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Amino acids of the biliary system in rats with tetracycline-induced hepatosis and the use of milk phospholipids

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Abstract. The relevance of the study is due to the high frequency of drug-induced liver injury in mammals and insufficient study of the issue of amino acid metabolism disorders in acute forms of hepatopathy of the corresponding genesis. In this regard, the study was aimed at identifying specific changes in the profile of free amino acids in the bile and liver of rats with tetracycline-induced fatty hepatosis, as well as determining the corrective effect of the phospholipid fraction of milk. The leading approach in studying this problem was an experiment on laboratory animals with modelling of fatty hepatosis due to the cytotoxic effect of tetracycline, followed by the selection of bile and liver samples, followed by studying the spectrum of free amino acids using thin-layer chromatography with ninhydrin staining. It was found that the general pattern of changes in the hepatobiliary system of diseased rats indicated a violation of bile acid conjugation processes and mitochondrial dysfunction, as well as a pronounced blockage of the use of free amino acids in metabolic processes. At the same time, the use of phospholipid-containing therapy in rats with tetracycline-induced fatty hepatosis activated the use of the intrahepatic reserve of amino acids involved in bile acid

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conjugation and antioxidant protection, in particular sulphur-containing representatives and the total fraction of glycine/serine/glutamine. It was determined that the bile of these rats also had a reduced content of glycine and taurine-containing amino acid fractions in the bile of these rats, with a simultaneous increase in the level of the total alanine/tyrosine/threonine and arginine/ornithine/lysine fractions, confirming the activation of detoxification processes in the liver involving free amino acids. It was found that the use of the phospholipid fraction of milk reduced the manifestations of disturbances in the intermediate metabolism of amino acids and contributed to the restoration of the content of leucine- and valine-containing fractions in the bile and liver of sick rats. The established patterns are of practical value for laboratory diagnosis and preclinical evaluation of hepatoprotective agents, since the amino acid profile of the hepatobiliary system can be used as a sensitive indicator of the severity of liver injury and as a criterion for determining the effectiveness of therapy

Keywords: chromatography; amino acid fractions; liver; bile; corrective therapy; tetracycline hydrochloride; laboratory diagnostics

Introduction

Drug-induced liver injury (DILI) is considered one of the most challenging problems in modern hepatology, as it is a common cause of acute liver failure, masquerades as other lesions, and requires clear diagnostic and monitoring algorithms. In practical guidelines, R.J. Fontana *et al*. (2023) noted that the management of patients with DILI should be based on a standardised approach that combines clinical assessment, biochemical markers, and analysis of potentially hepatotoxic drugs (including herbal and dietary supplements). Summarising current understanding of the pathogenesis of this hepatopathology, J. Skat-Rørdam *et al.* (2025) emphasised that the key mechanisms of DILI remain mitochondrial dysfunction, oxidative stress and immune-mediated injury to hepatocytes, and therefore risk assessment should be based on the evaluation of these links. At the same time, S. Thakur *et al.* (2024) emphasised that biomarker panels (rather than individual indicators) capable of reflecting various components of pathogenesis, from oxidative stress to cholestasis and inflammation, are becoming increasingly important for hepatotoxicity. Against this background, amino acid homeostasis is gaining increasing attention as the "central axis" of liver metabolism and its disorders.

In the context of metabolic hepatopathies, C. Fotakis *et al.* (2023) showed, based on metabolic profiling, that non-alcoholic fatty liver disease (NAFLD) is associated with reproducible metabolic patterns, including amino acid patterns, that reflect changes in energy and nitrogen metabolism. This is consistent with what M. Holeček (2024) described in detail, pointing to the role of alanine and glutamine as key carbon and nitrogen carriers for gluconeogenesis and the functional interaction of the liver with other organs. This finding makes amino acid shifts not only "markers" but also functionally significant components in pathology. Of particular interest are integral amino acid indices as tools for assessing metabolic imbalance and predicting the severity of liver injury. In particular, H. Enomoto *et al*. (2023) emphasised that the ratio of branched-chain amino acids to tyrosine is not only an indicator of amino acid imbalance but also a reflection of systemic metabolic shifts in chronic hepatopathies. A study

by J. Bugajska *et al*. (2023) demonstrated that the amino acid composition of bile can reflect the characteristics of pathological processes in the biliary tract. This reinforces the relevance of analysing the biliary spectrum of amino acids in elucidating metabolic changes in the hepatobiliary system. At the same time, A. Eguchi *et al*. (2025) showed that the profile of amino acid-conjugated bile acids in blood serum reflects the severity of chronic liver disease and has prognostic significance for the viability of the organism. In other words, changes in conjugation are not only a biochemical feature, but also a clinically and experimentally significant characteristic of the lesion.

