NON-ALCOHOLIC FATTY LIVER DISEASE AS A RISK FACTOR FOR ANEMIA OF CHRONIC INFLAMMATION (EXPERIMENTAL RESEARCH)

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ABSTRACT

The aim of the study. *In recent years, non-alcoholic fatty liver disease (NAFLD) has been considered a hepatic manifestation of the metabolic syndrome. The main consequence of NAFLD is chronic hepatic inflammation, which leads to dyslipidemia, inflammation, increased oxidative stress, and endothelial dysfunction. Immune activation in response to interaction with agents of a metabolic nature induces the release of pro-inflammatory cytokines in the liver, which subsequently cause iron homeostasis disorder. This leads to a frequent association of NAFLD with anemia of various etiology. In this regard, we considered it important to assess the severity of the systemic inflammatory response in NAFLD in the experiment in order to diagnose anemia of chronic inflammation.*

Materials and methods. *The study was carried out on 26 male Wistar rats, which were divided into control and experimental groups. In animals of the experimental group, NAFLD was modeled according to the generally accepted method. In order to assess metabolic disorders, we determined the main biochemical parameters, a complete blood count with the calculation of erythrocyte indices, the concentration of the main pro-inflammatory cytokines – interleukin (IL) 1, IL-6.*

Results. *In laboratory rats with NAFLD, a statistically significant increase of intrahepatic enzymes in blood serum was found. The state of the erythrocyte lineage of hematopoiesis in the experimental group progressively worsened and caused the development of anemic syndrome. Synchronously, a statistically significant increase in serum levels of IL-1, IL-6 was recorded, which confirms the correlation of NAFLD with anemia of chronic inflammation.*

Conclusions. *A high concentration of IL-1, IL-6 cytokines in NAFLD inhibits iron absorption in the duodenum, leads to the activation of macrophages, blocking the release of iron processed from aging erythrocytes into plasma.*
Further study of the mechanisms of anemia in NAFLD provides important therapeutic targets in the treatment of both NAFLD and its comorbidities.

Key words: *non-alcoholic fatty liver disease, anemia of chronic inflammation, rats*

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НЕАЛКОГОЛЬНАЯ ЖИРОВАЯ БОЛЕЗНЬ ПЕЧЕНИ КАК ФАКТОР РИСКА АНЕМИИ ХРОНИЧЕСКОГО ВОСПАЛЕНИЯ (ЭКСПЕРИМЕНТАЛЬНОЕ ИССЛЕДОВАНИЕ)

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РЕЗЮМЕ

Цель исследования. *В последние годы неалкогольная жировая болезнь печени (НАЖБП) считается печёночным проявлением метаболического синдрома. Основным последствием НАЖБП является хроническое воспаление печени, которое приводит к дислипидемии, воспалению, усилению окислительного стресса и дисфункции эндотелия. Иммунная активация в ответ на взаимодействие с агентами метаболической природы индуцирует в печени высвобождение провоспалительных цитокинов, которые впоследствии приводят к нарушению гомеостаза железа. Это приводит к частой ассоциации НАЖБП с анемиями различной этиологии. В связи с этим мы посчитали важным оценить выраженность системного воспалительного ответа при НАЖБП в эксперименте с целью диагностики анемии хронического воспаления.*

Материалы и методы. *Исследование проведено на 26 крысах-самцах линии Wistar, которые были разделены на контрольную и экспериментальную группы. У животных экспериментальной группы моделировалась НАЖБП по общепринятой методике. С целью оценки метаболических нарушений определяли основные биохимические показатели, общий анализ крови с подсчётом эритроцитарных индексов, концентрацию основных провоспалительных цитокинов – интерлейкина (ИЛ) 1, ИЛ-6.*

Результаты. *У лабораторных крыс с НАЖБП регистрировалось статистически значимое повышение в сыворотке крови внутрипечёночных ферментов. Состояние эритроцитарного ростка гемопоэза у животных экспериментальной группы прогрессивно ухудшалось, приводя к развитию анемического синдрома. Синхронно регистрировалось статистически значимое повышение в сыворотке уровней ИЛ-1, ИЛ-6, что подтверждает корреляцию НАЖБП с анемией хронического воспаления.*

Выводы. *Высокая концентрация цитокинов ИЛ-1, ИЛ-6 при НАЖБП ингибирует всасывание железа в двенадцатиперстной кишке, приводит к активации макрофагов, блокируя высвобождение железа, переработанного из стареющих эритроцитов в плазму.*

Дальнейшее изучение механизмов развития анемии при НАЖБП предоставляет важные терапевтические мишени в лечении как НАЖБП, так и сопутствующих заболеваний.

