Current Topics in Biophysics,2014, 37, 1-8 DOI: 10.2478/ctb-2014-0068

MECHANISM OF GALLSTONES FORMATION IN WOMEN DURING MENOPAUSE (EPR STUDY)*

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Received December 04, 2012; accepted January 24, 2014; published online January 30, 2014.

The worldwide incidence of gallbladder disease is highly variable. Identification of metabolic alterations like cholesterol metabolism or free radicals may provide insight into the formation of gallstones and provide a basis for prognostic markers. The aim of the study was to identify the pathogenesis and prognostic value of metabolic disorders in gallstone formation in menopausal women.

Methods: Menopause-aged women with (group I, 58 patients) and without gallstone disease (group II, 25 patients) were investigated. In each group blood lipid metabolism and blood redox parameters (free Mn^{2+}-ions) and antioxidant system activity (ceruloplasmine/Fe^{3+}-transferrin) were studied using micro enzymatic method and Electron Pramagnetic Resonance (EPR) spectroscopy. Surgically removed gall bladder stones were studied by routine laboratory methods (cholesterol and bilirubin content) and EPR spectroscopy. In model experiments *in vitro* purified bilirubin was irradiated with visible light in different conditions (in presence and without oxygen).

Results: Intensive signal of oxidized bilirubin free radical was detected in the EPR spectra of gallstones. Reduced activity of blood antioxidant ceruloplasmin/F^{3+}-transferrin system and increased content of prooxidants Mn^{2+}-ions were detected in menopausal women blood with gallstone disease. *In vitro* experiments demonstrated, that prolonged exposure of bilirubin to visible light in the presence of oxygen induces formation of the bilirubin free radicals (EPR signal g= 2.003 ΔH = 1.0 mT); irradiation of bilirubin in vacuum was not associated with generation of free radicals. Correlation analysis revealed statistically significant correlation between EPR signal intensity of bilirubin free radical in gallstones, activity of ceruloplazmin/F^{3+}-transferrin antioxidant system and content of free Mn^{2+} ions in patients' blood (r=-0.5725, p=0.0324; r=0.7805, p=0.0010, respectively). The tight correlation between marker of oxidative stress, Mn^{2+} ions EPR signal intensity and HDL-, LDL-cholesterol content in blood (r = 0.910629, p = 0.0324; r = 0.029477, p = 0.0010, respectively) was also revealed.

Conclusion: The results of the present study demonstrated the pathophysiological significance of alterations of blood redox-homeostasis during menopause in the gallstone formation. The risk of bilirubin oxidation, which plays a crucial role in the gallstones formation, increases with intensification of oxidative stress. Crystallization of cholesterol in polymeric network of oxidized bilirubin may contribute to gallstone formation.

INTRODUCTION

Gallstone disease is one of the most common pathologies of the digestive tract. The frequency of the gallstone disease varies in different countries: While Pima Indian women have the highest rate of gallstone disease in the world (reaching 32% of population), significantly high rate occurs in the United States, Europe, and Asia. Gallstone disease is characterized by low frequency in Japan (3.5%) and Australian aborigines (1%), while the Masai people of East Africa have none at all. In Georgia, this pathology occurs in 10-12% of the population (Stinton, Myers &Shaffer, 2010).

Gallstone development is not dependent on the food habits (meat and fat consumption) and is quite common in the vegetarian population. About 80 percent of all gallstones are cholesterol stones (yellow-green stones). They are the most common type of gallstones in individuals in Europe and the Americas. Cholesterol stones are associated with bile that is "supersaturated" with cholesterol. The "Western" cholesterol gallstone is often only 70% cholesterol by weight and almost invariably contains a pigmented nucleus (Been, Bills, Lewis, 1979). The other 20 percent of gallstones are pigmented stones (black and brown), mainly composed of bilirubin and other elements (in Asian cultures but rarely in U.S. and European patients).

