Original article

FOLLOW-UP OF BIOCHEMICAL PARAMETERS AND INTENSITY OF OXIDATIVE STRESS IN PATIENTS WITH EXTRAHEPATHIC CHOLESTASIS

Zoran Damnjanovic, Aleksandar Nagorni, Gordana Kocic, Zoran Blagojevic, Djordje Mancic and Ivana Damnjanovic

In cholestasis, due to impossibility of the gall flow into duodenum, there occurs a subsequent return of gall constituents to hepatocytes and circulation. Extrahepatic cholestasis is the result of mechanic obstacle of gall flow through ductus hepaticus, choledochus or papilla Vateri, leading to the occurrence of manifest icterus. In cases leading to liver damage, hydrophobic gall salts and non-conjugated bilirubins have the most important toxic effects.

The aim of this study was to follow up the biochemical parameters, enzyme activity AST, ALT, *-GT and AF, bilirubin concentration and albumins, and intensity of oxidative stress in blood plasma in patients with different types of extrahepatic cholestasis.

The study included 60 subjects divided into two groups. The first one was control group (30 healthy subjects), while II group involved 30 patients with intraluminar extrahepatic obstruction.

Significant increase of enzyme activity of AST, ALT, *-GT and AF in plasma of cholestatic patients was present in comparison to the control group (p< 0,001). The levels of total direct and indirect bilirubin in plasma of cholestatic patients increased (p<0,001) when compared to the control group. The level of albumin in plasma of cholestatic patients significantly decreased in comparison to the control group (p<0,05). The intensity of oxidative stress measured through the levels of malondialdehyde (MDA) and carbonyl group concentration in plasma of cholestatic patients increased (p<0,001) when compared to the control group.

Significant increase of cholestasis enzyme markers (AST, ALT, *-GT and AF) and bilirubin levels in blood plasma was noticed in patients with extrahepatic cholestasis. Cholestasis leads to significant disorders of synthetic function of the liver that are manifested by decrease of albumin concentration in plasma. *Acta Medica Medianae 2008;47(3):21-27.*

Key words: *cholestasis, choledocholithiasis, oxidative stress, bilirubin, albumin*

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Introduction

Cholestasis is a disorder of the liver functioning, brought about by cessation of gall flow through bile ways into the small intestine (1). The obstructive icterus is brought about as a consequence of the extrahepatic obstruction, due to a mechanical obstruction in the swelling of a bile through ductus hepaticus, ductus choledochus or papilla Vateri. It is a stopping, obstructive or extrahepatic cholestasis, occurring at any level, starting from the left and right hepatic duct all the way down to the in-flow of the mutual bile canal into the Vateri ampoule. Bile stones, tumors, inflammatory exudates or parasites can lead to a blockage of the bile ducts. The above mentioned obstruction in any part of the biliary tract leads to a biochemical syndrome of cholestasis and clinical manifestations of obstructive icterus (2). The most frequent cause of extra-hepatic cholestasis is the presence of calcus in the main bile duct (3).

In the clinical picture of extra-hepatic cholestasis there is a pain of colic type, yet at times that pain may be dull, localized in the upper-right part of the abdomen and in the area of epigastrium with the feeling of uneasiness and pressure or, yet, the pain is localized in the belt zone. The pain is usually accompanied by sickness, nausea and vomiting. Pain character can oscillate depending on the type of the obstruction, in form of attacks and remissions, and the disorders can also be displayed in the form of dyspepsia. If, in addition to this obstruction, cholangitis is also developed, there will be Charcot trias pain, elevated body temperature (chill, shiver), as well as jaundice (4).

Biochemical disorders are the consequence of the increase in the value of conjugated bilirubin. With partial obstruction due to the damaging hepatocytes, hyperbilirubinaemia is of mixed, conjugated or unconjugated type (4). During cholestasis, there is an increase in the activity of membrane enzymes in the plasma (glutamil-transferasis, alkal- phosphatase, 5- nucleotidases) upon the retention of bile salts which perform the membrane solubilization, thus leading to the out-flow of the above mentioned enzymes into the circulation. Moreover, in the plasma of cholestatic patients there is an increased activity of the hepatocyte enzymes (alanine aminotransferase- ALT, and asparate aminotransferase- AST) due to the increased permeability of the cell membrane or necrosis (5).

