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INTENSITY OF OXIDATIVE STRESS IN THE COMORBID COURSE OF NON-ALCOHOLIC STEATOGEPATITIS AND DIABETIC KIDNEY DISEASE IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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75 patients with non-alcoholic steatohepatitis with comorbid type 2 diabetes mellitus and stage I-IV diabetic kidney disease were examined in the treatment dynamics. It was found that the comorbid course of non-alcoholic steatohepatitis and diabetic kidney disease in patients with type 2 diabetes is accompanied by a significant increase in the intensity of oxidative stress, accompanied by an increase in blood intermediate and final products of lipid peroxidation and oxidative modification of proteins in the range of 1.9-2 times ($p < 0.05$). The damaging effect of oxidative stress in patients with type 2 diabetes leads to activation of hepatocyte apoptosis with an increase in blood cytokeratin-18 (7.5 times, $p < 0.05$), the content of which correlates with the degree of oxidative stress, the intensity of liver damage and the stage of diabetic kidney disease ($p < 0.05$). Oxidative stress increases the risk of endothelial damage by atherosclerotic process due to hyperproduction of homocysteine (3.9 times, $p < 0.05$), which contributes to the progression of diabetic kidney disease. The use of Quercetin in the complex therapy of non-alcoholic steatohepatitis and type 2 diabetes with diabetic kidney disease contributes to a probable decrease in the intensity of oxidative stress, increased activity of antioxidant protection factors (content of reduced glutathione in erythrocytes, glutathione peroxidase activity, catalase). 1.7 times) and endothelial damage (reduction of homocysteine in the blood by 1.9 times) ($p < 0.05$).

Key words: non-alcoholic steatohepatitis, type 2 diabetes mellitus, diabetic kidney disease, apoptosis, atherosclerosis, quercetin.

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ІНТЕНСИВНІСТЬ ОКСИДАТИВНОГО СТРЕСУ ПРИ КОМОРБІДНОМУ ПЕРЕБІГУ НЕАЛКОГОЛЬНОГО СТЕАТОГЕПАТИТУ ТА ДІАБЕТИЧНОЇ ХВОРОБИ НИРОК У ХВОРИХ НА ЦУКРОВИЙ ДІАБЕТ 2-ГО ТИПУ

Обстежено в динаміці лікування 75 хворих на неалкогольний стеатогепатит із коморбідними цукровим діабетом 2-го типу середньої важкості та діабетичною хворобою нирок I-IV стадій. Встановлено, що коморбідний перебіг неалкогольного стеатогепатиту та діабетичної хвороби нироку хворих на цукровий діабет 2-го типу супроводжується істотним зростанням інтенсивності оксидативного стресу, що супроводжується зростанням вмісту в крові проміжних та кінцевих продуктів пероксидного окиснення ліпідів та окиснювальної модифікації білків у межах 1,9-2,3 рази ($p<0,05$). Пошкоджуюча дія оксидативного стресу у хворих на цукровий діабет 2-го типу призводить до активації процесів апоптозу гепатоцитів із підвищенням вмісту в крові цитокератину-18 (у 7,5 рази, $p<0,05$), вміст якого корелює із ступенем оксидативного стресу, інтенсивністю пошкодження печінки та стадією діабетичної хвороби нирок ($p<0,05$). Оксидативний стрес підвищує ризик пошкодження ендотелію атеросклеротичним процесом через гіперпродукцію гомоцистеїну (у 3,9 рази, $p<0,05$), що сприяє прогресуванню діабетичної хвороби нирок. Застосування кверцетину у комплексній терапії неалкогольного стеатогепатиту та цукрового діабету 2-го типу із діабетичною хворобою нирок сприяє вірогідному зниженню інтенсивності оксидативного стресу, підсиленню активності чинників антиоксидантного захисту (вмісту в еритроцитах відновленого глутатіону, активності глутатіонпероксидази, каталази), наслідком чого є істотне зниження процесів апоптозу гепатоцитів (зниження вмісту цитокератину-18 у 1,7 рази) та пошкодження ендотелію (зниження вмісту в крові гомоцистеїну у 1,9 рази) ($p<0,05$).