Usually bile contains water (85-95%), sufficient amount of bile acids and a low concentration of cholesterol. An increase in the amount of cholesterol in bile or decrease in the total pool of bile acids induces the formation of large unstable multi-phospholipid vesicles,

*This paper was presented at IX International Workshop on EPR (ESR) in Biology and Medicine, 7-10 October, 2013, Kraków, Poland

which can be aggregated and produce cholesterol monohydrate crystals. Thus, the risk of gallstone formation increases with the decrease in the degree of solubility of cholesterol. However, the supersaturation of bile with cholesterol is possible even in the absence of cholesterol-containing gallstones. Cholesterol gallstones usually contain also a pigment (monomeric calcium salts of unconjugated bilirubin anions and/or an insoluble, black, network polymer of tetrapyrroles) nucleus. Bilirubin is also insoluble in water and its transport in human bile is bile acid dependent. However, there have been few studies of bilirubin metabolites in bile from patients with cholesterol gallstones, even though it has been proposed that unconjugated bilirubin may play an important complementary role in the initiation of cholesterol gallstone formation (Dutt, Murphy & Thompson, 2003). Mechanisms of deposition and crystallization of cholesterol in bilestones is still not established.

The goal of this study was to identify the pathogenesis and prognostic value of metabolic disorders in gallstone formation in menopausal women.

MATERIALS AND METHODS

This study is a case–control analysis in which lipid and redox homeostasis was compared between menopausal women with and without gallstones.

Patients

Menopausal women (20 women - surgical menopause (30 - 34 years), 19 women in early stage of menopause (39-56 years), 19 women in late stage of menopause (59-73 years)) with gallstone disease (group I), who had been admitted to the "1-st Clinic Ltd." (Tbilisi, Georgia) during 2009-2011, and 25 menopause-aged women without gallstone disease (control group II) were investigated. Determination and verification of menopause was provided based on the criteria of at least 12 months of amenorrhea. Gallstone disease was confirmed by transabdominal ultrasonography.

The following exclusion criteria were used: history of liver, pancreatic, or other gastrointestinal diseases, lipid disorders, diabetes, high blood pressure, cancer, surgical resection, thyroid, neurological, muscular, rheumatological and immunological diseases. Patients were also excluded if they were heavy drinkers, smokers or habitual drinkers of espresso coffee. Individuals enrolled were not receiving estrogen replacement therapy or any medication affecting lipid metabolism, and were not taking vitamin, mineral, or phytoestrogen supplements. None had overt haemolysis; haemoglobin and serum levels of haptoglobin and bilirubin were normal in all cases. All the patients had given their informed consent before any procedure.

All patients were subjected to a standard diagnostic protocol. In each group (I and II) we investigated lipid spectrum (high density lipoprotein (HDL), low density lipoprotein (LDL) and triglycerides in the patients' blood, and blood redox-status (free Mn^{2+}-ions and antioxidant system activity (ceruloplasmine/Fe^{3+}-transferrin).

Stones

In the surgically removed gall bladder stones, cholesterol and bilirubin content was measured by routine laboratory procedures **(**Duvaldestin**,** Mahu, Metreau, Arondel, Preaux & Berthelot, 1980). All cholesterol stones (cholesterol content > 70%) were studied by Electron Paramagnetic Resonance (EPR) method.