The key link between amino acid metabolism and the detoxification function of the liver is the conjugation of bile acids with glycine and taurine, which is provided by the enzyme bile acid-CoA:amino acid N-acyltransferase (BAAT). Importantly, S.A.J. Trammell *et al*. (2023) identified BAAT not only as a bile acid conjugation enzyme, but also as a hepatic N-acyltaurine synthase for polyunsaturated fatty acids, i.e., BAAT forms a broader spectrum of bioactive lipid derivatives potentially relevant to models of drug-induced steatosis and cholestatic components of injury. The regulatory mechanisms of this axis complement the data of T. Miyazaki *et al*. (2023), who used an experimental model to demonstrate enhanced taurine biosynthesis and conjugation of bile acids with taurine through farnesoid X receptor (FXR)-dependent mechanisms, emphasising that the balance of taurine/glycine conjugates is a controllable and therefore potentially decisive link in the pathogenesis of hepatopathy. Against the backdrop of the search for non-fragmentary approaches to the correction of drug-induced hepatopathies, there is growing interest in nutritional strategies, primarily in milk phospholipids. Experimentally, Z. Wu *et al*. (2022) showed that the addition of milk fat globule membranes (MFGM) alleviates colitis and secondary liver injury by improving the mucosal barrier and modulating the microbiota, which is important for the gut-liver axis. Additionally, Q. Zhang *et al*. (2021) showed that adding MFGM to the diet can reduce the manifestations of hepatic steatosis under experimental conditions of metabolic stress, which forms a rational basis for testing the hepatoprotective potential of milk phospholipids and in a model of tetracycline-induced hepatosis. That is why a comprehensive study of amino acids in the biliary system of rats under conditions of tetracycline-induced liver injury and against the background of milk phospholipid administration represents a relevant research direction that combines mechanistic analysis (mitochondrial dysfunction/oxidative stress/bile acid conjugation) with potential nutritional correction.

Thus, current data highlight two key unresolved issues. First, simultaneous changes in the profile of free amino acids in the liver and bile in a model of acute drug-induced steatosis, in particular tetracycline-induced steatosis, have not yet been sufficiently characterised. Second, there is limited information on whether the phospholipid fraction of milk is capable of modifying these amino acid shifts through its effect on bile acid conjugation, antioxidant pathways, and complexes associated with glutathione metabolism.

The aim of the study was to reveal specific changes in the profile of free amino acids in the bile and liver of rats with tetracycline-induced fatty hepatosis and to determine the corrective effectiveness of the phospholipid fraction of milk in restoring the amino acid balance at the level of the hepatobiliary system. The objectives were, first, to quantitatively characterise changes in the profile of free amino acids in the bile and liver of rats relative to the control group; secondly, to determine whether the phospholipid fraction of milk modifies the deviations of

glycine-taurine and sulphur-containing amino acid groups associated with bile acid conjugation and antioxidant protection; and, thirdly, to evaluate the informativeness of the amino acid spectrum as a potential indicator of the effectiveness of correction, comparing the "Self-rehabilitation" groups without therapy and in the case of oral administration of milk phospholipids "Tetracycline + biologically active supplement FLP-MD" to laboratory rats.

Literature Review

In both veterinary and human medicine, the toxic effect of drugs on the liver is one of the leading problems in hepatopathology. Several interrelated pathogenetic mechanisms are distinguished in the development of DILI, including mitochondrial dysfunction, oxidative stress, and bile acid homeostasis imbalance, which significantly complicates the treatment of sick animals. J. Skat-Rørdam *et al*. (2025) added the activation of cell death signalling pathways to the list of drug-induced liver injury disorders. The experts emphasised the need to use experimental models in combination with comprehensive approaches and genetic tools for in-depth study of the pathogenesis of DILI and the search for reliable biomarkers. This pathogenesis of drug-induced liver injury is consistent with the phenotype of tetracycline-induced microvesicular steatosis, where the suppression of mitochondrial β -oxidation of fatty acids, which leads to the accumulation of small fat droplets in hepatocytes. The key links in this phenotype are dysregulation of lipid metabolism, membrane disorders and oxidative stress, so it seems reasonable to consider milk polar lipids as potential corrective factors.