Ключові слова: неалкогольний стеатогепатит, цукровий діабет 2-го типу, діабетична хвороба нирок, апоптоз, атеросклероз, кверцетин

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Every year in Ukraine and the world the incidence of non-alcoholic steatohepatitis (NASH) in patients with obesity and type 2 diabetes mellitus (DM2) is significantly increasing [6]. These diseases have a large number of common etio-pathogenetic links and mechanisms of mutual burden. The intensity of damage factors increases with the development of diabetic kidney disease (DKD) [6, 12].

Oxidative stress (OS) occupies a leading place in the mechanisms of progression of NASH and DKD in patients with diabetes mellitus [6]. The increase in the intensity of OS under the influence of various inducers underlies the transformation of nonalcoholic steatosis of the liver into NASH, the development and progression of inflammatory-necrotic changes in the liver in NASH, as well as liver fibrosis [6]. Enhancement of lipid peroxidation (LPO) and oxidative modification of proteins (OMP) of organelle membranes is accompanied by swelling of mitochondria, increased permeability of lysosomal membranes, systemic disruption of cell membrane integrity [6]. LPO products stimulate collagen formation in the liver and kidneys [6], as well as cause the formation of Mallory cells, which involves the deposition of cross-linked cytokeratin monomers [2, 6]. Accumulation of endotoxins, intermediate and end products of LPO on the background of impaired

carbohydrate and lipid metabolism contribute to the induction of cytochrome P450 (Cyp2E1), which is accompanied by the release of large amounts of free radicals of oxygen (FRO) and nitrogen [10]. In NASH, there is increased activity of cytochrome P450 in the liver, which is able to generate FRO in the process of detoxification of free fatty acids (FFA), aldehydes, ketones, N-nitrosamines, and other endotoxins and exotoxins [6]. Initiation of necrotic processes in liver tissue is also a consequence of FRO hyperproduction [6]. An important role in the attachment of the inflammatory component is played by the processes of LPO of structural membrane lipids, which induce the processes of apoptosis and cytolysis of hepatocytes [6]. The entry of lysosomal hydrolases and other components of the hepatocyte into the intercellular space and into the systemic circulation is a signal to the induction of a cascade of reactions in response to damage [2]. A further scenario involves the activation of cell adhesion molecules (ICAM-1, ICAM-2), polymorphonuclear infiltration of liver tissue, microcirculation disorders, ie the progression of dystrophic and inflammatory-necrotic changes in liver tissue [4, 6]. By a similar mechanism, OS affects the endothelium, causing the development of endothelial apoptosis, its accelerated exfoliation and endothelial dysfunction (ED). Oxidative stress is counteracted by antioxidant defense systems (AODS). The leading system of the natural detoxification system and AODS is the glutathione system. Performing the functions of the universal redox system, glutathione and a number of enzymes that serve it protect cell membranes from the effects of FRO, nitrogen (peroxynitrite), hydroperoxides, and binds hydrophilic products of microsomal oxidation (first phase of detoxification) and provides the second phase conjugation) with the excretion of non-toxic compounds from the body [6]. In terms of counteracting free radical effects in clinical practice, the drug Quercetin – a flavonoid of plant origin, which inhibits the intensity of LPO and OMP membranes, stimulates the activity of catalase and superoxide dismutase (SOD) in cells [1,2,6]. Quercetin restores the ability of the endothelium to synthesize NO, which explains its cardioprotective effect in ischemic and reperfusion heart disease [1,3]. The drug has a powerful anti-inflammatory effect, inhibiting 5-lipoxygenase, cyclooxygenase, hyaluronidase, a number of proteases, calcium-dependent ATPase, synthesis of leukotrienes LTC4 and LTB4, has immunomodulatory properties, thus inhibits the production of to reduce the area of necrotized myocardium and enhance reparative processes [6,10]. There are a number of reports of hypolipidemic, choleretic, anticholestatic, hepatoprotective properties of quercetin, established in an experiment in obesity and in patients with NASH [1,7,11,12-15].