Electron Paramagnetic Resonance Spectroscopic studies

We used the EPR spectroscopy because it is the only technique that has the potential for the direct detection of free radicals in biological systems (Rice-Evans & Diplock, 1991; Nonaka, Manabe, Asano, Kyogoku, Imanishi, Tamura, Tobe, Sugiura & Makino, 1989; Kozlov, Szalay, Umar, Fink, Kropik, Nohl, Redl & Bahrami, 2003). The low-temperature (liquid nitrogen) EPR measurements using ESR-231 (X-band, "Akademie der Wissenschatten Der DDR", Germany) were carried out for the free radical direct detection in the patients' blood. The blood samples were placed into the polyethylene tubes, and then frozen in liquid nitrogen. EPR signals of oxidized ceruloplasmin ($g = 2.05$), Fe^{3+} transferrin ($g = 4.3$) and free Mn^{2+} ions were measured in the blood samples (Kozlov, et al., 2003; Shinkarenko, Kozlov, Gol'dshtein, Azizova & Vladimirov, 1987). EPR spectra of gallbladder stones were measured at room temperature. The magnetic field was calibrated with Mn^{2+}-doped MgO, giving a sextet of paramagnetic centers with g values of (2.14, 2.08, 2.03, 1.98, 1.93, and 1.86); the half width (ΔH – distance between maximum and minimum of free-radical EPR signal in gauss) of the free radical signal has been determined.

Gallstones were cut into pieces (with weight 50 mg) in aerobic atmosphere before the EPR measurement. Samples were not milled to powder to prevent the free radicals formation. The EPR spectra of gallstones were measured at room temperature.

In order to prevent the effects of different factors on the EPR signal intensity and to standardize the samples measurement conditions we used as a standard the EPR spectrum of Mn^{2+} ions in the powder MgO, calibrated by proton magnetic resonance magnetometer at the given microwave frequency. EPR signals intensity was measured in arbitrary units: ratio of the intensity of studied EPR signal to intensity of the standard's (Mn^{2+} ions in the powder MgO) 2-nd component.

Photoradiation of bilirubin in vitro

In order to establish the mechanisms of bilirubin free radical formation, we conducted EPR spectroscopic study of in vitro photoradiated bilirubin. Bilirubin (Sigma Chemical Co., St. Louis, USA) was purified on a chromatographic column, used as a silica gel sorbent (100/400 "Chemapol" Prague). After purification of the bilirubin oxidized powder a black mass insoluble in chloroform was obtained. 30 mg of purified bilirubin dissolved in 10 ml of chloroform was irradiated with visible light (light intensity 10mW/cm^2) at aerobic (atmospheric pressure) and anaerobic (in a glass vacuum cell at pressure 10^{-3} mmHg) conditions at room temperature (the distance to the fluorescent lamp ("Osram" L18W/67, Germany) was sufficient to prevent heating of the samples). The EPR spectra of irradiated bilirubin were measured.

S*tatistical analysis*

Analysis of data was carried out using the SPSS-11for Windows. Data were reported as mean ±SD. For normally distributed variables, the Student t test was applied. The Mann–Whitney test was used for not normally distributed variables. The comparison between categorical variables was evaluated by the χ^2 test.

RESULTS

Stone analysis

Stone composition. In 58 cases the cholesterol and bilirubin content in the gallstones was measured (Table 1). The cholesterol content in stones exceeded 70% of their dry weight, which allows us to assign them to cholesterol gallstones. Macroscopically the cross-section showed a pigment centre in 49 of the 58 cholesterol gallstones (67%).

Table 1. Gallstones physical characteristics.

Shape	Round
Color	Yellow
Surface	Smooth
Weight	4,7±1,4 mg
Cholesterol content	98.01±13,54%
Bilirubin	1,68±0,43%

EPR study of gallbladder stones. Following the results of ESR spectra measurements, the gallstones could be divided by their paramagnetic composition into cholesterol, brown pigment and black pigment stones (Chikvaidze, Tabutsadze, Gogoladze, Datuashvili & Iremashvili, 2009). Cholesterol stones don't contain paramagnetic impurities and the EPR spectrum reveals only the presence of free bilirubin radical. In brown pigment stones besides this signal it is observed a complicated EPR signal consisted of six intensive components that were attributed to the hyperfine (HF) Mn^{2+} ions structure. As per weaker components with 2.2 mT splitting it was supposed that they also belong to n^{2+} ions but only in other complexes and the actual spectrum is the superposition of spectra from different Mn2+ ion complexes. The same could be said about the EPR spectrum of black pigment stones – this spectrum is the superposition of Fe^{3+} ion signal (g=4.19) and the EPR signal characteristic for bioorganic complexes of Cu^{2+} ($g_{\parallel}$ = 2.37, $g_{\perp}$= 2.05; $A_{\parallel} \approx$ 18.6 mT) (Figure 1). An intense signal of bilirubin free radical (g=2.003, ΔH=1.0 mT) was detected in cholesterol gallstones removed from menopausal women (Figure1a).

