The Egyptian Journal of Hospital Medicine Vol., 18 : 66 - 72 March 2005
I.S.S.N: 12084
1687 - 2002

Ambivalent property of bilirubin in human bile juice

Anna Blázovics, Péter Sípos, Ferenc Örsi,* Mervat Abdel Rahman**
Semmelweis University, Budapest, Hungary, *Technical University of Budapest, Hungary, ** Student Hospital, Cairo University, , Giza

Abstract

Gallstones are formed as a result of many metabolic disorders e.g. chronic haemolytic anaemia, diabetes mellitus, ileal diseases, short bowel syndrome, gluten sensitive enteropathy, elevated serum lipids or Crohn's disease. The relationship between gallstone disease and free radical reactions is not known exactly even today. Free radicals are involved in many clinical conditions e.g. in hyperlipidemia and in fatty liver. Oxygen free radicals are produced and accumulated while the function of mitochondrial and microsomal electron transport or in peroxisomes and the activated arachidonic acid cascade. Spontaneous lipid peroxidation and oxygen free radical products of respiratory burst of Kupffer cells can be added to peroxide pool of liver tissue**.** Tissues, cells and subcellular particles exhibit different specific defence activities in pathological processes, which involve free radicals.

The activity of microsomal P450 enzyme system and the microsomal structure are changed during pathological free radical attack and the cholesterol/bile acid ratio in bile juice is also altered. At the same time bilirubin metabolism can also be modified.

Bile samples of 88 cholecystectomysed patients in both sexes (male: 29, female: 59) were examined. HPLC analysis (HP1090 liquid chromatograph with diode array detector) was used for the detection of free bilirubin and bilirubin derivates. HP5890 gas chromatograph and flame ionization detector was used for fatty acid analysis. The induced chemiluminescence intensity was also determined in bile juice with (Berthold Lumat 9501) luminometer.

As results show, the occurrence of C18:1ϖ 9, C18:2ϖ 6, C20:4ϖ 6 fatty acids were in high percentage in gallbladder bile in every case of randomly chosen 17 cholecystectomysed patients in both sexes suffered from cholecystitis chronica with gallstone. Lipid peroxidation products (diene conjugates and malondialdehyde) were detected in all cases of bile as well. Mathematical statistical analysis showed, that positive significant correlation was between low concentration of total bilirubin of gallbladder bile and chemiluminescent intensity in hydrogen peroxide - luminol system. Extra high chemiluminescence light could be detected in bile samples of patients with severe clinical state. Bilirubin pro-and antioxidant forms are justified in human gallbladder bile.

Key words: bilirubin, human bile, gallbladder, gallstones, free radicals.

Introduction

Due to deranged liver structure and function both mechanism of biliary passage and composition of bile juice are changed significantly (Cohen and Soloway 1985, Abdel Rahman 1995, 1996, Sipos 2001, Bosma *et al.,* 2003).)

Cholesterol supersaturation, gallbalder hypomotility and mucin hypersecretion are essential for the formation of cholesterol gallstones. Exogen arachidonic-lecithin stimulates the formation of gel matrix lining the gallbladder wall and accelerates the nucleation process (Levy *et al.*,1984, Cohen *et al.*,1985 Carey 1988, Trotman 1982). Supersaturation of bile with calcium hydrogen bilirubinate is essential for pigment gallstone formation. The gallbladder bile in brown or black pigment gallstone diseases is usually not saturated with cholesterol and contains an increased proportion of mono-and unconjugated bilirubin IX.α. Bilirubin hypersecretion can be observed in haemolytic disorders or enterohepatic cycling in nonhaemolytic

states (Trotman 1985, Nakao *et al.*, 1988). Bacterial infection of bile in brown pigment stone approaches 100%. Bacterial β-glucuronidases hydrolyse the conjugated bilirubin (Trotman 1982, Vitetta and Sali 1992). Bilirubin is the endproduct of hem catabolism, (formed in reticuloendothelial cells) coming from haemoglobin, myoglobin and many respiratory enzymes. The aim of this research is to study the role of bilirubin and free radicals- mediated mechanisms in gallstone formation.

Subjects and Methods

Bilirubin standard was purchased from SIGMA Aldrich Kft, HPLC grade chloroform and ethanol were obtained from MERCK, acetic acid (99-100%) and all other reagents were analytical quality from REANAL, Budapest.