The purpose of the study was to determine the intensity of the effect of a complex of metformin, rosuvastatin, essential phospholipids and quercetin on the state of oxidative-antioxidant homeostasis, as well as the intensity of apoptosis of hepatocytes in the blood cytokeratin-18, which are factors in the progression of non-alcoholic steatohepatitis with comorbid type 2 diabetes mellitus and stage I-IV diabetic kidney disease.

Material and methods. Studies in the dynamics of treatment in 75 patients with NASH with type 2 diabetes and stage I-III DKD. According to the prescribed treatment, the examined patients were divided into 2 groups: (1 group – control: 37 patients) received a low-calorie diet with dietary restrictions No. 9, essential phospholipids (Essentiale forte H (Sanofi-Avensis/Natterman and Cie mg GmbH) 2 caps. 3 times a day) 30 days for the treatment of active NASH, for concomitant type 2 diabetes and hyperlipidemia prescribed metformin hydrochloride (Metformin-Teva, LLC Teva Operations Poland) 1000 mg per day, rosuvastatin (Rozuvastatin-Teva, LLC Teva Operations Poland) (5 mg once daily) for 1 month. Group 2 consisted of patients (38 people) who, in addition to similar dietary recommendations, essential phospholipids (Essentiale forte H), similar to hypoglycemic and hypolipidemic therapy for a month, additionally received the drug quercetin and povidone (Corvitin (PC NVC "Borshchahivsky CFP", Ukraine) in 100 ml of isotonic sodium chloride solution) for 10 days. The mean age of patients was (54.7±3.56) years. Groups of patients were randomized by age, sex, duration of the disease. The comparison group for the presentation of the average reference values of homeostasis indicators consisted of 30 healthy individuals (PHP) of the appropriate age. The diagnosis of NASH was established in accordance with the unified clinical protocol approved by the order of the Ministry of Health of Ukraine No. 826 from 06.11.2014.Diagnosis of type 2 diabetes was carried out in accordance with the unified clinical protocol approved by the Order of the Ministry of Health of Ukraine No. 1118 of 21.12.2012.

The intensity of oxidative modification of proteins (OMP) in serum was determined by the method of Dubinina OE et al. in the modification of Meshchishen IF it contains aldehyde and ketondinitrophenylhydrazones (AKDPH) in the blood. The content in the blood of LPO products – isolated double bonds (IDP) in compounds, diene conjugates (DC), ketodienes and conjugated trienes (KCT) – according to Volchegorsky IA et al., Malonic aldehyde (MA) in blood plasma and Er – by Vladimirov YuA, Archakov AI. The content of reduced glutathione (RG) in the blood was determined by the titration method according to Travina OV in the modification of Meshchishena IF, Petrova IV. The activity of enzymes of the AODS: glutathione peroxidase (GP) was studied by Meshishenym IF, glutathione-S-transferase (GT) –by Meschishenim IF, catalase – by Korolyuk MA et al. The content of cytokeratin-18 (CC-18) in the blood was carried out by enzyme-linked immunosorbent assay (ELISA) using Elisa reagents. The content of homocysteine in the blood was performed by ELISA using a set of reagents Axis® Homocysteine Enzyme Immunoassay.

Before testing the statistical hypotheses, the analysis of the normality of the distribution of values in randomized samples was performed by determining the coefficients of asymmetry and excess using the Khan-Shapiro-Wilkie test. For statistical analysis of the obtained results we used software packages Statistica for Windows version 8.0 (Stat Soft inc., USA), Microsoft Excel 2007 (Microsoft, USA).