Previous studies have noted the high efficacy of milk phospholipids (polar lipids) in models of liver injury. Thus, Z. Wu *et al*. (2022) in a model of acute colitis (induced by DSS) with secondary liver injury showed that inflammatory aggression was accompanied by an increase in the activity of aspartate aminotransferase and alanine aminotransferase in blood plasma and liver, while prophylactic administration of MFGM reduced these indicators and mitigated the manifestations of hepatic oxidative stress. This indicated the pronounced hepatoprotective potential of milk polar lipids. Further studies detailed the effect of milk polar lipids on fat metabolism in hepatic steatosis. In particular, Q. Zhang *et al*. (2021) demonstrated that the offspring of mice whose mothers received a high-fat diet developed pronounced hepatic steatosis: small fat vacuoles accumulated in hepatocytes, the content of triacylglycerols in blood serum and liver increased, and aspartate aminotransferase activity increased. In contrast, the addition of MFGM in the early postnatal period contributed to the restoration of triacylglycerol levels and aspartate aminotransferase activity and improved antioxidant defence parameters (decrease in malondialdehyde content, increase in superoxide dismutase activity). This was consistent with the suppression of *de novo* lipogenesis and restoration of mitochondrial function in the liver of these animals. A.L. Zhou & R.E. Ward (2024) also showed that a diet enriched with milk polar lipids caused a decrease in absolute and relative liver weight and a decrease in the expression of liver genes associated with lipid metabolism, including cholesterol. This confirms the beneficial effect of milk phospholipids on hepatic lipid metabolism under stress conditions. In a review of clinical studies, A.P. Kanon *et al*. (2024) showed that adding MFGM to the diet of adults was associated with a decrease in total cholesterol and low-density lipoproteins in the blood. This hypolipidemic effect was consistent with the hypothesis of the cardiometabolic benefits of milk phospholipids. Taken together, these results significantly complement experimental data on the effective correction of the lipid profile of blood plasma,

bile and liver in rats using the components of the dietary supplement "FLP-MD" in a model of tetracycline-induced hepatosis.

In modern research, increasing attention is being paid to the amino acid profile as a marker of liver injury. For example, J. Zhao *et al*. (2023) used Mendelian randomisation to prove a causal link between genetically determined levels of certain amino acids and the risk of metabolically associated fatty liver disease (MAFLD). In particular, it was shown that genetically determined increases in alanine levels and decreases in glutamine content cause a higher risk of developing MAFLD. These data emphasised that changes in amino acid composition are not only a consequence but also a possible trigger for the progression of steatosis. In turn, N.Y.T. Tan *et al*. (2024) proposed an integrated glutamate-serine-glycine index as a non-invasive indicator of MAFLD activity stage and response to therapy. Overall, the results obtained indicate that the amino acid profile is a promising platform for assessing the severity and prognosis of hepatopathy. At the same time, most studies focus on the analysis of blood plasma and practically do not consider synchronous changes in the amino acid spectrum in the liver and bile.

A separate area of research is the assessment of the phospholipid fraction of MFGM as a source of bioactive lipids. Literature data have shown that MFGM components such as phosphatidylcholine, sphingomyelin and phosphatidylethanolamine are capable of regulating lipid and cholesterol metabolism, improving the barrier function of the intestine and exhibiting anti-inflammatory properties. The reduction in cholesterol and low-density lipoprotein levels in adults when consuming MFGM, demonstrated in clinical studies, is consistent with the hypolipidemic and general health-promoting effects of milk polar lipids. These facts are comparable to the above experimental data on the correction of lipid metabolism in the liver under the influence of milk phospholipids. In addition, it is worth noting the role of individual amino acids in the course and treatment of animals with hepatopathology. A decrease in branched-chain amino acids (BCAA) during the progression of chronic liver disease is associated with a worsening prognosis, while their additional administration is recommended in patients with cirrhosis (especially in the presence of hepatic encephalopathy) to improve nutritional status and clinical outcomes. Moreover, M.C. Trillos-Almanza *et al*. (2024) showed that BCAAs and their metabolites contributed to a decrease in the intensity of activation of hepatic stellate cells (the main effectors of fibrogenesis) in *in vitro* experiments. Thus, the available literature data have shown that tetracycline-induced hepatosis is an experimentally validated model for studying DILI. Milk phospholipids and other components of milk fat globule membranes have a clinically proven ability to correct structural and metabolic disorders in the liver. At the same time, the amino acid profile is considered a sensitive marker of liver injury. However, simultaneous changes in the amino acid composition of the liver and bile in conditions of drug-induced hepatosis and its correction with milk phospholipids remain insufficiently characterised, which explains the need for this study.