Hypo-albuminemia indicates liver damage and its reduced capacity to synthesize albumins. In cases of serious damages, hypo-glycaemia occurs due to lower glycogenolysis. Damages in the CNS in cholestasis conditions are the consequence of hyper-bilirubinaemia and hypoxia.

Oxidative stress is the process of tissue damaging as a consequence of free radicals impact. They are capable of damaging almost all biomolecules in a cell (6). In physiological conditions, in any organism there is a balance between the processes of creating free radicals and antioxidant defence, by means of which the emergence of oxidative stress is prevented. Lipid peroxidation of unsaturated fatty acids, by impact of free radicals, is an unfavourable and negative process followed by a massive damages of lipid cell membranes. The peroxidation causes an increase in the membrane permeability of epatocyte plasma membrane (7). Parol et al. (1996) detected an increased level of lipid peroxidation (MDA) and oxidative (carbonyl group) proteins modification in a rat liver, with the obstruction of the mutual bile canal (8). Moreover, many other studies have proven more intense oxidative stress in the liver plasma and tissue in the animals suffering from an experimentally induced cholestasis (9,10,11).

The basic methods in morphological diagnostics of extra-hepatically caused obstructive icterus are: ultrasound (US), computerized tomography (CT), and magnetic resonance (MR). Ultrasound is the cheapest, safest and very sensitive technique for visualization of hepato-bilipancreatic system and it is an ideal screening method (12).

Taking all this into consideration, a significant interest in this work is dedicated to illuminating the changes in cholestasis markers enzyme activity, bilirubin concentration and albumin in blood plasma, as well as the intensity of oxidative stress measured through the level of **malondialdehydes** (MDA- the secondary product of lipid peroxidation) and the concentration of carbonyl groups (oxidative protein modification) in the patients suffering from extra-hepatic cholestasis caused by choledocholithiasis.

Aims

For the purposes of this paper, the following was done:

- considering the damages in liver cells during extrahepatic cholestasis caused by choledocho-lithiasis, through the observation of the activities of hepatic enzymes (asparate aminotransferase- AST - ALT, alanine aminotransferase, glutamil-transferasis and alkal- phosphatase) and albumin concentration in blood plasma
- analyzing disorders of hepatobiliary liver function through observation of disorders regarding the bilirubin concentration in a cholestasis patient blood.
- determining the intensity and characteristics of oxidative stress in patients suffering from obstructive jaundice caused by choledo-cholithiasis.

Material and methods

The general methodological approach has been the comparison between the data received by means of prospective and retrospective analysis of anamnestic data and clinically-biochemical indicators.

60 examinees, divided into two groups, were included in the research: I group- 30 patients suffering from obstructive jaundice caused by choledocholithiasis; II group- 30 healthy examinees.

In order to avoid the influence of different pathological processes which can lead to obstructive jaundice, only the patients suffering from extrahepatic cholestasis brought about by a mechanical obstruction caused by choledo-cholithiasis participated in the research (bilirubin, cholesterol, mixed). Obstructions of the bile ways caused by any other factors were not taken into the consideration.

The diagnosis of obstructive icterus was established based on the anamnestic data, clinical picture, biochemical and clinical diagnostic methods (ultrasound examination of bile ducts).

All the examinees were anamnestically and clinically observed at the Internal Ward of the Military Hospital in Nis. The examination of the patients was conducted in 2007/2008. The basic biochemical indicators and parameters of oxidative stress were determined in the Biochemical laboratory at the Military Hospital in Nis and in the laboratory of the Biochemical Institute of the Faculty of Medicine in Nis.

The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamil-transferasis (y-GT and alkal- phosphatase (AP) were determined in the patients' plasma. Enzyme activity was expressed in units per litre of plasma (U/l).

The bilirubin level and the quantity of albumin were determined in the plasma of the patients with extrahepatic obstruction. Bilirubin concentration in plasma was expressed in μmol/l, and of albumin in g/l.

