallographic plane (002) gave the values which were close...
to the typical size of bone crystallites. It should be noted that the line, corresponding to the longitudinal size of the apatite crystallites (e.g. 002), was broadened considerably less than the line corresponding to the transverse size (e.g., 310). This suggests a certain crystals’ elongation along the hexagonal axis at a relatively small size of the transverse direction. Such crystals’ morphology of biological apatite (plate or rod like) is typical for bone tissue and similar to some synthetic biomaterials.

Structural studies of pathological deposits, which were located in the gallbladder, showed that the major phase of the formed deposit was calcium carbonate (CaCO$_3$, JCPDS № 83-577 and/or JCPDS № 88-1810) that is characterized by a high degree of crystallinity (Figure 6).

Figure 5. Patterns of X-ray diffraction of the GB wall mineral formations; vertical lines correspond to the angular positions and relative intensities of the lines of the standard JCPDS № 9-0432; line with the indices 002 and 310 were used to estimate the size of the crystallites.

Figure 6. X-ray diffraction patterns of PBM formations in the GB; A – the strongest line of additional trace calcium phosphate phase ($\beta$-(Ca,Mg)$_3$(PO$_4$)$_2$ and/or apatite) is marked by the arrow, B – *- lines of calcite, JCPDS № 88-1810, v - vaterite lines, JCPDS № 74-1867.
The trace amounts (a few percent) of another crystal phase or phases, presumably, β-tricalcium magnesium phosphate \((\beta-(\text{Ca,Mg})_3\text{(PO}_4)_2)\) and/or apatite, were detected. In the one sample, a mixture of two different structural (polymorphic) forms of calcium carbonate - vaterite (JCPDS № 74-1867) and calcite (JCPDS № 88-1810), with a predominant concentration of the last one, was found (Figure 6B).

**DISCUSSION**

There are two types of PGB, according to the calcification level: complete (covers the entire organ, penetrates the muscle layer) and incomplete (multifocal, point deposits) (5, 10). The combination of GBC and PGB with incomplete calcification type, according to various data, ranges between 0% and 5% (11). There was no information about the combination of complete type of PGB and malignant tumors. This can indicate that two types of calcification cause different risk of GBC development. Despite this, a preventive simple cholecystectomy is the variant of PGB treatment (12).

Consequently, we did not detect cancer in all examined PGB samples. The results of histological and histochemical examinations show similarity of pathological changes (presence of chronic inflammation). Obviously, the idea that PGB is a type of chronic cholecystitis has a morphological basis (8). It is also known that PGB is associated with cholelithiasis in 90% of cases (13).

Differential diagnosis between PGB and calcified carcinoma of GB requires more precise criteria, as the correct diagnosis has a decisive influence on further treatment of the patient and on the perspectives for recovery.

Historically, the idea about the link between the PGB and the risk of GBC has changed. First works from the 60-70’s in the 20th century described a rather high incidence of malignancy (malignant transformation) of GB - up to 61% (6, 14). Recent studies claim that the incidence of carcinomas in PGB ranges from 0% to 5% (5, 10). Also, it was found that the complete type of PGB is not associated with GBC (5, 8, 11).

The results of our study and literature review are presented in Table 2.

Table 2. Histopathological features of different forms of gallbladder calcification

<table>
<thead>
<tr>
<th></th>
<th>CCC</th>
<th>GBC</th>
<th>PGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>Enlarged</td>
<td>Enlarged</td>
<td>Reduced in size</td>
</tr>
<tr>
<td>Wall thickness</td>
<td>Slightly thickened</td>
<td>Thickened</td>
<td>Thickened</td>
</tr>
<tr>
<td>Morphology</td>
<td>GB wall with normal structure, fibrosis, hyalinosis, inflammation</td>
<td>Fibrosis, atypical glands with invasive growth</td>
<td>The number of cells is reduced, massive deposits of small and big biominerals</td>
</tr>
<tr>
<td>Localization of biominerals</td>
<td>In the lumen (stones)</td>
<td>In the wall (in tumor tissue) as single biominerals</td>
<td>Walls are totally calcificated, solid layer, sometimes it spreads to the whole organ</td>
</tr>
<tr>
<td>Mineral compound</td>
<td>Calcium carbonate, dolomite</td>
<td>Hydroxyapatite</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Hyalinosis</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
</tbody>
</table>

PGB and GBC have common features of PBM. The result of PBM is the formation of hydroxyapatite. The common element is the development of dystrophy and necrosis in the GB walls. They are accompanied by cell death and by the occurrence of the building material for mineralization (calcium and phosphates). The reason of cell death in the case of PGB is inflammation, and in the case of GBC the damaging factor is infiltrative growth and dissemination of tumor cells, which leads also to secondary inflammation.