Materials and Methods

An acute experiment on laboratory rats, followed by the collection of biological material samples and their preparation for amino acid composition analysis, was conducted in January 2024 at the vivarium of the Educational and Scientific Centre (ESC) "Institute of High Technologies" of the Taras Shevchenko National University (Kyiv), which is equipped with centralised water supply, sewage, ventilation and air conditioning. The research was conducted in

accordance with Directive 2010/63/EU (2010), Law of Ukraine No. 3447-IV (2006) and Order of the Ministry of Education and Science, Youth and Sports of Ukraine No. 249 (2012). The amino acid composition of the prepared bile and liver samples was studied in the scientific laboratory of the same research centre during 2024. The research project was reviewed and approved by the Bioethics Committee of the National University of Life and Environmental Sciences of Ukraine, as confirmed by the decision of the Bioethics Committee of the National University of Life and Environmental Sciences (NULES) of Ukraine dated 10 October 2023, protocol No. 10.

Each group of animals was kept in separate cages on a standard vivarium diet. They had free access to food and drinking water. Before the start of the experiment, the rats were kept in quarantine with clinical examination for two weeks. Monitoring of changes in body weight and feed consumption by the test animals was carried out for 9 days.

Using the author's method by V. Gryshchenko *et al*. (2019), fatty hepatosis was modelled in laboratory rats. For this purpose, using a soft silicone probe, a 4% solution of tetracycline hydrochloride was administered intragastrically to the animals daily at a rate of 250 mg/kg of body weight for 7 days. As a result of intragastric administration of high doses of tetracycline hydrochloride, rats developed fatty hepatosis. Animals that received intragastric administration of tetracycline hydrochloride without further therapy were assigned to the "Self-rehabilitation" group (n = 6). Rats in the "Tetracycline + "FLP-MD" dietary supplement" group (n = 6) were administered an intragastric dose of a 1% solution of the FLP-MD dietary supplement (experimental series, NULES Ukraine), which contains milk phospholipids as the main active ingredient. The drug was administered intragastrically for 9 days – one hour before the administration of the antibiotic, as well as for two days after the completion of the course of antibiotics. The daily therapeutic dose of "FLP-MD" dietary supplement was 13.5 mg/kg of body weight (Patent of Ukraine No. 8651, 2009). Rats that were administered distilled water in a volume equivalent to the volume of the antibiotic and dietary supplement were included in the "Control" group (n = 6). The next group of animals, the "Preparation" group, consisted of clinically healthy animals that additionally received milk phospholipids as part of the dietary supplement "FLP-MD" (n = 6). During the experiment, the body weight of the rats was monitored daily using ORION OS-0K22 electronic scales (ORION ELECTRONICS LTD, Europe), adjusting the dosage of the drugs according to the established changes. On day 10, bile and liver samples were taken from rats in all groups for further analysis of their amino acid composition.

The spectrum of free amino acids was determined by the method of paper chromatography with ninhydrin staining, modified on the basis of the classical methods of R. Consden *et al.* (1944), S.M. Partridge (1948) and taking into account the approaches of S. Moore & W. H. Stein (1948) and R.J. Block *et al.* (1958), which allowed for a quantitative assessment of the amino acid composition of biological material. This method facilitated the separation of amino acids on chromatographic paper due to differences in the distribution coefficients between the mobile and stationary phases and is suitable for quantitative analysis in biomaterials, with the exception of proline and hydroxyproline, which give an atypical reaction with ninhydrin.

Bile sampling. Rats were fasted for 18 hours prior to surgery. Anaesthesia was achieved by intraperitoneal administration of thiopental sodium at a dose of 60 mg/kg. Approximately 5 minutes after administration of the anaesthetic, the animal was immobilised on its back. A median laparotomy was performed along the

midline of the abdomen, starting approximately 0.5 cm caudal to the xiphoid process; the length of the incision was 2.0-2.5 cm. The common bile duct and portal vein were identified at the hepatic hilum. At a distance of about 0.5 cm distal to the bifurcation of the duct, two ligatures were applied: one to hold the duct during manipulation, the other to fix the cannula. After delicate incision of the duct wall, a cannula was inserted and connected to a 200 µL micropipette. Bile collection began 30 minutes after cannulation and was performed every 30 minutes for 3 hours (a total of six samples: at 30, 60, 90, 120, 150, and 180 minutes), as described by D.O. Melnychuk *et al.* (2015).