The above mentioned biochemical methods were determined by a ready-made test of Ellitech Company, on the biochemical analyzer BTS-370 (BioSystems).

The quantity of malondialdehydes (MDA), the ultimate product of lipid peroxidation in the liver tissue was determined by means of tiobarbitur acid (TBA) (13).

Carbonyl groups concentration, as the measure for oxidative protein modification, was determined by means of spectrographic method, by using 2.4 dinitrophenilhydrazine (DPNH)-of traditional carbonyl reagents (14).

The data was processed by means of standard descriptive statistical methods (mean value and standard deviation). In the paper, the Student's t-test was applied for paired and unpaired samples. The processing of the obtained data was done by statistical programming package Statistical Package for Social Science (SPSS) software, version 11.0 in Windows 2000 surrounding, and the results were presented in tables and graphs.

Results

The final number of examined patients was: 30 patients with extrahepatic cholestasis caused by choledocholithiasis and 30 control examinees. The basic characteristics are shown in Table 1.

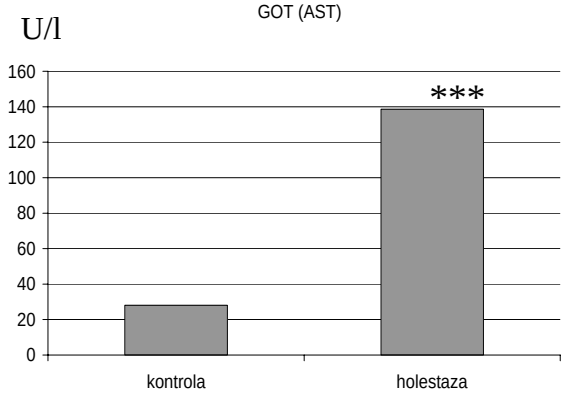
Table 1. Basic characteristics of examinees

	No	%	men	women	Age (years)
cholestasis	30	50	16	14	60,2±14,6
control	30	50	19	11	54,5±15
total	60	100	35 (58,3%)	25 (41,7%)	57,3±14,8

NS. for all the parameters

Out of the patients examined, 35 (58,3%) were male, and 25 (41.7) were female. The statistical analysis showed an even sex distribution in the examined groups. The average age of the examinees was 57.35+-14.8 years. There was not any statistically significant difference in the age between the sexes.

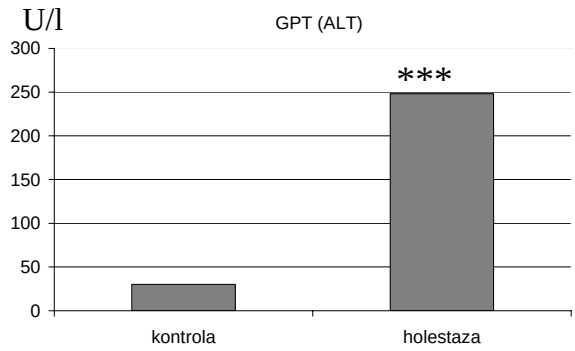
Examination of cholestasis markers included the analysis of the enzymes GOT (AST), GPT (ALT), AF and γ-GT, as well as the level of total and direct bilirubin.



*** p<0.001 in relation to control

The activity of enzyme GOT (AST) in blood plasma and the examined groups is displayed in Graph 1. In the patients suffering from extrahepatic cholestasis caused by choledocholithiasis, there was a significant increase in the activity of this enzyme in relation to the control group (138.61+- 81.55 U/l in relation to 28.07+-8.07 U/l ; p<0.001) (Graph 1).