There was no statistically reliable differences in the bilirubin radical EPR signal intensity of gall-bladder stones from different patient groups (surgical, early and late menopause) (Table 2).

Table 2. Intensity (I) of bilirubin (Br) free radical's EPR signal in gall bladder stones.

	N	Br (I)
Surgical menopause	20	2,8±1,7
Early menopause	19	3,8±2,5
Late menopause	19	3,7±2,8

Blood analysis

Lipid profile. Results showed a significant elevation in both HDL and LDL-cholesterol content in the patient's blood with gallstones when compared with controls (without gallstones). The triglycerides and total cholesterol content in serum in the gallstones patients increased statistically not significantly (see Table 3).

Table 3. Blood lipid profile in menopausal women with and without gallstones.

	LDL	HDL	TG	Total Cholesterol
Without gallstones	86,5±18,3	42,0±10,6	105,1±14,5	145,2±36,0
With gallstones	122.9±32.7*	58.96±12.8**	121.4± 45.67	178±24.20

*statistical significance $p<0.05$

**statistical significance $p<0.001$

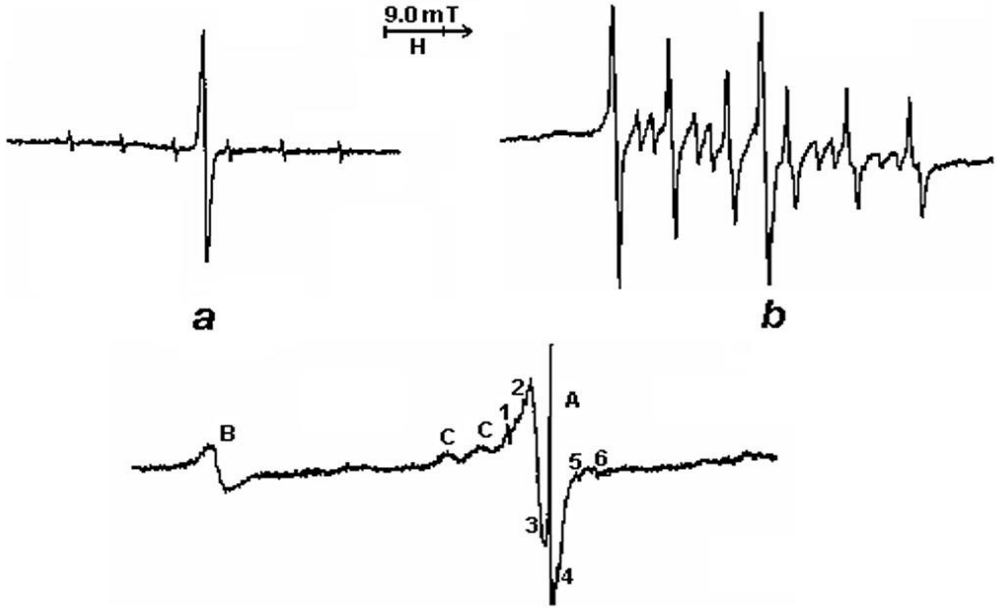

Fig. 1. Typical EPR spectra of gall bladder stones. *a*) Cholesterolical stones: the singlet with g=2.003 corresponds to free a radical of bilirubin; six hyperfine (HF) components correspond to peaks of Mn^{2+} in MgO used as a calibration standard with splitting $\Delta H = 8.7$ mT. *b*) Brown pigment stones: the central intensive line with g=2.003 corresponds to free radical of bilirubin; six hyperfine (HF) components with splitting $\Delta H = 8.7$ mT corresponds to Mn^{2+} complexes with organic compounds . *c*) Black pigment stones: the central intensive line with g= 2.003 corresponds to free radical of bilirubin; a wide line with $g_{\parallel} = 2.37$, $g^{\perp} = 2.05$ and HF structure $A_{\parallel} \approx 18.6$ mT corresponds to Cu^{2+}-complexes with organic compounds. Numbers in spectrum (c) indicate the HF components of standard Mn^{2+} in MgO. Conditions: modulation - 100 kHz/1 G, microwave power - 5 mW. amplitude of high field modulation - 0,1 mT, field scan - 10 mT, sweep time -200 sec, time constant - 80 ms; room temperature.

Redox parameters. In the blood of menopausal women with gallstones, the EPR signal intensities of oxidized form of the blood serum antioxidant enzyme, ceruloplasmin, and the ferrum-transport protein, Fe^{3+}-transferrin, were measured. The blood ceruloplasmin/Fe^{3+}-transferrin system is responsible for the maintenance of redox balance in blood. In our investigation EPR signal intensity of oxidized ceruloplasmin (Cp) increased by 13% and Fe^{3+}-transferrin (Fe^{3+}-Tr) decreased by 10% in comparison to the same parameters of menopausal women blood without gallstones. In blood samples of menopausal women with gallstones EPR signals of low-molecular Mn^{2+}-containing complexes was detected (Table 4). This signal was not detected in blood samples of menopausal women without gallstones. The data indicate an alteration of the blood redox-balance in women during gallstones formation.