Ключевые слова: *неалкогольная жировая болезнь печени, анемия хронического воспаления, крысы*

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Excessive consumption of foods containing fast-digesting carbohydrates, such as fructose and sucrose, leads to the development of metabolic disorders in the liver. Non-alcoholic fatty liver disease (NAFLD) is a striking example of such a disease [1, 2].

A 2016 meta-analysis with a sample size of 8,515,431 people from 22 countries found that 25 % of the world's adult population suffers from NAFLD. Thus, currently, NAFLD is the most common liver disease and one of the main causes of metabolic syndrome [1].

The increasing incidence of NAFLD leads to an increased risk of mortality from associated cardiovascular disease, obesity, type 2 diabetes mellitus and hepatocellular carcinoma [1–3].

The increasing prevalence, especially in recent decades, has made NAFLD the second most common cause of liver transplantation in the United States. The hallmark of NAFLD is primary hepatic steatosis, subsequently exacerbated by non-alcoholic steatohepatitis (NASH), which is characterized by liver inflammation, hepatocyte damage and fibrosis, highlighting the potentially progressive nature of the disease [4–6]. The severity of cirrhosis is the most reliable predictor of long-term clinical outcomes, with marked fibrosis indicating a high risk of hepatocellular carcinoma and death [2, 6]. Metabolic dysfunctions, such as insulin resistance, dyslipidemia, and cardiovascular disease, are directly correlated with hepatic steatosis and appear to be more related to hepatic fat accumulation and NAFLD than to obesity per se [4–6].

The first stage of NAFLD, hepatic steatosis, is the earliest and most common response to excessive ethanol consumption and/or high-calorie and high-carbohydrate diet [4–6]. It is characterized by the accumulation of fat (more than 5 %), mainly triglycerides (TG), in the liver. Excessive lipid accumulation results in multiple parallel blows to the liver: pro-inflammatory action of leptin, release of inflammatory mediators, endoplasmic reticulum stress, Kupffer cell activation, etc. Progressive fatty dystrophy leads, among other things, to mitochondrial dysfunction due to disruption of mitochondrial membrane integrity. The release of free oxygen radicals exacerbates lipid peroxidation, activates liver cell inflammation and apoptosis, which eventually progresses from steatosis to NASH [4–7].

Thus, in addition to lipogenic effects, excessive consumption of fast-digesting fructose triggers inflammatory processes in hepatocytes due to mitochondrial dysfunction and oxidative stress [7].

According to many authors, chronic liver disease is often associated with hematologic abnormalities. Anemias of diverse etiologies occur in approximately 75 % of patients with chronic liver disease [8, 9]. The frequent association of anemia with chronic liver disease provides a rationale for investigating the role of the liver in red blood cell formation and destruction [2, 10], to find out whether the liver itself may indeed be involved in many different mechanisms that contribute to anemia in patients with NAFLD [10].

In this regard, we considered it important to assess the severity of the systemic inflammatory response in NAFLD in the experiment in order to diagnose anemia of chronic inflammation. The study results will provide more information on the pathogenesis and therapeutic strategies for NAFLD.

MATERIALS AND METHODS

The study was conducted on 26 male Wistar rats with body weight of 250–300 g at the time of inclusion in the study in the research laboratory of the Department of Pathological Physiology with a Course of Immunopathology, St. Petersburg State Pediatric Medical University. The animals were obtained from the Nursery for Laboratory Animals of the Branch of the Institute of Biological Chemistry of the Russian Academy of Sciences (IBCh RAS), Pushchino (Moscow Region). Before starting the study, animals were isolated in a special box to undergo a 14-day quarantine.

The study design, standardized operating procedures, and accompanying documentation underwent ethical review by the Local Ethics Committee of the St. Petersburg State Pediatric Medical University (Minutes No. 09/04 dated 11.02.2022).

A total of 2 groups of laboratory animals were formed:

1. "Control" ($n = 13$) – healthy intact rats in which metabolic parameters were investigated to calculate background reference values ("normal values").