Results of the study and their discussion. The analysis of the obtained data showed that before treatment in patients of both groups of comparison a significant degree of OS was established, which was accompanied by a significant accumulation in the blood of intermediate and final products of LPO and OMP. Thus, before treatment, the content of MA in blood plasma exceeded the reference values by 2.1 times ($p<0.05$), the content of FRO – 1.9 times ($p<0.05$), the content of AKDPH OC – by 2.3 times ($p<0.05$) (table 1).

Table 1

Indices of oxidative stress intensity, antioxidant protection factors and markers of hepatocyte apoptosis in patients with a combined course of non-alcoholic steatohepatitis, type 2 diabetes mellitus and diabetic kidney disease in the dynamics of treatment (M±m)

Indices	PHP, n=30	Groups of examined patients			
		1, control, n=37		2, basic, n=38	
		before	after	before	after
MA plasma, μmol/hour hl	2.22±0.09	4.71±0.09 *	3.18±0,07 */**	4.73±0,07 *	2.35±0.05 **/#
IDP, E220/ml	2.89±0.02	5.53±0.06 *	4.76±0.05 */**	5.52±0.08 *	3.28±0.04 */**/#
AKDPH OC, o.od.g/l of protein	1.37±0.03	3.17±0,08 *	2.75±0.04 */**	3.19±0.05 *	1.70±0.03 */**/#
GR, μkmol/l	0.93±0.04	0.56±0.05 *	0.65±0.04 *	0.55±0.06 *	0.83±0.02 **/#
GP, nmol VG/min × gNb	152.22± 3.46	120.31±5.45 *	131.64±5.14 *	122.18±5.36 *	149.85±3.25 **/#
Cytokeratin-18, Ed/l	57.62 ± 5.37	428.34±17.87 *	385.83±15.83 *	430.52 ± 18.45 *	249.28±12.19 */**/#
Homocysteine, μkmol/l	9.93 ± 0.42	38.27±1.51 *	32.62±1.37 *	39.23±1.43 *	20.42±1.31 */**/#

Note: * – the difference is probable in comparison with the indicator in PHP ($p<0.05$);** – the difference is probable in comparison with the indicator before treatment ($p<0.05$); # – the difference is probable in comparison with the indicator in patients of the control group after treatment ($p<0.05$).

At the same time, the state of the antioxidant defense system was significantly unbalanced. Thus, the content of GR in the blood was lower than in PHP 1.7 times ($p<0.05$), the activity of G3 – was inhibited by 1.3 times ($p<0.05$), which explains the high intensity of OS in the subjects patients.

These products of LPO and OMP on the background of significant insufficiency of the ADS system led to the activation of hepatocyte apoptosis. Evidence of this is a significant increase in the content of CC-18 in the blood – by 7.5 times ($p<0.05$) compared with PHP.

Intensive OS and metabolic intoxication also resulted in an increase in the blood content of homocysteine in patients with NASH with DKD by 3.9 times ($p<0.05$), which poses a risk of endothelial damage and progression of DKD.

The correlation analysis indicates a strong and medium correlation between the intensity of OS and the content of CC-18 and homocysteine in the blood of patients with NASH with DKD on the background of diabetes mellitus2, as well as weak and medium relationship with markers of liver damage in NASH and stage DKD ($p<0.05$) (table 2).

Table 2

Matrix of correlations of morpho-functional parameters of the liver, kidneys, blood cytokeratin-18 and homocysteine with indicators of oxidative-antioxidant homeostasis in patients with NASH and DKD, DM2 (r, p)