Table 4. EPR signal intensity (I) of the blood of menopausal women with and without gallstone disease (Cp –ceruloplasmine; Tr – transferrine).

Parameters/Groups	N	Cp g=2,05	Fe^{3+}-Tr g=4,2
Healthy women without gallstones	40	18,8±0,8	27,8±0,9
Women with gallstone disease	25	21,5±0,7* $p_{12}<0,01$	25,0±0,3* $p_{12}<0,02$

*statistical significance p<0.01

EPR study of in vitro photo-irradiated bilirubin

In order to determine EPR signal parameters of bilirubin free radical – g-factor and half-wildness (ΔH), we studied the EPR spectrum of *in vitro* photo radiated bilirubin. Purified bilirubin EPR signal intensity was negligibly small (Figure 2). The EPR signal intensity of purified bilirubin in chloroform solution irradiated with visible light in aerobic conditions (Figure 3) increased noticeably with increasing of radiation time exposure (Figure 4). Long-term (20 hours) irradiation of the bilirubin chloroform solution with visible light under anaerobic conditions (vacuum) was not accompanied by

the generation of free radicals (data not shown). These data suggest the involvement of molecular oxygen in the photo induced bilirubin free radical formation.

DISCUSSION

The key molecular abnormalities underlying gallstones formation are not yet fully understood. It has been postulated that abnormal regulation of hepatic cholesterol and bile acid synthesis, or esterification, deposition of cholesterol monohydrate crystals and gall bladder dysfunction would be the primary metabolic abnormalities of gallstone disease. Results of our investigation showed a significant elevation in both high density lipoprotein (HDL) and low density lipoprotein (LDL) in the patients blood with gallstones when compared with controls (without gallstones). Cholesterol is only slightly soluble in water; it can dissolve and travel in the water-based bloodstream at exceedingly small concentrations. In general it is transported in the circulatory system within lipoproteins (chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL)); although some cholesterol is carried as the "free" alcohol and cholesterol esters. Lipoproteins have cell-targeting signals that direct the lipids they carry to certain tissues. IDL molecules, contain an even higher percentage of cholesterol. Therefore, LDL are the major carriers of cholesterol in the blood. HDL and LDL particles are thought to transport cholesterol to the liver for excretion or to other tissues that use cholesterol for cellular metabolism. VLDL molecules are produced by the liver and contain excess triacylglycerol and cholesterol that is not required by the liver for synthesis of bile acids. Thus the relatively low elevation of total cholesterol content against the statistically significant increase in the LDL- and HDL-cholesterol concentration during gallstone disease can be caused by the uneven distribution of cholesterol between carriers and indicates the preferential uptake of cholesterol by the liver tissue where it may under certain conditions included in the cholesterol gallstones.

