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MICROBIOCENOSIS DISORDERS PROVOKE OXIDATIVE STRESS AS A SIGNIFICANT PATHOGENETIC FACTOR IN NON-ALCOHOLIC STEATOHEPATITIS COMBINED WITH ISCHEMIC HEART DISEASE

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The study presents the results of our own research evaluating the impact of intestinal microbiocenosis disorders on oxidative stress activity and the degree of dyslipidemia in patients with combined non-alcoholic fatty liver disease and ischemic heart disease. The study included 44 patients with non-alcoholic steatohepatitis and concurrent ischemic heart disease. Patients were divided into two groups based on the presence of intestinal microbiocenosis disruptions. The findings demonstrate that under these comorbid conditions, microbiocenosis disturbances are characterized by: reduced lactobacilli counts in 95 % of patients, decreased bifidobacteria levels in 90.9 %, lower total Escherichia coli counts in 50 % of patients. A decrease in the total amount of Escherichia coli is associated with the emergence of opportunistic microflora. Under conditions combining non-alcoholic steatohepatitis and ischemic heart disease, impaired gut microbiota composition exacerbates the severity of dyslipidemia and triggers the production of reactive oxygen species with depletion of antioxidant defense enzymes. This is characterized by an increase in thiobarbituric acid-reactive substances (TBARS/TBA-reactants) levels alongside a simultaneous decrease in superoxide dismutase activity compared to normal gut microbiota composition.

Key words: non-alcoholic steatohepatitis, ischemic heart disease, gut microbiota (microbiocenosis), oxidative stress, antioxidant defense, dyslipidemia.

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ПОРУШЕННЯ МІКРОБІОЦЕНОЗУ ПРОВОКУЮТЬ ОКСИДАТИВНИЙ СТРЕС ЯК ВАГОМИЙ ПАТОГЕНЕТИЧНИЙ ФАКТОР НЕАЛКОГОЛЬНОГО СТЕАТОГЕПАТИТУ У ПОЄДНАННІ З ІШЕМІЧНОЮ ХВОРОБОЮ СЕРЦЯ

У статті наведені результати власних досліджень з оцінки впливу порушень мікробіоценозу кишечника на активність оксидативного стресу та ступінь дисліпідемії у хворих із поєднанням неалкогольної жирової хвороби печінки та ішемічної хвороби серця. У дослідження включено 44 хворих на неалкогольний стеатогепатит у поєднанні із ішемічною хворобою серця. Пацієнти були розподілені на дві групи залежно від наявності порушень мікробіоценозу кишечника. Показано, що порушення мікробіоценозу за умов даної коморбідності характеризується зниженням кількості лактобацил у 95 % хворих, біфідобактерій у 90,9 %, загальної кількості кишкової палички – у 50 % хворих. Зниження загальної кількості кишкової палички асоціюється із виникненням умовно-патогенної мікрофлори. За умов поєднання неалкогольного стеатогепатиту та ішемічної хвороби серця порушення мікробіоценозу кишечника потенціює тяжкість дисліпідемії та провокує продукцію агресивних форм кисню із виснаженням ферментів антиоксидантного захисту, що характеризується зростанням рівня ТБК-реактантів за одночасного зниження активності супероксиддисмутази порівняно із нормальним складом мікрофлори кишечника.

Ключові слова: неалкогольний стеатогепатит, ішемічна хвороба серця, мікробіоценоз кишківника, оксидативний стрес, антиоксидантний захист, дисліпідемія.

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In recent decades, non-alcoholic fatty liver disease (NAFLD) has emerged as a significant public health concern, driven by a marked increase in its global prevalence, including in Ukraine [1, 6]. The rising incidence of NAFLD is closely associated with the growing number of individuals affected by overweight and obesity [4].