Bile samples of 88 cholecystectomysed patients (aged between 40-60 years) in both sexes (male: 29, female: 59) were examined. All patients suffered from cholecystitis chronica. The stones were of mixed types.

Bile samples of randomly chosen 17 cholecystectomysed patients in both sexes (male: 4, female: 13) were examined with HPLC and GC.

HP 5890 gas chromatograph with flame ionization detector and split capillary injector was used for fatty acid analysis of bile. Labinform A/D converter and data station was employed to collect the data and processed the chromatograms. Colonna: Carbovax 20 M (25 m x 0.2 mm, 0.25 µm film thickness), column oven: 180 ^{o}C, injector 280 ^{o}C, detector 205 ^{o}C, injector: split split ratio: 1:50, carrier gas: nitrogen 0.3 cm^3/min, detector gas: hydrogen 30.0 cm^3/min, air: 400 cm^3/min, nitrogen: 30 cm^3/min. The sample injections were 10 µl in hexane. The peaks were identified by comparison with retention behaviour of known standards of FAME mixtures (Alexander, 1985).

Diene conjugates were measured by the recommendation of AOAC method (1984) with isooctane fractionated extraction. Volume of bile samples was 500 µl in each measurement.

The TBA reactive products (malondialdehyde /MDA/) were monitored by the thiobarbituric acid test of Pyles *et al.*, 1993. We controlled the bilirubin TBA reaction. The find equation, to be used to correct for absorbance due to interfering substances in measuring the MDA-TBA chromogen is as follow: MDA_{532} = 1..22 $((A_{532})-(0.56)(A_{510}) + (0.44)(A_{560}))$

A recently developed chemiluminescence assay adapted to a Berthold Lumat 9501 instrument was applied for determination of the total scavenger capacity or free radical activity of the bile (Blázovics *et al.*, 1999).

Raw data operation mode was used. The built-in microprocessor performed the data processing and printed out the final results in relative light unit (RLU). Procedure: The first trigger solution of H_2O_2 (0.30 ml, 10^4dilution), the second trigger solution of microperoxidase (0.30 ml, 1 mmol/l) as a catalyst. Light emission is initiated by addition of the alkaline luminol solution (pH 9.8) (in 0.050 ml of $7x10^{-5}$ mol/l). Photon output is accumulated for 30 sec. The volumes of bile samples were 0.5 ml respectively. Luminol solution was added to the bile samples and was mixed with a vortex (10 sec) before fixing the tube in a holder. Each measuring point represents five parallel data in luminol-dependent chemiluminescence experiments when cv% was < 5 %. (Watanabe *et al.*,1992)

Free bilirubin was determined from chloroform extraction with Muraca method (1983). The bilirubin and bilirubin derivates of collected bile samples were analysed by HP 1090 M liquid chromatograph with diode-array detector. Colonna CHROSIL 10 µm, 250 x 4,6 mm, eluent: chloroform-ethanol 99:1 + 0,5 % acetic acid, separation: isocratic, flow: 1 cm^3/perc, detection: 440 nm, temperature: 40 ^{o}C.

Results and Discussion

In vitro studies strenghtened, that free radicals influence the gallstone formation (Eder *et al.*,1996). In animal experiments free radical reactions were observed in the

flow bile as well as gallbladder bile (Blázovics *et al.*,1996, 1997; Sipos 2003). Occurrences of C18:1ω9, C18:2ω6 and C20:4ω6 fatty acids were present in high percentages in the gallbladder biles of the examined cholecystectomysed patients. In every case the patients suffered from cholecystitis chronica with gallstones (Table 1.).

Unsaturated fatty acids are precursor molecules for diene conjugates. There was no significant correlation between diene conjugates and total bilirubin in human gallbladder bile (Figure 1.) It was not surprising, since diene conjugates are transient lipid peroxidation forms. It was also interesting, that high diene conjugate concentration could be detected in high bilirubin concentration too Figure 2. shows the correlation between MDA, the end-product of lipid peroxidation process and total bilirubin concentration.

Both antioxidant and free radical formation of bilirubin (concentration dependently) can be present in the gallbladder and in enterohepatic circulation of the bile. Figure 3. shows the ambivalent property of bilirubin molecule in human gallbladder bile. Bilirubin is a free radical in low concentration range 100-800 μmol/l, and antioxidant in higher concentrations in the bile.

It can be supposed, that oxygen free radicals and bilirubin reactions contribute the bile lipid peroxidation pool and gallstone formation, thus, not only biological, biochemical but physicoch-emical factors may play an important role in the formation of gallstones.