In menopausal women with gallstomes revealed the positive correlation between alterations of lipids content in the blood and bile. A strikingly frequent relationship between elevated serum HDL- and LDL-cholesterol concentration and gallstone disease suggests the important role of lipids metabolism disorders in the formation of cholesterol gallstones (de Bari, Neuschwander-Tetri, Liu, Portincasa, Wang & Ezetimibe, 2012).

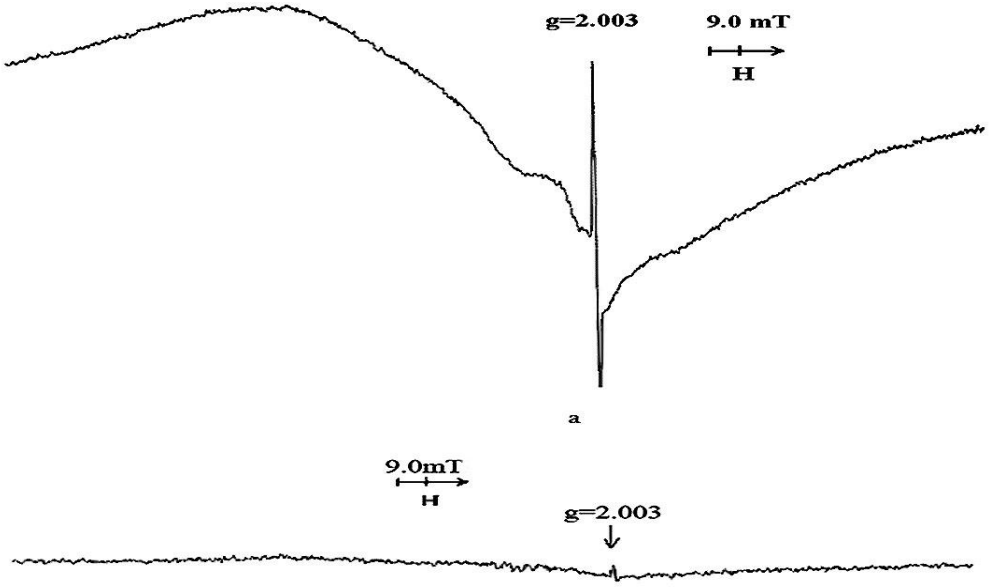

Fig. 2. EPR spectrum of the sample of bilirubin after the chromatographic purification, isolated from fraction bilirubin. Conditions:, modulation- 100 kHz/1 G, microwave power- 5 mW. amplitude of high field modulation - 0,1 mT, field scan - 10 mT, sweep time -200 sec, time constant - 80 ms; room temperature.

In EPR spectra of gallstones removed from menopausal women an intense signal of bilirubin free radical (g=2.003, ΔH=1.0 mT) was detected. Using *in vitro* model experiments the participation of the bilirubin

free radicals in the cholesterol crystallization process was demonstrated (Lightner, Linnane, Ahlfors, 1984; Ohkubo, Ostrow, Carr & Rege, 1984). These data indicate the possibility of involvement of bilirubin free radicals in the gallstones formation.

To validate this hypothesis we performed the model experiments *in vitro* with photo radiation of purified bilirubin in different conditions. The results of the study demonstrated that prolonged exposure of bilirubin to visible light in the presence of oxygen induced formation of the bilirubin free radicals with EPR signal g= 2.003 ΔH = 1.0 mT. It was shown that EPR signal intensity of bilirubin free radical increases with increasing time duration of light exposure. Irradiation of bilirubin in vacuum did not accompanied with generation of free radicals.