2. "NAFLD" ($n = 13$) – rats, which throughout the experiment for 30 days as feed received briquettes containing food components in the following ratios (by weight): 26 % – protein, 10 % – animal fat, 50 % – fructose, 8 % – cellulose, 5 % – minerals, 1 % – vitamins. This diet is standard for experimental modeling of NAFLD [11] and allows to obtain morphological and metabolic changes in hepatocytes of laboratory animals characteristic of this pathology in a fairly short period of time.

Drinking restrictions, hypodynamic conditions were not imposed. The duration of the experiment was 30 days.

Blood was collected from the animals on the 30th day of the experiment by percutaneous puncture of the rat heart into 6 ml Monovette vacuum system (Germany). In the control group, blood collection from all rats was performed on the first day of the experiment.

In order to assess metabolic disorders in experimental animals, the following biochemical parameters were evaluated: biochemical parameters – activity of enzymes alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT).

To detect signs of an impaired red hematopoietic lineage, the hematocrit (HCT), red blood count (RBC), reticulocytes (RTC), hemoglobin level (HGB) were determined

in rats of the tested groups using an Automatic Abbott I Stat Blood Analyzer (Abbott Laboratories, USA) and i-STAT CG8+ cartridges.

The content of the main pro-inflammatory cytokines: interleukin (IL) 1, IL-6 was determined in the blood serum of experimental animals by enzyme immunoassay (EIA) using ELISA diagnostic kits (Cloud-Clone Corp., USA).

To assess morphologic changes and make a final diagnosis, animals were decapitated to collect autopsy material for histologic verification of liver damage in the studied groups of animals. Histologic examination was performed using hematoxylin and eosin staining by light microscopy at ×20 and ×40 magnification.

Statistical processing of the study results was performed using Prism 8 software (GraphPad, USA) and MS Excel 2016 (Microsoft Office Corp., USA). Results are reported as arithmetic mean ± arithmetic mean error (M ± SE). The Kolmogorov – Smirnov test was used to determine the nature of data distribution. The Mann – Whitney U test was used to compare the mean data of independent sample populations (in case of variant distribution other than normal). A statistically significant level of difference was taken as $p < 0.05$ (probability not less than 95 %), which is standard for biomedical experiments.

RESULTS

In the group of animals with NAFLD, the results of biochemical markers of liver damage revealed a statistically significant increase in ALT (46.23 ± 1.19 U/L) and AST (123.3 ± 7.691 U/L) compared to the control (30.96 ± 1.16 U/L, $p = 0.005$ and 101.5 ± 2.404 U/L, $p = 0.005$, respectively). Increased AST and ALT are considered the two most important indicators of liver hepatocyte damage characterizing the development of cytolytic syndrome (Fig. 1).

There was also a statistically significant difference in the level of alkaline phosphorus ($p = 0.005$) in the experimental (22.99 ± 1.092 U/L) and control (14.51 ± 0.81 U/L) groups (Fig. 1).

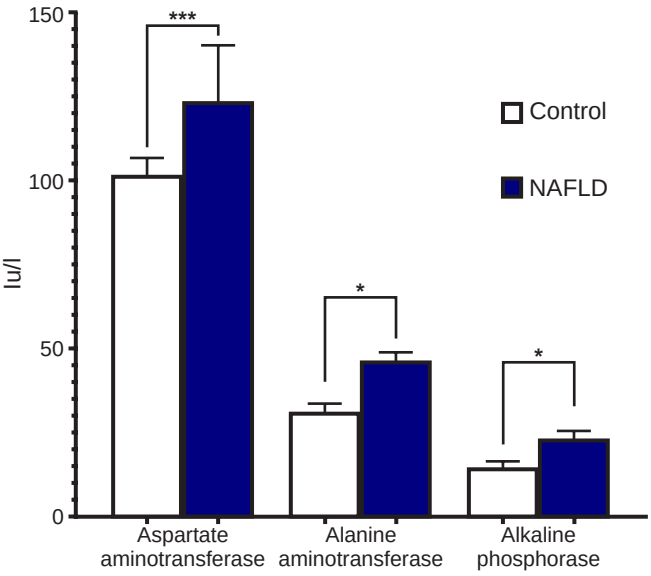
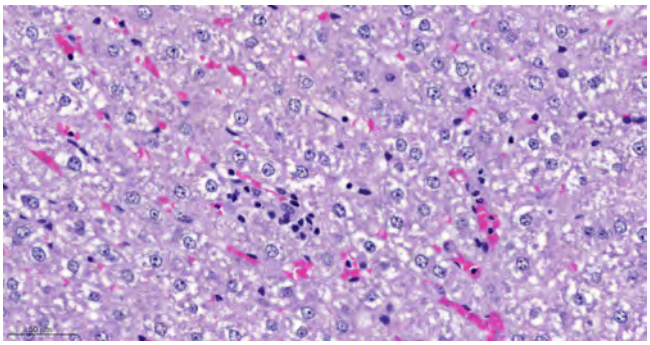


FIG. 1.

