Original article **UDC: 577.1:616.36-008.6]:616.366-003.7**
doi:10.5633/amm.2014.0102

CONNECTION BETWEEN MARKERS OF CHOLESTASIS AND INTENSITY OF OXIDATIVE MODIFICATION OF PROTEINS IN PATIENTS WITH CHOLEDOCHOLITHIASIS

Zoran Damnjanović[1], Milan Jovanović[1,2], Aleksandar Nagorni[2,3], Dušan Sokolović[2], Milan Radojković[2,4], Goran Damnjanović[5], Boris Đinđić[2], Dragutin Grozdanović[1], Mlađan Golubović[1]

The aim of this study was to examine the connection between cholestatic markers and the oxidative protein modification intensity in patients with choledocholithiasis. All the participants were subjected to clinical, laboratory and ultrasonic check-up at the Internal Department of the Military Hospital in Niš, Serbia. The parameters of oxidative stress: carbonyl groups, a measure of oxidative protein modification, and biochemical markers of cholestasis were determined by standard biochemical methods. The concentration of total ($r=0.41$, $p<0.05$), direct ($r=0.49$, $p<+0.01$) and indirect ($r=0.41$, $p<0.05$) bilirubin was in statistically significant positive linear correlation with the intensity of oxidative modification of proteins, while the other biochemical markers of cholestasis did not show such correlation. Total, direct and indirect bilirubins showed a significant positive correlation with oxidative protein modification, assessed through the levels of carbonyl groups in patients with choledocholithiasis. *Acta Medica Medianae 2014;53(1):10-14.*

Key words: *cholestasis, intensity of oxidative modification of proteins, choledocholithiasis*

Vascular Surgery Clinic, Clinical Center Niš, Serbia[1]
Faculty of Medicine, University of Niš, Serbia[2]
Clinic of Gastroenterology and Hepatology, Clinical Center Niš, Serbia[3]
General Surgery Clinic, Clinical Center Niš, Serbia[4]
Department of Internal Medicine, Military Hospital Niš, Serbia[5]

Contact: Zoran Damnjanović
Vascular Surgery Clinic, Clinical Center of Niš
Bulevar Zorana Đinđića 48, 18000 Niš, Serbia
E-mail: damnjanovicz@yahoo.com

Introduction

Numerous experimental and clinical researches indicate the significance of cholestasis, inflammation and oxidative stress, mutually connected by pathophysiological mechanisms in patients with choledocholithiasis (1-5).

Results of our previous prospective study confirmed the positive correlation of neutrophils, total, direct and indirect bilirubin with the intensity of lipid peroxidation in patients with choledocholithiasis (6).

There are no literary data on connection between cholestatic parameters and the oxidative protein modification intensity in patients with choledocholithiasis. Therefore, the aim of this study is to examine the connection between cholestatic markers and the oxidative protein modification intensity in patients with choledocholithiasis.

Patients and methods

The study included 70 subjects divided into two groups: the choledocholithiasis group (CHDL) - 40 patients with obstructive jaundice caused by choledocholithiasis and the control group - 30 healthy individuals.

The patients with extrahepatic cholestasis due to mechanical obstruction caused by choledocholithiasis were included in the study. The obstruction of biliary ducts caused by other factors was not considered.

The diagnosis of obstructive icterus was made according to anamnestic data, clinical features, and biochemical and ultrasound examination of biliary ducts. For the ultrasound examination of biliary ducts in the supine position, Sono et Medison Co. Ltd ultrasound was used.

All the patients were anamnestically and clinically observed at the Internal Department of Military Hospital in Niš, Serbia. Basic biochemical indicators and parameters of oxidative stress were determined in Biochemical Laboratory of Military Hospital in Niš and the Laboratory of the Biochemistry Institute at the Faculty of Medicine in Niš.

All the patients with choledocholitiasis were tested in the first three days since the occurrence of cholestasis syndrome and before surgery or endoscopic retrograde cholangiopancreatography (ERCP) with papillotomy.