Under physiological conditions the concentration of oxygen in tissues and blood plasma is an order of magnitude lower when compared to the concentration of oxygen in the air (~21%). This concentration, however, is sufficient for the oxidation of bilirubin (Eder, Miquel, Jongst, Paumgartner & von Ritter, 1996) in oxidative stress conditions, promoted by different endogenous (alterations of hormonal metabolism, infections) and exogenous (stress factors, sun bathing, light exposure) factors. Free-radical oxidation is involved in the pathogenesis of many pathological processes (Halliwell, Gutterigge & Cross, 1992), however, only a limited number of studies have been devoted to understand the role of oxidative processes in gallstones formation (Shiesh, Chen, Lin, Liu & Tsao 2000; Lightner et al., 1984). As a result of testing blood redox parameters of menopausal women with gallstone disease reduced activity of blood antioxidant ceruloplazmin/F^{3+}-transferrin system and increased content of prooxidants - Mn^{2+}-ions were revealed. This data and results of our model experiments indicated the possibility of bilirubin oxidation in oxidative stress conditions and involvement of bilirubin free radicals in the gallstones formation.

Increased content of Mn^{2+}-ions in the blood was detected also by other investigators during aggressive and chronic hepatitis (Chikvaidze, Kirikashvili,

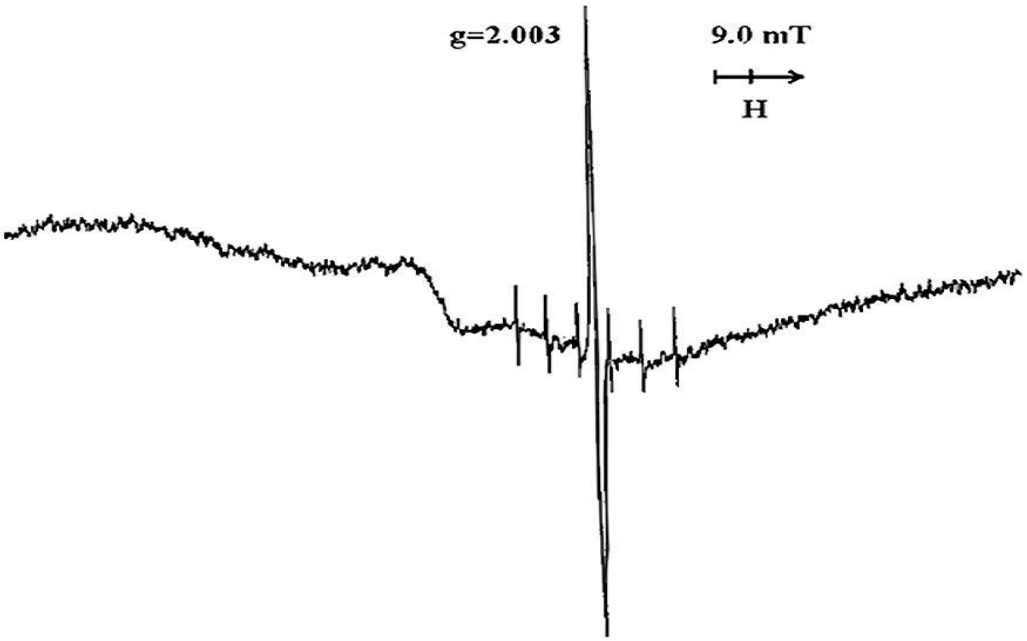

Fig. 3. EPR spectrum of the sample of purified bilirubin irradiated with visible light (λmax=450 nm) during 4 hours. (The six weak components around the signal of the bilirubin radical belong to the standard Mn^{2+} in MgO). Conditions:, modulation- 100 kHz/1 G, microwave power- 5 mW. amplitude of high field modulation - 0,1.