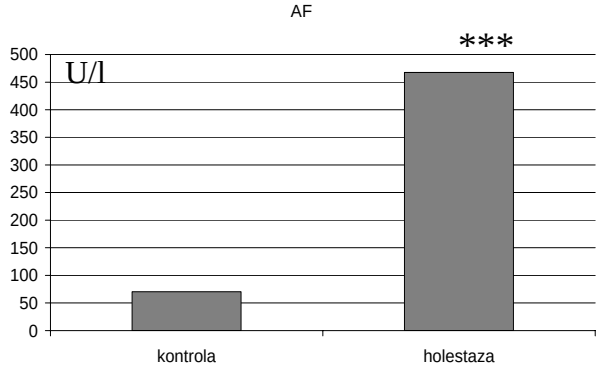
In Graph 2, the GPT activity (ALT) in the plasma of the patient with extrahepatic cholestasis is shown. In the patient suffering from cholestasis caused choledocholithiasis there was a significant increase in the activity of this enzyme in relation to the control group (248.24+-154.23 U/l in relation to 30.2+-10.49 U/l; p<0.001) (Graph 2).



***p<0.001 in relation to control

Graph 2. The GPT (ALT) Enzyme Activity in the Examined Groups

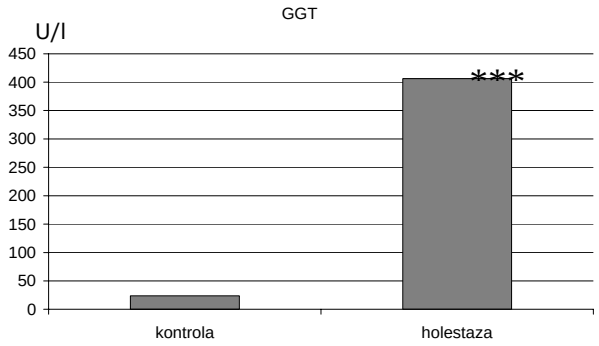
In Graph 3, the activity of alkal phosphatase is shown (AF) in the plasma of a patient with cholestasis. In the patient suffering from cholestasis there was a multiple increase in the activity of the above mentioned enzyme in relation to the control group (467.62+- 141.32 U/l in relation to 70.43+-18.66 U/l; p<0.001) (Graph 3).



***p<0.001 in relation to control

Graph 3. The alkal phosphatase (AF) enzyme activity in the examined groups

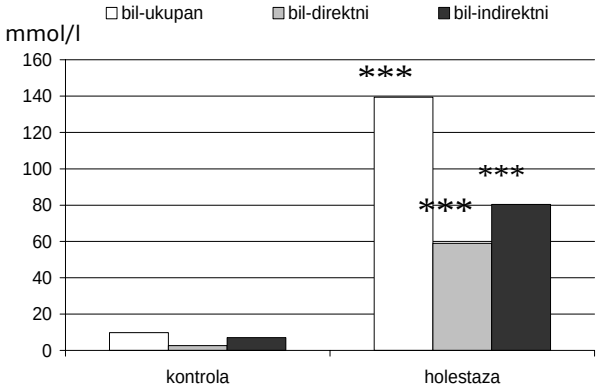
The activity of γ-GT enzyme in the patient plasma suffering from extrahepatic cholestasis caused by choledocholithiasis was shown in the Graph 4. The results show that there was a significant increase in the activity of this enzyme in the plasma of patients suffering from cholestasis (406.45+-266.17 U/l in relation to the control 23.78+- 8.45 U/l; (p<0.001) (Graph 4).



*** p<0.001 in relation to control

Graph 4. γ-Glutamil-Transferase (GGT) enzyme activity in the patient`s blood plasma

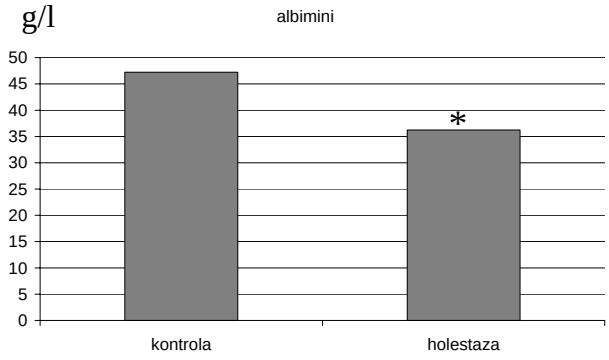
The levels of total, direct and indirect bilirubin in blood plasma in the patients suffering from extrahepatic cholestasis are significantly higher (p<0.001) in relation to the values with control patients (total-139.4+-72.60 μmol/l in relation to 9.8+-2.1 μmol/l, direct- 59.0+-34.33 μmol/l in relation to 2.7+- 1.03 μmol/l, indirect- 80.4+-34.63 μmol/l in relation to 7.1+-1.44 μmol/l) (Graph 5).