NAFLD encompasses a broad spectrum of conditions, including non-alcoholic hepatic steatosis (characterized by liver fat accumulation without inflammation or hepatocellular injury) and non-alcoholic steatohepatitis (NASH), which involves steatosis, inflammation, and hepatocellular damage with or without liver fibrosis [4, 9, 11]. Recently, significant attention has been focused on the role of NAFLD in the development and progression of cardiovascular diseases, particularly ischemic heart disease (IHD). Numerous epidemiological studies have demonstrated that NAFLD – especially NASH – is strongly associated with an increased risk of atherosclerosis and related disorders [5]. Evidence confirms that NASH progression directly contributes to the development of IHD, congestive heart failure, and cardiovascular mortality [2, 5–8, 10]. At the same time, the development of cardiovascular diseases plays a leading role in determining the prognosis and clinical course of NAFLD [12].

NAFLD and IHD share common risk factors and pathophysiological mechanisms, including disorders of carbohydrate and lipid metabolism, endothelial dysfunction, and development of vascular wall inflammation [12].

Lipid profile abnormalities underlie both NAFLD and IHD. The development of dyslipidemia is strongly associated with oxidative stress. Cholesterol serves as an essential component of membranes and lipoproteins, playing a crucial role in their stabilization. Oxidative modification of low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) represents a key pathogenetic mechanism that promotes atherosclerosis [7].

Recent evidence has confirmed the role of gut microbiome and its dysbiosis in the pathogenesis and progression of cardiovascular diseases. Several studies demonstrate the impact of intestinal dysbiosis on the development and clinical course of specific cardiovascular disorders, particularly myocardial infarction, atrial fibrillation, and heart failure [11, 13].

However, the specific alterations in gut microbiota composition in patients with combined NAFLD/NASH and IHD, as well as their impact on lipid peroxidation processes – a key mechanism driving this comorbidity – remain insufficiently studied**.**

The purpose of the study was to evaluate intestinal microbiota alterations and their impact on oxidative stress development and lipid metabolism disorders in patients with non-alcoholic steatohepatitis and comorbid ischemic heart disease.

Materials and methods. We examined 42 patients with NASH and comorbid IHD treated at the Poltava Regional Clinical Cardiology Center, including 24 men (57.1 %) and 18 women (42.9 %) aged 36-67 years. Participants had the following IHD forms: stable exertional angina (functional class I-II) and diffuse cardiosclerosis. IHD diagnosis and baseline therapy followed Ukraine's Unified Clinical Protocol "Stable Ischemic Heart Disease" (MoH Order No.152, 02.03.2016). All patients received β-blockers, ACE inhibitors, Calcium antagonists, Aspirin, Statins. The clinical trial included patients with NAFLD with a minimal degree of activity. Diagnosis of NAFLD and treatment of patients was performed according to a unified clinical protocol of primary and secondary (specialized) medical care "Nonalcoholic steatohepatitis" (Order of the Ministry of Health of Ukraine No.826 of November 06, 2014). Patients were excluded from having viral hepatitis B and C.

All patients underwent a fecal bacteriological examination at the Clinical Diagnostic Laboratory of the Poltava Central Clinical Hospital, which estimated the total number of full-grown E. coli, the number of E. coli with altered properties, lactose-negative E. coli, microorganisms that form hemolysis, lactobacilli, bifidobacteria, opportunistic flora (bacillus and coccus forms), staphylococci, Candida fungi, streptococci. Depending on the presence of dysbiosis, patients were divided into two groups:

Group I (n=20) – Patients with NASH (non-alcoholic steatohepatitis) combined with CAD (coronary artery disease) who do not have intestinal microbiocenosis disorders;

Group II (n=22) – Patients with NASH combined with CAD who have intestinal microbiocenosis disorders.

The average age of patients in Group I was 55.65±7.74 years, with a male-to-female ratio of 11 (55 %) men and 9 (45 %) women. The average age of patients in Group II was 56.91±6.62 years, with a gender distribution of 13 (59.1 %) men and 9 (40.9 %) women. The control group of practically healthy individuals consisted of 20 people aged 27.01±5.78 years, including 12 (60 %) men and 8 (40 %) women.

All patients underwent a lipid profile study, including measurements of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG). (The tests were performed in the clinical laboratory of the Poltava Regional Clinical Cardiological Dispensary.)

All patients were evaluated for their prooxidant-antioxidant status by measuring: the concentration of thiobarbituric acid reactive substances (TBARS), the activity of superoxide dismutase (SOD), the concentration of medium sized molecules (MSM), oxidatively modified proteins (OMP) in blood serum (the study was conducted at the Department of Biological and Bioorganic Chemistry, Poltava State Medical University).

Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad Software, Inc., San Diego, CA, USA, license number U1048-12MC), which enables both parametric and non-parametric statistical analysis. The distribution was assessed as non-normal. Results were presented as arithmetic means (M) with their standard deviations (SD). We applied the paired non-parametric Wilcoxon signed-rank test (W). Correlation analysis between study parameters was performed using Pearson's correlation coefficient (r). Relative risk assessment was conducted by calculating χ^2 (chi-square). Statistical significance was set at $p<0.05$, where: p_1 represents significant differences between Group I and healthy

controls; p_2 represents significant differences between Group II and healthy controls p_3 represents significant differences between Group I and Group II.

Results of the study and their discussion. To evaluate the impact of gut microbiota disturbances on prooxidant-antioxidant status and lipid profile parameters, we identified Group II consisting of patients with NASH combined with CAD. Analysis of fecal dysbiosis test results in Group II patients revealed that the most frequent alterations in intestinal luminal microbiota were: reduced counts of lactobacilli, decreased levels of bifidobacteria (Table 1).

Table 1

Characteristics of gut microbiota dysbiosis in patients with non-alcoholic steatohepatitis and comorbid ischemic heart disease (Group II)

Microflora	Number of patients, n	Percentage of patients, %.
The total number of of E. coli	11/22	50 %
E. coli with altered enzymatic properties	0/22	0
Lactose-negative of E. coli	0/22	0
Microorganisms that form hemolysis	0/22	0
Lactobacilli	21/22	95.5 %
Bifidobacteria	20/22	90.9 %
CPF	9/22	40.9 %
Staphylococci	2/22	9.09 %
Fungi of the genus Candida	1/22	4.5 %
Streptococci	0/22	0

Note: CPF – conditionally pathogenic flora (rod-shaped and coccal forms).

Thus, a decrease in the number of lactobacilli was recorded in 95.5 % (21/22) of patients, and bifidobacteria – in 90.9 % (20/22) of patients. A decrease in the total number of Escherichia coli was detected in 50 % (11/22) of patients in group II. Violation of the microbiota composition in this category of patients was also characterized by the development of conditionally pathogenic microflora. Thus, the presence of Klebsiella was detected in 13.6 % (3/22) of patients, Citrobacter – in 18.2 % (4/22), Enterobacter – in 9.1 % (2/22) of the examined patients of group II. At the same time, Staphylococcus aureus was detected in 18.2 % (4/22) of patients. Thus, it can be assumed that a decrease in the number of lactobacilli and bifidobacteria contributes to the emergence of opportunistic microflora. It should be noted that, according to the results of our study, the most significant role in the emergence of opportunistic infections and Staphylococcus aureus was played by a decrease in the total number of Escherichia coli. Thus, Klebsiella, Citrobacter, Enterobacter, and Staphylococcus aureus were detected in 81.8 % (9/11) of patients with a decrease in the total number of E. coli. In conditions of normal E. coli count, the presence of Staphylococcus aureus and Citrobacter was recorded only in 18.1 % (2/11) of patients. Thus, a decrease in the total number of Escherichia coli increases the risk of developing opportunistic pathogens (X^2=8.91; p=0.002).

The presence of gut microbiota disturbances significantly influenced lipid metabolism alterations in patients with non-alcoholic steatohepatitis (NASH) and ischemic heart disease (IHD). Compared to healthy controls, serum analysis revealed (Group I and II Patients vs. Controls): total cholesterol (TC) 1.9-times increase (p_1<0.0001, p_2<0.0001); HDL-C 1.2- times (p_1=0.0017) and 1.1-times (p_2=0.04) increase, respectively; LDL-C: 1.8- times (p_1<0.0001) and 1.9-times (p_2<0.0001) increase, respectively; triglycerides (TG): 3.4-times (p_1<0.0001) and 3.5-times (p_2<0.0001) elevation, respectively.

Patients with gut dysbiosis (Group II) demonstrated: 1.1-times higher serum LDL-C levels compared to Group I (Table 2).