Free bilirubin and two oxidable derivates can be seen in Figure 4. The basic structure of bilirubin has six intracellular stable hydrogen bonds, whereby the bilirubin is insoluble in aqueous phase. Solubility of bilirubin in acids and alkalies is better, than in normal pH (Bonnett 1976). The potentially cytotoxic lipid soluble bilirubin is transported in the plasma tightly bond to albumin. Conjugated bilirubins (bilirubin esters) are formed from bilirubin and glucuronic acid by microsomal enzymes of liver. Sulphate, xylose and glucose conjugation also occur to a small extent and may be increased in cholestasis (Chowdhury 1983). In general the small rate transport of free bilirubin molecules from the microsomes can be observed parallel with conjugated bilirubins (Nakao *et al.*,1988, Okta *et al.*,1992).

The bilirubin content of the human gallbladder bile is cc. 5 mmol/l. Bilirubin can be found in mono-and diglucuronide forms, but free bilirubin molecules can be detected in the gallbaldder bile as well. Sources of free bilirubin molecules are in the microsomal P450 system in the liver and bacterial enzymatic transformation in gastrointestinal tract. Bilirubin reduced forms: urobilinogen, urobilin and stercobilin absorb in the terminal ileum and in large intestine (Chowdhury and chowdhurg 1983, Bosma *et al.*,2003).

In this study, diene conjugates and malondialdehyde were detected in gallbladder bile after cholecystectomy. Free bilirubin and bilirubin derivatives and fatty acids were also detected in randomly chosen bile samples. Bilirubin pro-and antioxidant forms are justified in human gallbladder bile.

Positive correlation is found in the literature between bacterial β-glucuronidase activity and the proportion of unconjugated bilirubin in bile (Vitetta and Sali 1992). In cholangitis, pH of bile becomes lower toward the optimal pH of bacterial β-glucuronidase activity. Papillary dysplasia and or severe dysplasia of the bile duct epithelium may be caused by aerobic and anaerobic bacterial infection of the biliary tract in contribution with bile stasis. Appearance of Escherichia coli and Clostridium. perfringens β-glucuronidase activity in bile duct is a key factor in primary bile stone formation (Nakao, et al 1988, Okta *et al.*,1992). Free bilirubin interferes with free fatty acid metabolism in pathological conditions (Bayes Garcia *et al.*,1989). Unsaturated fatty acids in food seem to increase the risk of gallstone formation (Cohen, 1985). Ultraweak chemiluminesc-ence from bilirubin occurs in the presence of triplet oxygen and it can be stimulated by aldehydes (Watanabe 1992). Bilirubin is a known photodyinamic agent producing O_2 in tbe presence of O_2

and light (Yang 1992). Free radical forms of bilirubin are justified by ESR technique (Shen *et al.*, 1996). The kinetic curve of the regeneration of bilirubin radicals has been determined and the reaction follows a zero order mechanism (Yang *et al.*,1992). Linear correlation was found between the amplitude of the free radical signal of the electron spin resonance spectrum of paraffin embedded liver blocks and the number of bile casts in the histological section made from the same blocks. ESR signal intensity also correlated linearly with the content of calcium bilirubinate in pigment gallstone samples (Shen *et al.*, 1996).

At the same time antioxidant properties of conjugated bilirubin and biliverdin have been discussed in several studies. In vivo, the plasma antioxidant capacity of jaundiced newborn infants is related to the level of bilirubin (Belanger *et al.*,1997).

Conjugated bilirubin at low micromolar concentrations inhibits luminol chemiluminescence response of stimulated human polymorphonuclear leucocytes. Bilirubin can scavenge peroxyl radical. Furthermore under 2% of oxygen in liposomes bilirubin suppresses the oxidation more than α tocopherol (Stocker *et al*. 1987,Stocker and Peterhans 1989).

Table 1.

Fatty acid composition in human gallbladder bile with cholecystitis chronica		
Fatty acids		Gallbladder bile (N=17) (%±SEM)
C14:0	miristic acid	1,79 ± 0,64
C16:0	palmitic acid	15,90 ± 1,46
C16:1	palmitoyl acid	10,54 ± 1,56
C18:0	stearic acid	7,38 ± 1,18
C18:1	oleic acid	28,05 ± 2,19
C18:2	linoleic acid	28,05 ± 1,15
C20:0	arachin acid	2,51 ± 0,80
C20:4	arachidonic acid	6,07 ± 0,89

Figure 1.