*Change in the concentration of hepatic biochemical markers in the blood serum of rats with experimental NAFLD and in control group: * – $p < 0.05$; *** – $p < 0.0003$ in comparison with the intact control group*

The histological study of liver autoptates from animals of control and experimental groups revealed morphological changes of various severity degrees. In rats of the experimental group, marked hyperemia of sinusoids, violation of the beam structure and infiltration by mononuclear cells were registered (Fig. 2a, b).



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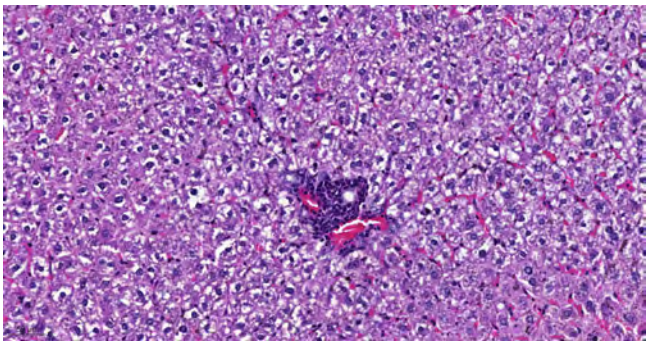

b

FIG. 2.

*Non-alcoholic fatty liver disease (liver autopsy, hematoxylin and eosin staining): lobular lymphocytic infiltration, small droplet fatty degeneration of hepatocytes. Magnification ×40 (**a**), ×20 (**b**)*

In rats' liver autoptates with reproduced NAFLD, large droplet fatty degeneration of hepatocytes was observed: large lipid droplets in the cytoplasm, the nucleus was displaced to the periphery of the cell. Fibrosis of different localization and degree is observed: in some loci – pericellular, pericentral and even in some places bridging portal veins with central veins. All these structural changes indicate a powerful inflammatory process, which may lead to activation of various extrahepatic functions of hepatocytes, such as synthesis of acute phase inflammatory proteins such as ferritin, C-reactive protein, and hepcidin.

Assessment results of erythrocytic hematopoietic lineage in animals with NAFLD confirm the development of anemic syndrome of mild severity by the end of the experiment. A statistically significant decrease in hematocrit (p = 0.016), RBC (p = 0.021) and reticulocytes (p = 0.038) as well as hemoglobin concentration (p = 0.041) per unit of blood volume was registered compared to the control group (Table 1).

TABLE 1

THE STATE OF THE ERYTHROCYTE LINEAGE OF HEMATOPOIESIS IN RATS WITH EXPERIMENTAL NAFLD AND IN THE CONTROL GROUP

Test item	Control group	Experimental group
HCT, %	47.3 ± 0.94	39.1 ± 1.08*
RBC, × 10^{12}/L	7.8 ± 0.18	6,7 ± 0,20*
RTC, %/RBC	15.5 ± 0.85	11.1 ± 1.02*
HGB, g/L	127.3 ± 1.31	95.6 ± 4.77*

Note. * – $p < 0.05$ in comparison with the intact control group

During EIA of concentrations of pro-inflammatory cytokines IL-1 and IL-6 in blood serum of animals belonging to the control and experimental groups, statistically significant differences between these groups were observed, which confirms the important role of cytokines in the response of the liver to pathological effects.

For example, the IL-1 content on the 30th day of the experiment in the group of animals with NAFLD (5.27 ± 0.20 pg/ml) significantly exceeded the initial control (0.84 ± 0.06 pg/ml) (p = 0.011); even more significant differences on the 30th day of the study were observed in the dynamics of IL-6 (12.04 ± 0.4 pg/ml) compared to the animals of the control group (1.54 ± 0.07 pg/ml; p = 0.012) (Fig. 3).