***p<0,001 compared to control

Graph 5. Concentration of bilirubin (mmol/l) in blood plasma of control and cholestasis patients

The level of albumin in blood plasma in the patients suffering from extrahepatic cholestasis caused by choledocholithiasis is statistically significantly lowered in relation to the control group (36.25+-6.81 g/l in relation to 47.24+-4.22 g/l; p< 0.05) (Graph 6).



*p<0,05 compared to control

Graph 6.Llevel of albumin (g/l) in blood plasma of control and cholestasis patients

The intensity of oxidative stress was measured through: the ultimate product of lipid peroxidation (malondialdehyde-MDA) and the quantity of oxidative modified proteins (carbonyl group), which is shown in Table 2.

Table 2. Intensity of oxidative stress in the examined groups

oxidative stress parameters	extrahepatic cholestasis	control
MDA (μmol/l)	55,1±25,3***	20,15±2,4
Carbonyl groups (μmol/g prot.)	9,2±2.55***	5,3±1,2

***p<0,001 compared to control

MDA and carbonyl group values were significantly higher in patients suffering from extrahepatic cholestasis compared to the control group of examinees (p<0.001) (Table2).

Discussion

In cholestasis syndrome, hydrophobic bile salts and unconjugated bilirubin have the most significant toxic effects on liver parenchyma (1). The highest percentage of intramural obstructions of bile canals belongs to choledocholithiasis. The most frequent causes for intraluminal obstruction are acute cholecystis-pancreatitis and the tumours of ductus-choledochus, whereas the most frequent reasons for extraluminal obstruction of bile ducts are acute calculous cholecystis and tumors of the pancreas head.

In the examined sample of the patients with extrahepatic obstruction caused by choledocholithiasis, there was no significant difference in sex distribution (Table 1). According to the results of Taiwan populational studies, the total calculosis prevalence of the bile is 5.0% in which case it is approximately in a similar way present in men -4.6% and women -5.4% (15). Similar results were obtained in the studies performed in America, which estimate the prevalence of calculosis to 5.9-21.9%, whereas this percent is even higher in European countries for the whole 10 to 20%, but without a significant difference between the sexes (16). The most recent studies done in Germany indicate that the highest prevalence of this disease occurs between 70-79 years of age (17).

The course of choledocholithiasis has not been studied enough. Even though the complications are often very frequent and serious, it is believed that less than 50% of the patients with calculosis of bial ducts develops the symptoms and that more than 20 % of these stones pass spontaneously (18,19).

The activities of AST and ALT enzyme in the patients with extrahepatic cholestasis are significantly increased in relation to the control group (p<0.001) (Graphs 1 and 2). The obtained results point to the fact that in the cholestasis conditions toxic bile acids accumulated in the

liver damage hepatocyte membrane (by their detergent qualities) and lead to the outpour of cytozolar ALT into the circulation (20). This is in keeping with the findings and results of some other authors who point out that the activity of transaminases is significantly increased in all the conditions with cholestasis (21). A slightly greater increase of Alt is explained by the fact that ALT is a more specific liver enzyme, because in other organs it can be found only in smaller quantities, differently from AST. ALT is a cytozolary hepatocyte enzyme, thus it goes out into the circulation even with smaller damages in the liver. Whereas AST is present in mitochondria (60-70%) and cytozole (30-40%) of hepatocyte; thus, it is necessary to cause a more serious damage of hepatocyte for the increase of this enzyme in plasma to be visible (22).