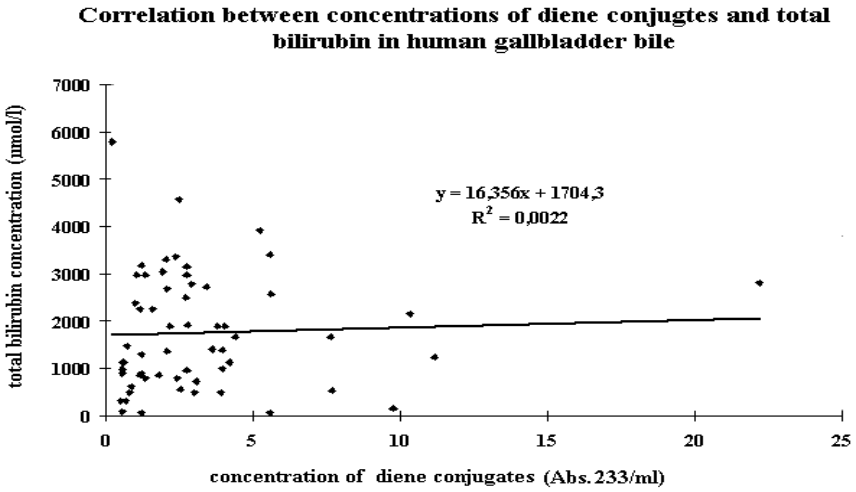


Figure 2.

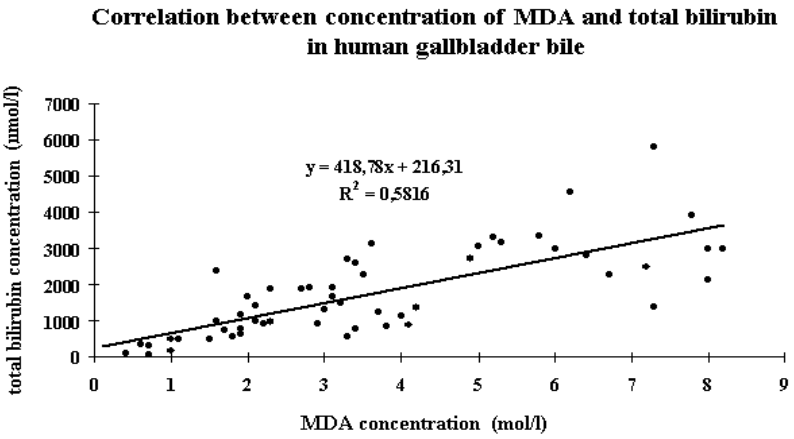


Figure 3.

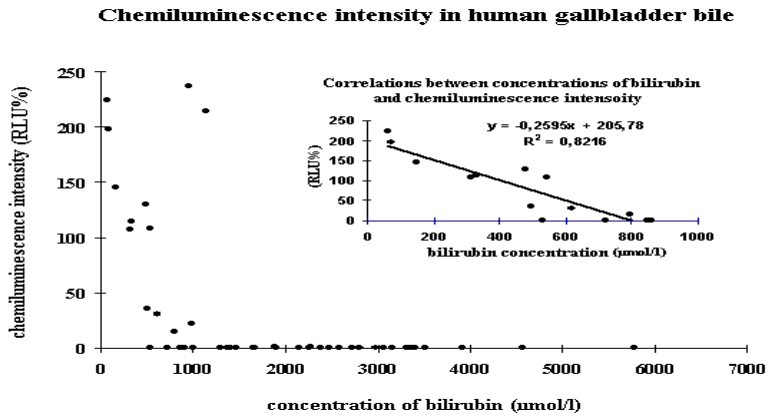
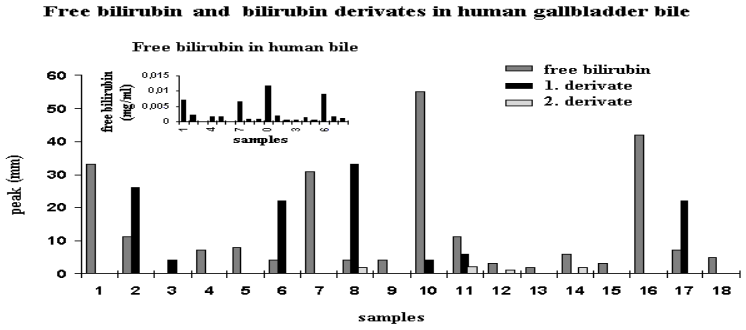


Figure 4.