The prevalence of hydroxyapatite was found in our previous studies of biomineral formations of aorta and heart valves, prostate, and eye (15-17). We can assume that the similar feature for all these cases is a local tissue damage with the collagen fibers denudation, that is a matrix for bone mineralization. Therefore, we suggest that bioapatite crystals are similar to bone and synthetic biomaterials (15).
Unlike PGB and GBC, mineralization develops in organ cavity in conditions of CCCh. Calcium-containing concretions are more resistant to mechanical destruction in comparison to stones, which are organic or have a significant organic component. Calcium stones can have a different shape, their color ranges from snow-white (calcium palmitate) to dark brown (calcium carbonate - laterite), even black (calcium bilirubinate) (3). Due to the results of our research, calcium carbonate with the signs of small amounts of calcium phosphate phases (vaterite, dolomite) forms mainly in CCCh.

We have also studied the pathological biominerals of non-apatite nature in the pancreas (18). Biominerals with calcium carbonate develops in the pancreatic tissue and ducts as well as in the lumen of GB. Apparently, this is due to the functions of bicarbonate buffer systems.

The difference of mineral content of biominerals can be caused by various conditions and formation mechanisms. It is obvious that different pH and the environment are in GB wall and cavity. It is caused by the influence of various pathological conditions. One of the possible reasons could be an insufficient amount of phosphorus and lack of collagen matrix in the GB cavity.

CONCLUSIONS

Different crystal phases of biominerals were found in the wall (PGB and GBC) and in the GB cavity (CCCh) during pathology development. Intraparietal biominerals where represented by hydroxyapatite, stones from the GB cavity consisted predominantly of calcium carbonate with phosphate additives. These results indicate different conditions, causes and mechanisms of their formation.

Acknowledgements

This research has been performed with the financial support of grants of the Ministry of Education and Science of Ukraine No. 0117U003937 “The development of tumor diagnosis method of reproductive system organs using cellular adhesion molecules of cancer-embryonic antigen” and No. 0118U003570 “The efficiency of “liquid biopsy” and tissue biopsy in the diagnosis and treatment of malignant tumors”, Erasmus+ Project 2017-1-SE01-KA107-03486 between Sumy State University (Sumy, Ukraine) and Umeå University (Umeå, Sweden).

Conflicts of interest

Authors have no conflict of interest to declare.
References


Morfološke i hemijske kristalne karakteristike biomineralizacije žučne kese

Roman Moskalenko¹, Sergiy Danilchenko², Artem Piddubnyi³, Oleksandr Kravets³, Inna Chorna⁴, Olena Kolomiets¹, Anatolii Romaniuk¹

¹Državni univerzitet u Sumiju, Departman za patologiju, Sumi, Ukrajina
²Institut za primenjenu fiziku nacionalne akademije za nauku, Sumi, Ukrajina
³Državni univerzitet u Sumiju, Departman za opštu hirurgiju, Sumi, Ukrajina
⁴Državni univerzitet u Sumiju, Departman za biofiziku, biohemiju, farmakologiju i biomolekularni inženjering, Sumi, Ukrajina

SAŽETAK

Patološka biomineralizacija vida se kod nekih bolesti žučne kese, poput hroničnog kalkuloznog holecistitisa, karcinoma žučne kese i porcelanske žučne kese.

Cilj rada bila je analiza morfologije patološke biomineralizacije u tkivu žučne kese kod hroničnog kalkuloznog holecistitisa, karcinoma žučne kese i porcelanske žučne kese.

Pet slučajeva porcelanske žučne kese, deset uzoraka hroničnog kalkuloznog holecistitisa i pet slučajeva porcelanske žučne kese sa biomineralizacijom, uključeno je u ovu studiju. Svi uzorci su pregledani histološki, histohemijski i pomoću skenirajuće elektronske mikroskopije sa difrakcijom x-zraka.

Difrakcija x-zraka mineralnih depozita zida porcelanske žučne kese ukazala je na različit sastav minerala. Kod svih uzorka porcelanske žučne kese i karcinoma žučne kese, uočeno je prisustvo hidroksiapatita. Kalcifikati žučne kese sastoje se od kalcijum-karbonata, sa prisustvom ostalih faza kalcijum-fosfata u tragovima (veterit, dolomit).

Karcinom nije utvrđen u svim slučajevima porcelanske žučne kese. Različite kristalne faze biomineralu uočene su u židu žučne kese (porcelanska žučna kesa i karcinom žučne kese) i u žučnoj kesi (hronični kalkulozni holecistitis) za vreme patološkog razvoja. Razlika između mineralnog sastava biomineralu može biti uzrokovanu različitim stanjima i mehanzmima njihovog formiranja.

Ključne reči: holecistitis, karcinom žučne kese, porcelanska žučna kesa, hidroksiapatit, kalcijum-karbonat