Alkal phosphatase enzyme (AF) belongs to the group of enzymes which constitute the integral part of the cell membrane. The AF values with the patients suffering from extrahepatic cholestasis caused by choledocholithiasis are almost 7 times more increased compared to the control examinees ($p<0.001$) (Graph 3). This is in keeping with the findings that the increase in alkal phosphatase is much more reliable indicator of biliary obstruction, especially in case of incomplete and segmented obstruction, where bilirubin values remain normal, so that the alkal phosphatase value and not transaminase (AST and ALT) shows bigger leap with obstructive jaundice (23). The liver and bile canal diseases are accompanied by the increase of AF activity which is characteristic of cholestasis syndrome. It was proved earlier that in cholestasis bile salts induce the synthesis of new molecules AF (24).

γ-GT enzyme is localized on plasma membranes (especially on the epithelium of bile canals), whereas it is less present in cytozole and hepatocyte microzomes. From the clinical point of view, the activity in γ-GT in serum is the most sensitive indicator of hepato-biliary system damage. In the patients suffering from obstructive cholestasis caused by choledocholithiasis, the activity of this enzyme in serum shows a multiple increase (almost 20 times) compared to the control $p<0.001$ (Graph 4). This significant increase in the activity in γ-GT in the patient with extrahepatic cholestasis plasma in the course of this study can be explained in several ways. Hydrophobic bile acids by means of their detergent-like quality of impact onto the hepatocyte membrane, lead to releasing of this enzyme into the circulation. In cholestasis, due to the regurgitation of bile constituents into the blood, there is an increased activity of γ-GT in serum. The earlier studies showed that in the conditions of cholestasis in hepatocytes, the activity of γ-GT in serum is de novo increased. As in cholestasis there comes to the necrosis of bile ducts epithelial cells (rich in γ-GT), the activity of this enzyme in circulation is increased. Finally, due to the proliferation of biliar canal epithelium cells, rich in γ-GT, its activity in serum is significantly increased (22,25).

Due to the mechanical impediment in bile canals, a retention of bile colours into the circulation occurs and there is hyperbilirubemia of conjugated type (26).

Since the excretion of bilirubins from hepatocyte is the limitating stage in its metabolism in relation to the conjugation, at the beginning of cholestasis, with the damages of lower degree, hyperbilirubemia of conjugated type is always predominant. Later on, along with the advancement and the deterioration of the pathological process, the concentration of unconjugated bilirubin in blood plasma is increased (27). As can be seen from Graph 5, in the patient with extrahepatic cholestasis there occurred a significant increase of the total, conjugated, as well as unconjugated bilirubin in the plasma in relation to the controlled examinees ($p<0.001$). It is believed that the increase in the value of direct bilirubin in plasma in case of cholestasis is the consequence of the increased concentration gradient between the cells and plasma, and excretion of bilirubin due to the damage caused by the obstruction of the bile swelling (27).

The results of this research showed that the level of albumin in blood plasma of cholestasis patients was lower compared to the control examinees ($p<0.05$) (Graph 6). It can be assumed that in the liver damaged by cholestasis there is a particularly lower degree of albumin and other proteins with longer polypeptide chain synthesis. The explanation for this process is related to the crisis of energetic metabolism in hepatocytes and lowering the quantity of ATP in mitochondrias. The oxidative phosphorization process is altered due to the disruption in the structural organization of mitochondrial membrane by the attack of hydrophobic bile salts.

The results obtained in this study showed a statistically significant increase of the unconjugated bilirubin (Graph 5) and lowering of the level of albumin (Graph 6) in plasma of cholestasis patients, in relation to the same values with the control examinees. We can conclude, that lowering of the albumin level in the plasma, a part of unconjugated bilirubin can be found in free state having toxic effects.

The intensity of oxidative stress measured through the values of MDA and carbonyl groups was significantly higher in the patients with cholestasis ($p<0.001$) (Table 2). The obtained results in this experimental research are in keeping with the literary data in the already mentioned similar studies of experimantally caused cholestasis. Hydrophobic bile acids, retained in hepatocytes during cholestasis, lead to the production of free radicals, which brought about the lipid peroxidation and the consequential loss of vitality and the function of liver cells (29,30,31). Sokol et al. in 2001, showed that those toxic bile acids retained in the hepatocytes in cholestasis increase the intensity of oxidative stress and cause the permanent loss of cells capability for life (9). This is a consequence of breaking the respiratory chain and the inhibition of ATP synthesis, accompanied by necrosis and apoptosis (29). Yerushalmi et al. in 2001 noted that even relatively

low concentrations of bile acids lead to oxidative stress in the cells (prior to apoptosis), and a significant linear correlation between the apoptosis and the the level of MDA was also noticed (10).