Table 2

Characteristics of lipid profile disorders in patients with non-alcoholic steatohepatitis combined with ischemic heart disease depending on microbiocenosis disturbances (Mean±SD)

Patient groups	TC, mmol/l	HDL-C, mmol/l	LDL-C, mmol/l	TG, mmol/l
HC (n=20)	3.91±0.41	1.16±0.16	2.28±0.36	1.08±0.25
I (n=20)	7.37±0.70	1.37±0.23	4.02±0.77	3.7±1.1
II (n=22)	7.62±0.82	1.3±2.6	4.45±0.51	3.81±1.2
p	p_1<0.0001 p_2<0.0001 p_3>0.05	p_1=0.0017 p_2=0.04 p_3>0.05	p_1<0.0001 p_2<0.0001 p_3=0.039	p_1<0.0001 p_2<0.0001 p_3>0.05

Note: p (p<0.05) – statistically significant difference between the indicators; HC – healthy controls.

We have evaluated the impact of microbiocenosis disorders on the nature of prooxidant-antioxidant status. It was found that the combination of NASH and IHD is accompanied by the development of oxidative stress. Thus, in this category of patients, the increased production of aggressive free radicals was observed, which was characterized by a 2-times increase in the serum of patients of Groups I and II in the content of TBA-reactants ((0.04±0.008) vs. (0.02±0.008) mmol/L; p1<0.0001)) and 5.5 times ((0.11±0.11) vs. mmol/L; p2=0.0008) respectively, compared with the norm. It is important to note that the presence of microbiocenosis disorders potentiated the severity of oxidative stress. In patients of Group II, the concentration of TBA reactants in the blood serum increased 2.8 (p3=0.005) times compared to patients of group I without changes in the composition of the intestinal microflora (Fig. 1).

High oxidative stress activity under the influence of NASH combined with IHD is confirmed by a 3-times increase in serum OMP concentration in patients of Group I ((0.12±0.14) vs. (0.04±0.02) U/L; p_1=0.02) and Group II ((0.12±0.09) vs. (0.04±0.02) U/L; p_2=0.0009) compared to the normal range, with no statistically significant difference between the comparison groups (Fig. 1). The OMP level is influenced by oxidative stress activity as a key mechanism in the pathogenesis of NASH and IHD.

Simultaneously, we found that the serum MSM level in Group I patients increased 2-times ((0.38±0.17) vs. (0.18±0.04) U/mL; p_1<0.0001), while in Group II patients, it rose 2.5-times ((0.48±0.24) vs. (0.18±0.04) U/mL; p_2<0.0001) compared to healthy controls (Fig. 2). Moreover, Group II patients with intestinal microbiocenosis disturbances exhibited a trend toward a 1.3-times increase in serum MSM levels (p_3>0.05) compared to Group I patients (Fig. 2).

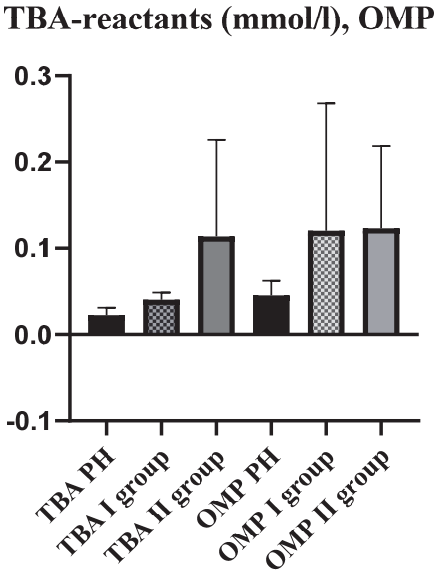

Fig. 1 Concentration of TBA-reactants and oxidative modification of proteins in the blood serum of patients with nonalcoholic steatohepatitis in combination with coronary heart disease depending on the presence of microbiocenosis disorders.