Table 1. The results of laboratory parameters in control group and patients with choledocholitiasis

Parameter	Control	CHDL
Alkaline phosphatase (U/L)	81.4±37.7	385.0±459.0***
γ-glutamyl transferase (U/L)	24.1±6.0	364.0±382.0***
Total bilirubin (mmol/L)	9.5±2.8	123.2±101.1***
Direct bilirubin (mmol/L)	3.01±1.09	55.1±39.4***
Indirect bilirubin (mmol/L)	6.6±2.4	73.6±61.8***
Carbonyl groups (μmol/g protein)	5.7±1.8	8.8±3.0***

***p < 0.001 compared to the control

Table 2. Correlation between biochemical parameters of cholestasis with the intensity of oxidative modification of proteins in the control group and the patients with choledocholitiasis

Parameter	Control correlation with CG (r)	CHDL correlation with CG (r)
Alkaline phosphatase (U/L)	0.05	0.16
γ-glutamyl transferase (U/L)	0.24	0.07
Total bilirubin (mmol/L)	0.02	0.41*
Direct bilirubin (mmol/L)	0.1	0.49**
Indirect bilirubin (mmol/L)	-0.04	0.41*

r – Pearson's correlation coefficient
*p < 0.05; **p < 0.01 compared to the control

Participants of both groups did not differ in gender and age structure. Out of the total number of studied subjects, 37 (53%) were men and 33 (47%) women. The average age of the patients was 58.8±15.9 years.

Biochemical analysis

Cholestasis parameters: activity of γ-glutamyl transferase (γ-GT), alkaline phosphatase (AP) and the level of bilirubin were determined. The previously mentioned biochemical parameters were determined by the ready tests produced by Ellitech Company, on the biochemical analyzer BTS–370 (Bio–system).

Assessment of oxidative protein modification products in form of carbonyl groups (CG), was made by using a colorimetric reaction with 2,4 dinitrophenyl-hydrazine (2,4 DNPH) and TCA (7). Assessment of carbonyl groups in amino acid residue is an important marker of oxidative protein modification. Concentration of carbonyl groups was expressed in μmol/g plasma protein.

Statistical analysis

The data were analyzed by means of the commercially available statistic software package (SPSS® for Windows, v. 9.0, Chicago, USA) using the Student's t-test and Chi-square test. The results were presented as means ±/SD. Statistical significance was set at $p<0.05$. To determine the correlation of biochemical markers of cholestasis with the intensity of oxidative modification of proteins the Pearson's correlation coefficient (r) was used.

Results

The level of cholestasis, measured via biochemical indicators, and the intensity of oxidative modification of proteins measured in the form of CG are shown in Table 1.

Using statistical analysis of data, the following was shown: significant increase ($p<0.001$) of alcal phosphatase, γ–GT, as well as the increase in total, direct and indirect levels of plasma bilirubin in patients with extrahepatic cholestasis caused by choledocholithiasis. Intensity of oxidative protein modification was followed by a change in levels of carbonyl groups, which were statistically significantly higher in patients with choledocholithiasis ($p<0.001$) compared to the control group of patients.

The correlation of biochemical parameters of cholestasis with the intensity of oxidative modification of proteins in the patients with choledocholithiasis is shown in Table 2.

The concentration of total ($r=0.41$, $p<0.05$), direct ($r=0.49$, $p<0.01$) and indirect ($r=0.41$, $p<0.05$) biliruin was in statistically significant positive linear correlation with the intensity of oxidative modification of proteins, while the other biochemical markers of cholestasis did not show such corre-lation.

Discussion

The cholestatic markers, as well as CG levels, were statistically significantly increased in group of patients with choledocholithiasis compared to control group (Table 1). Conducted research confirmed results presented by other authors about increased inflammation, cholestasis and oxidative protein modification in patients with choledocholithiasis (3-5).

Experimental literary data show that the increase of carbonyl groups level is followed by biliar duct proliferation and neutrophil infiltration, and therefore represents an important pathophysiological mechanism for increased oxidative stress in patients with choledocholithiasis (8,9).

Obstructive jaundice and endotoxemia cause the expression of hemostatic factors and an increase in liver/biliar tract – infiltrating neutrophils. Cascade reaction in which neutrophils have a leading role is a source of a great amount of free radicals which lead to damage of aforementioned structures and an increase in carbonyl groups concentration (10,11).

Noted in this research is a connection between total, direct and indirect bilirubin and CG, while other cholestatic markers did not present with such connection. Laboratory research indicates a significant bond between obstructive jaundice and immune function, especially in experimental models with biliar obstruction. Mice with ligated bile ducts have increased expression of toll-like receptors (TLR) 4 and increased level of hepatotoxic cytokines, such as tumor necrosis factor alfa (TNF-a), as well as the increased level of protective anti-inflammatory cytokines (interleukin-6), as a response to endotoxic effect. Laboratory research shows that bilirubin and other hem metabolic products are closely connected to inflammatory response and risk of infection (12).