In *in vitro* study of Seejayan et al. in 1999, it was proven that taurodezoksilhol hydrophobic bile acid lead to the increase of the level of lipid peroxidation caused by FE $^{2+\cdot}$ This increase was triple during the initial phase of the incubation, which can suggest that bile acids can cause initiation and chain propagation of peroxidation. Hydrophobic bile acids promote creation of hydroxile radicals and they intensify the lipid peroxidation (6).

Conclusions

1. In the patients suffering from extrahepatic cholestasis caused by choledocholithiasis, the multiple increase of cholestasis marker activities (ALT, AST, AF and γ-GT) was noticed, along with the drop in the albumin concentration.
2. Due to extrahepatic cholestasis hyperbilirubinaemia of mixed type occurs.
3. In the patients suffering from obstructive icterus caused by choledocholithiasis, there is increased intensity of oxidative stress (lipid peroxidation and oxidative protein modification) in plasma.

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PRAĆENJE BIOHEMIJSKIH PARAMETARA HOLESTAZE I INTENZITETA OKSIDATIVNOG STRESA KOD BOLESNIKA SA HOLEDOHOLITIJAZOM

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Ekstrahepatična holestaza je posledica mehaničke prepreke u oticanju žuči kroz ductus hepaticus, choledochus ili papilu Vateri, što dovodi do pojave manifestnog ikterusa. Najčešće je izazvana holedoholitijazom. Pračena je oštećenjem hepatocita zbog nagomilavanja hidrofobnih žučnih soli i nekonjugovanog bilirubina.

Za ciljeve ovog rada postavljeno je sagledavanje oštećenja ćelija jetre, analiziranje poremećaja hepatobilijarne funkcije i određivanje intenziteta i karakteristika oksidativnog stresa kod bolesnika sa opstruktivnom žuticom, izazvanom holedoholitijazom.

U ispitivanje je bilo uključeno 60 ispitanika podeljenih u dve grupe. Prva je kontrolna grupa (30 zdravih ispitanika), a drugu čini 30 bolesnika sa intraluminalnom ekstrahepatičnom opstrukcijom izazvanom holedoholitijazom. Od biohemijskih parametara holestaze određivani su AST, ALT, GGT i AF, nivoi ukupnog, direktnog i indirektnog bilirubina i koncentracija albumina.

Došlo je do značajnog porasta aktivnosti enzima AST, ALT, GGT i AF u plazmi holestaznih bolesnika, u odnosu na kontrolu ($p<0,001$). Nivoi ukupnog, direktnog i indirektnog bilirubina u plazmi bolesnika sa holestazom su povišeni ($p<0,001$) u odnosu na kontrolu. Nivo albumina u plazmi kod bolesnika sa holestazom je značajno snižen u odnosu na kontrolu ($p<0,05$). Intenzitet oksidativnog stresa, meren kroz nivo lipidne peroksidacije (MDA) i količine oksidativno modifikovanih proteina (karbonilne grupe), značajno je veći kod bolesnika sa ekstrahepatičnom holestazom u poređenju sa kontrolnom grupom ispitanika ($p<0,001$).

Kod bolesnika sa ekstrahepatičnom holestazom izazvanom holedoholitijazom primećeno je višestruko povišenje aktivnosti markera holestaze (ALT, AST, AF i GGT) i pad koncentracije albumina, pri čemu se javlja hiperbilirubinemija mešovitog tipa. Ujedno, javlja se povećan intenzitet oksidativnog stresa. *Acta Medica Medianae 2008;47(3):21-27.*

Ključne reči: *holestaza, holedoholitijaza, oksidativni stres, bilirubin, albumini*