Liver sampling. Biological material samples were collected under thiopental anaesthesia in rats. Liver samples were collected immediately after euthanasia of the rats. A portion of the organ weighing 300 mg was measured using torsion balances (model VT-500 torsion balance, Ukraine), after which 700 µL of physiological saline was added. The samples were homogenised using a (Lankai FSH-2A, China) over ice twice for 15-20 seconds at medium speeds, with a 30-second break between approaches. The homogenate was centrifuged in a high-speed microcentrifuge (Biosan Microspin 12, Latvia) after pre-cooling the rotor at 14.5 thousand rpm for 10-12 minutes.

Sample preparation. Since some amino acids have very similar *Rf* values and are not completely separated during one-dimensional chromatography, preliminary concentration and extraction of the sample was performed. Each sample (20 µL of liver sample homogenate) was applied to a small square of desulphurised filter paper (3.5×3.5 cm) – 20 µL three times per paper (each layer was dried before applying the next). The paper saturated with the sample was cut into small pieces and placed in a test tube with 2 mL of an extracting mixture of acetone:ethanol:water (7:2:1, by volume). This allowed the amino acids to be extracted (desorbed) from the paper into the solution and simultaneously separated from macromolecules and salts that could interfere with the analysis. After extraction, the solution was evaporated to dryness (in a stream of air at room temperature), and the residue was dissolved in 15 µL of distilled water. The resulting concentrate was applied to the starting line of the chromatographic paper in the form of three consecutive drops of 5 µL each (with a 15-minute interval between applications for drying). In this way, a compact concentrated spot was formed from each sample. The "cut paper elution" approach was adapted for amino acids as an author's modification (Hackman & Lazarus, 1956).

Chromatographic separation. Filtrak FN-1 chromatography paper (Germany) was used as the stationary phase. The mobile phase was a six-component mixture of solvents: isoamyl alcohol (Carlo Erba, France): n-butanol (Carlo Erba, France): glacial acetic acid (Merk, Germany): formic acid (BASF, Germany): distilled water: butylbenzyl ether (Thermo Fisher Scientific, USA) in a ratio of 21:8:10:1:10:1 (by volume) with the addition of 0.2% ninhydrin (Thermo Fisher Scientific, USA). Chromatography was performed in a sealed chamber using the ascending paper chromatography method, pre-saturated with mobile phase vapours (Consden *et al*., 1944; Williams & Kirby, 1948). The starting zones for sample application were located on a line 2-3 cm from the bottom edge of the paper. The development of the chromatogram lasted approximately 7 hours (the solvent passed through almost the entire length of the paper strip). If necessary, multiple solvent passes or extended development time (up to 12-20 hours) can be used to improve separation. However, in this case, a single pass over 7 hours was sufficient to separate most fractions.

Chromatogram development. After completion of chromatographic separation, the

paper strip was removed from the chamber and dried in a fume hood at room temperature (1 hour, until complete removal of solvent vapours). To visualise the amino acids, the chromatogram was placed in a drying oven (Labexpert 3015, Ukraine) at a temperature of 45°C for 120 minutes. During this time, ninhydrin (Thermo Fisher Scientific, USA), added to the mobile solvent, reacted with amino acids, forming coloured products directly on the paper. Thus, additional spraying with the reagent was not required. This gentle heating (temperature 45°C, 2 hours) ensured the gradual development of amino acid spots. For comparison, in standard methods, the paper chromatogram is immersed in a ninhydrin solution for several seconds after drying and then dried, followed by heating for 15 minutes at 60°C to reveal purple spots (Block *et al*., 1958). The approach used in this work, with the addition of ninhydrin directly to the eluent, simplified the procedure with a similar development effect.

Quantitative analysis. After development, the amino acid spots on the chromatogram were quantitatively evaluated by reflectance densitometry. The intensity of each spot's colour was measured on a DO-1M densitometer (Ukraine) in reflected light. The use of spectral reflectometry to evaluate ninhydrin staining on chromatograms is a well-known approach in the quantitative analysis of amino acids. For quantitative determination, calibration curves were constructed in advance: known amounts of standard amino acids were applied to chromatographic paper and, after the entire procedure (separation and development), the optical density of their spots was measured. Within the linear range of the ninhydrin reaction, the optical density of the colour is proportional to the amino acid content. The measured optical density of the spots of the test samples was compared with the calibration graph of the corresponding amino acid and used to determine the amount of this amino acid in the sample, taking into account the dilution made during sample preparation. If some amino acids did not separate (co-eluted) due to the proximity of *Rf*, their total content was noted together, or separation required additional measures (e.g., solvent change or two-dimensional chromatography).