Obstructive jaundice induces intestinal oxidative stress in people, which may be the key factor in intestinal barrier loss and development of septic complications in these patients (13).

It is presumed that hydrophobic bile acid, withheld in hepatocytes during cholestasis, induce production of reactive oxygen radicals (ROS) in mitochondria, which leads to lipid peroxidation and loss of liver cells vitality. There is experimental proof that hydrophobic bile acid stimulates production of ROS in isolated liver cells and in purified mitochondria in rat hepatocytes (14). Bile acid may disturb transport of electrons in respiratory chain and therefore lead to ″leakage″ of electrons in the third complex of respiratory chain (ubiquinone), thus stimulating the formation of superoxid anion radicals (O_2-) and their metabolites (15).

In cholestatic conditions, bile acid cause change in mitochondrial membrane permeability (MPT), and consequently lead to loss in mitochondrial membrane potential ($\Delta\Psi m$), therefore interrupting respiratory chain and inhibiting ATP synthesis. Complete chain of reactions that potentially change mitochondrial membrane permeability is a result of ROS production, which are the main stimulating factors in hepatocyte necrosis and apoptosis (16). Yerushalmi et al. (17) noted that even relatively low concentration of bile acid leads to oxidative stress in hepatocytes, and also noted a significant positive linear correlation between apoptosis and magnitude of oxidative stress.

Lately, there have been numerous clinical cases in which patients with cholestasis have lethal hepatocyte damage due to increased concentration of hydrophobic bile acid that induce apoptosis. Apoptosis is a form of cellular death, followed by numerous morphological and biochemical changes and it represents a form of „programmed cellular death". Mitochondrial dysfunction and loss of mitochondrial membrane potential can be an important mechanism that stimulates cholestatic bile acid induced apoptosis of hepatocytes (18).

Conclusion

Based on the results of this research, we can conclude that total, direct and indirect bilirubin show a significant positive correlation with oxidative protein modification, assessed through levels of carbonyl groups, in patients with choledocholithiasis.

Hyperbilirubinemia analyzed in this manner represents an important parameter that can additionally clarify pathophysiological aspects of liver tissue damage in choledocholithiasis, and can also be treated as a prognostic factor for existing liver oxidative stress.