Acknowledgements

Authors express their thanks to Ms. Edina Pintér and Ms. Sarolta Bárkovits for their excellent technical assistance. This study was supported by ETT 02/517 and ETT 002/2003

References

1. **Abdel Rachman M.(1996) :** Free radical reactions in bile secretion and gallstone disease, 1996, Ph.D Thesis, Semmelweis University, Hungary
2. **Abdel Rahman M, Blázovics A, Ágoston M, Asztalos I, Prechl J, Vallent K, Fehér J (1995) : A** chemiluminescent study for detection of free radicals in gallbladder bile in gallstone diseases, Ces. a Slov Gastroent., 1995, 49, 7-13.

3. **Alexander LR, Justice JB (1985) :** Fatty acid composition of human erythrocyte membranes by capillary gas chromatogr-aphy mass spectrometry, J. Chromatogr., 1985, 342, 1-12.
4. **AOAC (1984):** Official Methods of Analy-sis 28054 B. 14th ed.Arlington USA, 1984.
5. **Bayes Garsia R, Maldonado Sorano J, Molina Font JA (1989)** : Interrelation of bilirubin and free fatty acids in newborn infants with pathologic conditions, An. Exp. Pediatr., 1989, 30, 27-31.
6. **Belanger S, Lavoie JC, Chessex P (1997):** Influence of bilirubin on the antioxidant capacity of plasma in newborn infants, Biol. Neonate, 1997, 71, 4, 233-238.
7. **Blázovics A, Fehér E, Abdel Rahman M, Prechl J, Ágoston M, Fehér J (1996):** Free radical in connection of bile and liver, Ces. a Slov. Gastoent., 1996, 50, 73-78.
8. **Blázovics A, Örsi F, Kemény T, Fehér E, Barta I, Fehér J (1997)**: Lipids and lipid peroxidation in connection of bile and liver, Progr. Hepato-Pharmacol., 1997, 2, 103-117.
9. **Blázovics A, Kovács Á, Lugasi A, Hagymási K, Bíró L, Fehér J (1999):** Antioxidant defence in erythrocytes and plasma of patients with active and quiescent Crohn's disease and ulcerative colitis: A chemiluminescent study, Clin. Chem., 1999, 45, 6,895-896.
10. **Bonnett R, Davies JE, Hursthouse MB (1976):** Structure of bilirubin, Nature, 1976, 262, 326-327.
11. **Bosma P. Inherited disorders of bilirubin metabolism (2003):** a review, J. Hepatol., 2003, 38, 107-117.
12. **Carey MC(1988):** Formation of pigment gallstones. In: Paumgartner G, Stiehl A and Gerock W (ed) Trends in bile acid research, Dordrecht. 1988, 259-281.
13. **Chowdhury JR, Chowdhury NR (1983):** Conjugation and excretion of bilirubin, Semin. Liv. Dis. 1983, 3, 11-15.
14. **Cohen S, Soloway RD (1985):** Gallstones, Churchill Livingstone, New York 1985.
15. **Eder IM, Miquel JF, Jüngst D, Paumgartner G, von Ritter C (1996):** Reactive oxygen metabolites promote cholesterol crystal formation in model bile: role of lipid peroxidation, Free Rad. Biol. Med., 1996, 20, 743-749.
16. **Levy PF, Smith BF, LaMont JT (1984):** Human gallbladder mucin accelerates nucleation of cholesterol in artificial bile, Gastroenterol., 1984, 87, 270-275.
17. **Muraca M, Blanckaert N (1983):** Liquid-chromatographyc assay and identification of mono–and diester conjugates of biliru-bin in normal serum, Clin. Chem., 1983, 29, 1767-1771.
18. **Nakao T, Tabata M, Nakayama F (1988)**: Unconjugated bilirubin in hepatic bile with brown pigment gallstone and cholangitis, Dig. Dis. Sci., 1988, 33, 1116-1120.
19. **Okta T, Nagakawa T, Tsukioka Y, Sanada H, Miyazaki I, Terada T (1992)**: Proliferative activity of bile duct epithelium after bacterial infection in dogs, Scand J. Gastroenterol., 1992, 27, 845-851.
20. **Pyles IA, Strejskal J, Eizing S (1993):** Spectrophotometric measurement of plasma 2-thiobarbituric acid-reactive substances in the presence of hemoglobin and bilirubin interference, (4355) Proc. Soc. Exp. Biol. Med., 1993, 4, 407-419.
21. **Shen T, Lin C, Yang Z, Weng S, Lu J, Shen G, Gu Y, Xu Z, Fu X, Wang K, Wu J, Zhou X (1996):** Presence of free radicals in pigment gallstone in vivo, Chin. Med. J. Engl., 1996, 109, 6, 446-449.
22. **Sípos P (2001):** Free radical reactions in the bile. Doctor of Philosophy, 2001, Semmelweis University, Hungary,