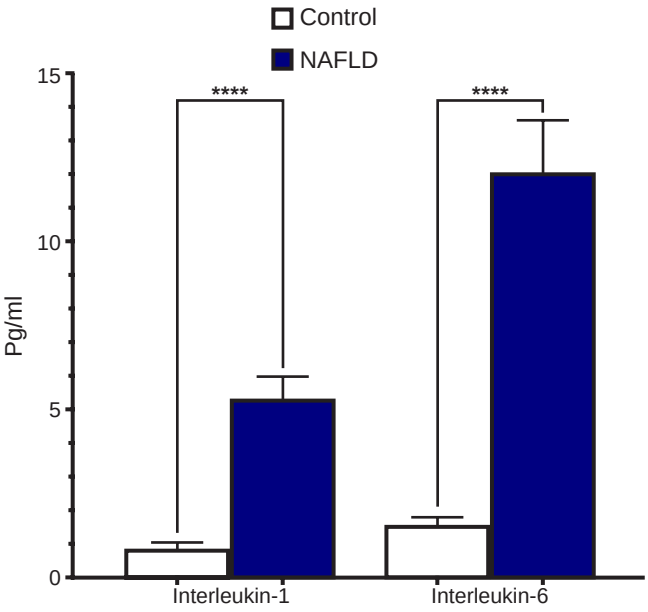

FIG. 3.

*Change in the concentration of pro-inflammatory cytokines in the blood serum of rats with experimental NAFLD and in control group: **** – $p < 0.0001$ compared with intact control*

DISCUSSION

Chronic liver diseases are often associated with hematologic abnormalities. Anemias of various etiologies occur in approximately 75 % of these patients [12].

The underlying causes of anemia associated with chronic liver disease are: gastrointestinal bleeding due to portal hypertension; clotting factor deficiencies [13]; immune-mediated aplasia of red bone marrow [14]; and pharmacological effects of drugs used to treat viral hepatitis [15, 16].

It is well known that one of the leading functions of the liver is protein-synthetic. All blood protein fractions, acute phase proteins, clotting factors, transport proteins, in particular proteins transporting and storing iron (ferritin, transferrin) are formed in the liver [8, 17].

Based on the results of this study, the anemia developed by modeling of NAFLD is hyporegenerative (RTC – 11.1 ± 1.02 % vs. control – 15.5 ± 0.85 % (p = 0.038)), hypochromic (HGB – 95.6 ± 4.77 g/l vs. control – 127.3 ± 1.31 g/l (p = 0.041)), which confirms the fact of iron deficiency development.

Immune activation in response to interaction with agents of microbial, immune, tumor, and metabolic nature induces the release of pro-inflammatory cytokines that subsequently lead to disruption of iron homeostasis. Although it is impossible to fully disentangle the iron-regulatory influences of multiple cytokine networks, IL-6 appears to be the most important, at least in animal models [18]. One of the effects of IL-6 associated with iron metabolism in laboratory animals and humans is its stimulatory effect on increased hepcidin production by hepat-

ocytes [19, 20]. Hepcidin, a major systemic regulatory factor of iron metabolism, binds to the protein ferroportin 1 and induces its lysosomal degradation. This reduces circulating iron concentration by decreasing the release of recycled iron from macrophages and deposited iron in hepatocytes [8, 17, 19, 21].

It is known that most iron is deposited in hepatocytes and macrophages of the reticuloendothelial system as part of ferritin, while hepatocytes obtain iron mainly by absorption of transferrin. Hemosiderin, another iron-storage protein, is formed when ferritin is depleted and is mainly found in cells with iron overload and mobilizes iron irregularly and slowly [10, 18].

Iron sequestration in macrophages also plays a significant role, as recycling of iron from aging erythrocytes by macrophages accounts for > 90 % of the daily iron requirement for hemoglobin synthesis and erythropoiesis [8, 22].

Liver disease is often associated with hematologic abnormalities. A large number of patients with NAFLD have anemia of varying severity, and its pathogenesis is complex. Timely diagnosis and therapy of anemic syndrome in NAFLD can prevent complications of the underlying disease.

CONCLUSIONS

Thus, there is reason to believe that the increased concentration of pro-inflammatory cytokines IL-1, IL-6 in NAFLD inhibits iron absorption in the duodenum, where ferroportin is required for absorption of dietary iron into the bloodstream, and they also act on macrophages to block the release of iron recycled from aging erythrocytes into plasma.

Further study of anemia development mechanisms as one of the links in the pathogenesis of NAFLD may provide important therapeutic targets in the treatment of both NAFLD and comorbidities.

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Conflict of interest

The authors declare no conflict of interest.

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