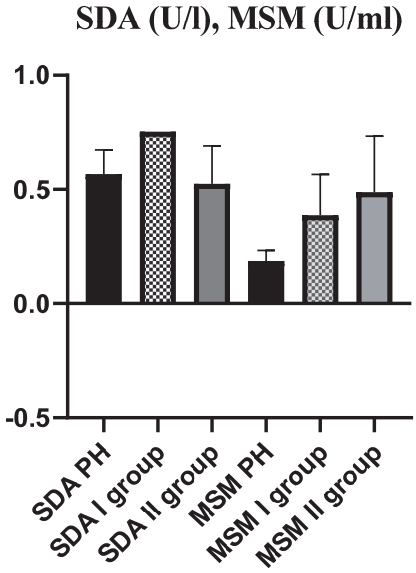

Fig. 2. Superoxide dismutase activity and medium seized molecules levels in serum of patients with non-alcoholic steatohepatitis combined with ischemic heart disease depending on microbiocenosis disturbances.

In response to free radical production activation in Group I patients, serum SOD activity increased 1.3-times ((0.75±0.18) vs (0.56±0.18) U/L; p_1=0.004) compared to healthy controls. Simultaneously, in Group II patients with intestinal microbiocenosis disturbances, no significant increase in antioxidant defense enzyme production was observed despite oxidative stress activation, which may be considered a risk factor for both NASH and IHD progression. In Group II patients, SOD activity decreased 1.4-times ((0.52±0.16) vs (0.75±0.18) U/L; p_3=0.0001) compared to Group I and showed no significant difference from normal values (p_2>0.05).

In Group II patients with NASH and comorbid IHD, we identified a direct correlation between TBA-reactants and OMP levels (r=+0.62; p=0.002) and between OMP and SOD (r=+0.62; p=0.002)

Accordingly, our findings demonstrate that patients with combined NASH and IHD exhibit significant reductions in lactobacilli, bifidobacteria, and total Escherichia coli counts. The disruption of normal intestinal microbiota composition, particularly in total E. coli levels, is associated with the emergence of opportunistic pathogens. Our results align with other studies that clearly confirm microbiota's role in maintaining human health status.

According to current understanding, dysbiosis development potentiates the progression of obesity, type 2 diabetes, atherosclerosis, arterial hypertension, heart failure, and other diseases. Modern research

has proven that decreased Firmicutes (which include lactobacilli) and altered Firmicutes/Bacteroidetes ratio increases cardiovascular disease risk [5, 11].

Our study results revealed lipid metabolism disorders in all patients, regardless of whether they had microbiocenosis disturbances or normal gut microbiota composition. The observed dyslipidemia was characterized by elevated levels across all lipid profile parameters compared to healthy controls.

Several studies, including experimental models, have demonstrated that gut dysbiosis leads to impaired lipid metabolism, promoting atherogenic dyslipidemia and increasing cardiovascular disease risk [4, 11]. Currently, investigating the pathogenetic mechanisms through which dysbiosis contributes to cardiovascular system diseases remains an important focus for future research.

Our study established that the combination of NASH with IHD is associated with oxidative stress development. Serum levels of TBA-reactants, OMP, and MSM were elevated in both Group I and II patients compared to healthy controls. However, intestinal dysbiosis in patients with combined NASH and IHD specifically led to significantly increased production of reactive oxygen species compared to patients with normal microbiota composition.

Importantly, intestinal microbiocenosis disturbances were accompanied by depletion of antioxidant defense enzyme synthesis. Our findings are consistent with other studies demonstrating NASH's role in atherosclerosis development and related disorders [3, 4, 9, 13]. These results confirm that oxidative stress serves as a common pathogenetic mechanism for both NASH and IHD [3, 6].

Conclusions

1. In patients with NASH combined with IHD, intestinal microbiocenosis disturbances were characterized by: lactobacilli reduction in 95.5 % of patients; bifidobacteria reduction in 90.9 % of patients; total Escherichia coli count reduction in 50 % of patients.

2. The reduction in total Escherichia coli count was associated with an increased risk of opportunistic pathogen overgrowth (χ^2=8.91, p=0.002).

3. An important pathogenetic mechanism of intestinal microbiocenosis disruption in patients with NASH and comorbid IHD is oxidative stress, characterized by a prooxidant-antioxidant imbalance. This manifests as: 5.5-times increase in serum TBA-reactant levels; 3-times increase in OMP levels; 2.5-times increase in MSM levels compared to normal values.

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