23. **Sipos P, Szentmihályi K, Fehér E, Abaza M, Szilágyi M, Blázovics A (2003):** Some effects of lead contamination on liver and gallbladder bile, Acta Biol. Szegediensis, 2003, 47, 1-4, 139-142.
24. **Stocker R, Peterhans E (1989):** Antio-xidant properties of conjugated bilirubin and biliverdin biologically relevant scave-nging of hypochlorous acid. Free. Rad. Res. Com., 1989, 6, 57-66.
25. **Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN (1987):** Bilirubin is an antioxidant of possible physiological importance, Science, 1987,235,1043-1046.
26. **Trotman BW (1992):** Formation of pigment gallstones, In: Cohen S and Soloway RD (ed) Gallstones, New York. 1985, 299-300.
27. **Vitetta L, Sali A (1992):** Primary bile duct stones and bacterial activity HPD. Surg. 1992, 6, 23-32.
28. **Watanabe H, Usa M, Kobayashi M, Agatsuma S, Inaba H (1992):** Weak chemiluminescence of bilirubin and its stimulation by aldehydes, J. Biolum. Chemilum., 1992, 7:1-11.
29. **Yang ZH, Wang K, Liu XT (1992):** studies of electron spin resonance on bilirubin free radicals, Sci. China B., 1992, 35, 1093-1100.

الخاصية المزدوجة للبليروبن في السائل المراري للإنسان

أنا بلازوفيتش* ، بيتر شيبوش ، فرنس أورشى ، ميرفت عبد الرحمن*****

* كلية الطب – بودابست – المجر ، **جامعة القبته – بودابست – المجر ، *** مستشفى الطلبة – جامعة القاهرة – الجيزة

تتكون الحصوات المرارية نتيجة لأسباب كثيرة منها زيادة تكسر كريات الدم الحمراء، زيادة مستوي الدهون بالدم، مرض السكر. بالرغم من أهمية الدور الذي تقوم به تفاعلات العوامل الحرة المسببة للأكسدة في الأمراض المختلفة مثل ترسب الدهون في خلايا الكبد وارتفاع مستوي الدهون بالدم إلا أن العلاقة بين العوامل الحرة المسببة للأكسدة و تكون الحصوات المرارية غير معروف علي وجه الدقة لليوم. في هذا البحث تم تحليل عينات من السائل المراري لعدد 88 من مرضي الحصوات المرارية والذين اجري لهم عملية استئصال الحويصلة المرارية. استخدم جهاز التحليل الكروماتوجرافي عالي الأداء (HP1090) في تحديد البليروبن ومشتقاته في السائل المراري. تم استخدام جهاز التحليل الكروماتوجرافي عالي الأداء (HP5890) لتحليل الأحماض الدهنية. بالإضافة الي ذلك تم قياس العوامل الحرة المسببة للأكسدة باستخدام luminometer. أوضحت النتائج وجود الأحماض الدهنية C18:1ϖ9, C18: 2ϖ6, C20:4ϖ6 بنسب عالية في السائل المراري في كل حالة من 17 عينة عشوائية من المرضي الذين عانوا من التهاب مزمن بالحويصلة المرارية ومن الحصوات المرارية وتم استئصال الحويصلة المرارية لهم. كذلك تم العثور علي نواتج أكسدة الأحماض الدهنية في كل العينات التي تم تحليلها من السائل المراري. اثبت التحليل الإحصائي وجود علاقة طر دية بين مستوي البليروبن المنخفض والعوامل الحرة المسببة للأكسدة. لوحظ وجود مستوي عال من العوامل الحرة المسببة للأكسدة في عينات السائل المراري من المرضي الذين عانوا من أعراض شديدة من الحصوات المرارية. يستنتج من البحث وجود بليروبن مساعد للأكسدة وبلروبن مضاد للأكسدة في السائل المراري للإنسان.