References

1. Kosters A, Karpen SJ. The role of inflammation in cholestasis: clinical and basic aspects. Semin Liver Dis 2010; 30(2): 186-94. [CrossRef] [PubMed]
2. Allen K, Jaeschke H, Copple BL. Bile acids induce inflammatory genes in hepatocytes: a novel mechanism of inflammation during obstructive cholestasis. Am J Pathol 2011; 178(1): 175-86. [CrossRef] [PubMed]
3. Woolbright BL, Jaeschke H. Novel insight into mechanisms of cholestatic liver injury. World J Gastroenterol 2012; 18(36): 4985-93. [CrossRef] [PubMed]
4. Kim ND, Moon JO, Slitt AL, Copple BL. Early growth response factor-1 is critical for cholestatic liver injury. Toxicol Sci 2006; 90(2): 586-95. [CrossRef] [PubMed]
5. Zollner G, Trauner M. Nuclear receptors as therapeutic targets in cholestatic liver diseases. Br J Pharmacol 2009; 156(1): 7-27. [CrossRef] [PubMed]
6. Damnjanović Z, Jovanović M, Nagorni A, Radoj ković M, Sokolović D, Damnjanović G, et al. Correlation of inflammation parameters and bio chemical markers of cholestasis with the intensity of lipid peroxidation in patients with choledo cholithiasis. Vojnosanit Pregl 2013; 70(2): 170-6. [CrossRef] [PubMed]
7. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994; 233: 346-57. [CrossRef] [PubMed]
8. Woolbright BL, Antoine DJ, Jenkins RE, Bajt ML, Park BK, Jaeschke H. Plasma biomarkers of liver injury and inflammation demonstrate a lack of apoptosis during obstructivecholestasis in mice. Toxicol Appl Pharmacol 2013; 273(3): 524-31. [PubMed]
9. Hatano E. Tumor necrosis factor signaling in hepatocyte apoptosis. J Gastroenterol Hepatol 2007; 22 (1): 43-4. [CrossRef] [PubMed]
10. Kono H, Asakawa M, Fujii H, Maki A, Amemiya H, Yamamoto M, et al. Edaravone, a novel free radical scavenger, prevents liver injury and mortality in rats administered endotoxin. J Pharmacol Exp Ther 2003; 307(1): 74-82. [CrossRef] [PubMed]
11. Yang M, Ramachandran A, Yan HM, Woolbright BL, Copple BL, Fickert P, et al. Osteopontin is an initial mediator of inflammation and liver injury during obstructive cholestasis after bile duct ligation in mice. Toxicol Lett 2014; 224(2): 186-95. [CrossRef] [PubMed]
12. Overhaus M, Moore BA, Barbato JE, Behrendt FF, Doering JG, Bauer AJ. Biliverdin protects against polymicrobial sepsis by modulating inflammatory mediators. Am J Physiol Gastrointest Liver Physiol 2006; 290(4): 695-703. [CrossRef] [PubMed]
13. Assimakopoulos SF, Thomopoulos KC, Patsoukis N, Georgiou CD, Scopa CD, Nikolopoulou VN, et al. Evidence for intestinal oxidative stress in patients with obstructive jaundice. Eur J Clin Invest 2006; 36(3): 181-7. [CrossRef] [PubMed]
14. Sokol RJ, Straka MS, Dahl R, Devereaux MW, Yerushalmi B, Gumpricht E, et al. Role of oxidant stress in the permeability transition induced in rat hepatic mitochondria by hydrophobic bile acids. Pediatr Res 2001; 49(4): 519-31. [CrossRef] [PubMed]
15. Shivaram KN, Winklhofer-Roob BM, Straka MS, Devereaux MW, Everson G, Mierau GW, et al. The effect of idebenone, a coenzyme Q analogue, on hydrophobic bile acid toxicity to isolated rat hepatocytes and hepatic mitochondria. Free Radic Biol Med 1998; 25(4-5): 480-92. [CrossRef] [PubMed]
16. Rodrigues CM, Steer CJ. Mitochondrial membrane perturbations in cholestasis. J Hepatol 2000; 32(1): 135-41. [CrossRef] [PubMed]
17. Yerushalmi B, Dahl R, Devereaux MW, Gumpricht E, Sokol RJ. Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrialpermeability transition. Hepa tology 2001; 33(3): 616-626. [CrossRef] [PubMed]
18. Solá S, Amaral JD, Aranha MM, Steer CJ, Rodrigues CM. Modulation of hepatocyte apoptosis: cross-talk between bile acids and nuclear steroid receptors. Curr Med Chem 2006; 13(25): 3039-51. [CrossRef] [PubMed]

POVEZANOST MARKERA HOLESTAZE SA INTENZITETOM OKSIDATIVNE MODIFIKACIJE PROTEINA KOD BOLESNIKA SA HOLEDOHOLITIJAZOM

Zoran Damnjanović, Milan Jovanović, Aleksandar Nagorni, Dušan Sokolović, Milan Radojković, Goran Damnjanović, Boris Đinđić, Dragutin Grozdanović, Mlađan Golubović

Cilj ovog istraživanja bio je ispitati povezanost markera holestaze sa oksidativnom modifikacijom proteina kod bolesnika sa holedoholitijazom. Svi ispitanici sagledani su klinički, laboratorijski i ultarzvučno na Internom odeljenju Vojne bolnice Niš u Srbiji. Parametri oksidativnog stresa, karbonilne grupe, merilo oksidativne modifikacije proteina i biohemijski marker holestaze ispitivani su standardnim biohemijskim metodama. Koncentracija totalnog ($r=0.41$, $p<0.05$), direktnog ($r=0.49$, $p<0.01$) i indirektnog ($r=0.41$, $p<0.05$) bilirubina pokazala je statistički značajnu pozitivnu linearnu korelaciju sa intenziteteom oksidativne modifikacije proteina, dok ostali biohemijski parametri holestaze nisu pokazali takvu povezanost. Na osnovu nivoa karbonilnih grupa, totalni, direktni i indirektni bilirubin pokazuje značajnu povezanost sa oksidativnom modifikacijom proteina kod bolesnika sa holedoholitijazom. *Acta Medica Medianae 2014;53(1): 10-14.*

Ključne reči: *holestaza, intenzitet oksidativne modifikacije proteina, holedoholitijaza*