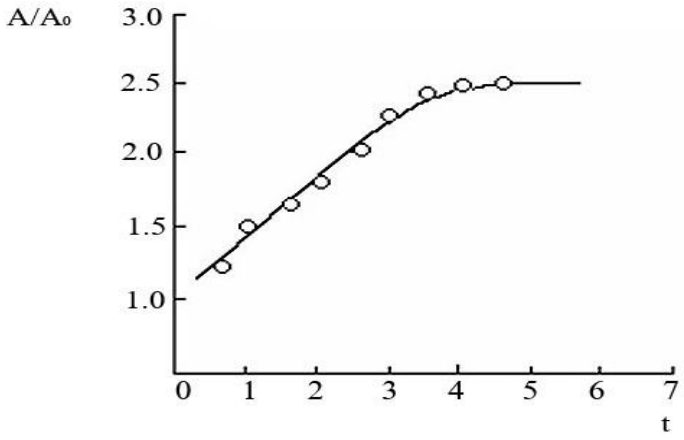

Fig. 4. Alteration of intensity of EPR signal of bilirubin powder irradiated by visible light. A/Ao is the ratio of intensity of EPR signal of bilirubin free radical to intensity of HF of the second component Mn^{2+} ions of standard.

Gogoladze & Chikvaidze, 2002; Marx G, 1984). It was shown that Mn^{2+}-ions may release from blood albumin cation-bridges complexes containing bivalent metal which transport bilirubin to the liver (Chikvaidze, et al., 2009). Mn^{2+}-ions can induce disorder of cholesterol biosynthesis and its degradation to bile acids (regulate activity of cholesterol rate limiting microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase); bilirubin blocks enzyme cholesterol-7α-hydroxylase responsible for cholesterol degradation to bile acids (Goering, 2003; Ohkubo et al., 1984). In experiments it was found that the combined action of the Mn^{2+}-ions and bilirubine leads to the increase intensity of hepatic cholesterogenesis (Rao, Jarari, Awaml & Patil, 2012) and cholesterol accumulation in liver (Goering, 2003). This mechanism jointly with participation of oxidized bilirubin in the polymerization and crystallization of cholesterol in bile perhaps contributes to gallstone formation. According to our correlation analysis results it was revealed statistical significant correlation between EPR signal intensity of bilirubin free radical in gallstones, activity of ceruloplazmin/F^{3+}-transferrin antioxidant system and content of free Mn^{2+} ions in patients' blood ($r=-0.5725$, $p=0.0324$; $r=0.7805$, $p=0.0010$, respectively). The tight correlation between marker of oxidative stress, Mn^{2+} ions EPR signal intensity and HDL-, LDL-cholesterol content in blood ($r = 0.910629$, $p = 0.0324$; $r = 0.029477$, $p = 0.0010$, respectively) was also detected.

CONCLUSION

The results of the present study demonstrated the pathophysiological significance of alterations of blood redox-homeostasis during menopause in the gallstone formation. The risk of bilirubin oxidation, which plays a crucial role in the gallstones formation, increases with intensification of oxidative stress. Crystallization of cholesterol in a polymeric network of oxidized bilirubin may contribute to gallstone formation.

FOOTNOTES

Ethics approval: The local ethics committee approved the protocol, and informed consent was obtained from all participants or their surrogates (according the Helsinki Declaration of the World Medical Association).
Funding: This work was supported by Tbilisi State Medical University. Competing interests: None.
Contributorship: All of the authors have contributed to the conception and design, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content and final approval of the version to be published. All results and conclusions were discussed with Prof. P. Kuppusamy, who was the main consultant of the research.

ACKNOWLEDGMENTS

The authors would like to thank B. Kiladze and A. Megreladze at the "1-st Clinic Ltd.", Tbilisi, Georgia, for providing the gallbladderstones.

ABBREVIATIONS

Cp – ceruloplasmine; EPR - Electron Paramagnetic Resonance; Tr – transferine; Br - bilirubin; HDL - high density lipoprotein; LDL - low density lipoprotein; VLDL - very low density lipoprotein; IDL - intermediate density lipoprotein; HF – hyperfine; HMG-CoA - 3-hydroxy-3-methylglutaryl coenzyme A.

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