Index	MA	IDP	DC	AKDPH	GR	GP	Catalase
Bilirubin	0.32*	0.43*	0.41*	0.38*	-0.45*	-0.21	-0.23
ALT	0.53*	0.57*	0.58*	0.44*	-0.69*	-0.34*	-0.37*
AST	0.51*	0.53*	0.51*	0.39*	-0.64*	-0.33*	-0.38*
GGT	0.49*	0.44*	0.47*	0.32*	-0.57*	-0.20	-0.25
AP	0.41*	0.43*	0.42*	0.33*	-0.43*	-0.28*	-0.12
Thymol test	0.48*	0.49*	0.47*	0.45*	-0.68*	-0.35*	-0.37*
Albumins	-0.34*	-0.41*	-0.42*	-0.34*	0.59*	0.43*	0.45*
Creatinine	0.58*	0.59*	0.60*	0.63*	-0.67*	-0.50*	-0.53*
GFR	-0.61*	-0.63*	-0.65*	-0.62*	0.62*	0.32*	0.33
Steat test	0.60*	0.62*	0.63*	0.51*	-0.65*	-0.49*	-0.50*
NASH- test	0.63*	0.65*	0.66*	0.52*	-0.68*	-0.53*	-0.56*
Fibrotest	0.54*	0.57*	0.59*	0.57*	-0.67*	-0.55*	-0.57*
CC-18	0.,63*	0.68*	0.72*	0.70*	-0.75*	-0.64*	-0.65*
Homocysteine	0.51*	0.53*	0.57*	0.44*	-0.61*	-0.43*	-0.48*

Note. * - statistically significant correlation coefficient ($p<0.05$).

Analyzing the indices after treatment should indicate the higher effectiveness of therapy, which additionally contained Quercetin. Thus, significantly increased content of MA in the blood before treatment

under the influence of therapy decreased in group 1 by 1.5 times ($p<0.05$), in group 2 – by 2.0 times ($p<0.05$). The increased content of the intermediate product LPO IDP decreased by 1.2 and 1.7 times, respectively ($p<0.05$). The prescribed therapy also had a significant effect on the increased content of AKDPH OC in the blood (2.3 times): yes, the decrease was by 1.2 and 1.9 times, respectively ($p<0.05$). That is, after treatment we found a decrease in the intensity of OS as relative to the oxidation of structural lipids of cell membranes, including endothelium, hepatocytes and podocytes, and relative to structural proteins, due to the established increase in the activity of AODS[12, 14]. This is evidenced by the recovery of more glutathione in erythrocytes: in group 1 – by 1.2 times ($p>0.05$), in group by 2 –1.5 times ($p<0.05$) and a probable increase in the activity of GP after treatment – only in patients of group by 2 1.2 times ($p<0.05$).

The obtained research results indicate that a significant decrease in the intensity of apoptosis processes after treatment was registered only in patients of group 2[11, 15]. Thus, the average blood content of CC-18 in patients with NASH with DKD group 2 after treatment probably decreased by 1.7 times ($p<0.05$), while in patients with group 1 changes were unlikely.

The effect of the proposed therapy with the addition of Quercetin was also more significant on the content of homocysteine in the blood – the decrease was 1.9 times ($p<0.05$), and in patients of the control group the indicator only tended to decrease ($p>0.05$).

The complex effect of Quercetin on the functional state of the LPO-AODS, the intensity of apoptosis and the factors that regulate them in patients with NASH and DKD on the background of diabetes mellitus 2 has been studied in limited patients or experimentally.

Conclusions

Comorbid course of nonalcoholic steatohepatitis and diabetic kidney disease in patients with type 2 diabetes mellitus is accompanied by a significant increase in the intensity of oxidative stress, accompanied by an increase in the content of intermediate and final products of lipid peroxidation and oxidative modification times ($p <0.05$). The damaging effect of oxidative stress in patients with type 2 diabetes leads to activation of hepatocyte apoptosis (increase in blood cytokeratin–18 by 7.5 times, $p<0.05$) with the progression of NASH, and an increased risk of endothelial damage due to atherogenesis (hyperproduction of homocysteine by 3.9 times, $p<0.05$) with the progression of DKD. The use of quercetin in the treatment of non-alcoholic steatohepatitis and type 2 diabetes with DKD contributes to a probable reduction in the intensity of oxidative stress, increased activity of antioxidant defense factors (reduced glutathione, glutathione peroxidase), resulting in a decrease by 1.7 times) and endothelial damage (reduction of homocysteine in the blood by 1.9 times).

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