Statistical processing of results. The experimental data obtained were processed using methods of variational statistics in the Microsoft Excel 2016 software environment. For each indicator, the arithmetic mean (*M*), standard deviation (*SD*) and standard error of the mean (*m*) were determined using the following formulas:

$$M = \frac{\sum x_i}{n}, \quad (1)$$

$$SD = \sqrt{\frac{\sum (x_i - M)^2}{n-1}}, \quad (2)$$

$$m = \frac{SD}{\sqrt{n}}, \quad (3)$$

where n – number of observations.

Calculations were performed using built-in Excel functions: = AVERAGE(range) – for the mean value; = STDEV.S(range) – for the standard deviation; =STDEV.S(range)/SQRT(COUNT(range)) – for the standard error of the mean. The significance of differences between means was tested using Student's t-test for independent samples with unequal variances (t-test, two-sample unequal variance) with a statistical significance level of $P < 0.05$.

Results and Discussion

To determine the features of the effect of tetracycline-induced hepatosis on the functioning of the hepatobiliary system of the organism and to clarify the corrective effectiveness of milk phospholipids, the amino acid composition of bile in rats was analysed. The obtained indicators of total amino acid fractions in the bile of experimental rats from different groups are

presented in Table 1. This made it possible to determine the direction of shifts in the amino acid profile of bile in the experimental rats under different experimental conditions.

Table 1. Concentration of free amino acids in the bile of rats with tetracycline-induced hepatosis and the use of milk phospholipids, mg% (M ± m, n = 6)

Total amino acid fraction	Clinically healthy rats, "Control" group	Rats with tetracycline-induced hepatosis, "Self-rehabilitation" group	Rats with tetracycline-induced hepatosis treated with the dietary supplement "FLP-MD", "Tetracycline + "FLP-MD" supplement" group	Clinically healthy rats treated with the dietary supplement "FLP-MD", "Preparation" group
Cysteine/ Cystine	0.48 ± 0.03	0.71 ± 0.02*	0.40 ± 0.02*	0.65 ± 0.06*
Arginine/ Ornithine/ Lysine	1.55 ± 0.07	2.77 ± 0.06*	1.95 ± 0.80*	2.20 ± 0.10*
Histidine/ Taurine/ Asparagine	0.97 ± 0.06	1.33 ± 0.08*	0.63 ± 0.04*	1.48 ± 0.09*
Glycine/ Serine/ Glutamine/ Aspartic acid	1.44 ± 0.09	3.16 ± 0.05*	1.03 ± 0.09*	2.30 ± 0.09*
Methionine/ Glutamic acid	1.00 ± 0.05	2.17 ± 0.08*	0.82 ± 0.05*	1.18 ± 0.05*
Alanine/ Tyrosine/ Threonine	1.50 ± 0.03	2.10 ± 0.07*	2.15 ± 0.09*	1.50 ± 0.07
Valine/ Norvaline/ Tryptophan	1.07 ± 0.05	1.67 ± 0.03*	0.85 ± 0.04*	0.98 ± 0.08
Leucine/ Norleucine/ Phenylalanine	0.88 ± 0.01	1.64 ± 0.10*	0.82 ± 0.18	0.97 ± 0.09

***Note**: * – P < 0.05 compared to the control group*
***Source**: authors' own work*

In the bile of rats in the "Self-rehabilitation" group, the cysteine/cystine fraction was 48% higher than the corresponding values in the "Control" group. This pattern reflected the activation of the methionine-homocysteine cycle and enhanced cysteine resynthesis during the recovery phase after tetracycline-induced oxidative stress. Cysteine is a limiting substrate for the synthesis of glutathione and taurine. These pathways are critical for the detoxification of reactive oxygen species and the conjugation of bile acids. Recent data from T. Miyazaki *et al*. (2023) confirmed that activation of farnesoid X receptors or NR1H4 stimulates both taurine synthesis and bile acid conjugation under the catalytic influence of BAAT, which requires sufficient supply of sulphur-containing amino acids. Thus, the increased content of cysteine/cystine in bile during the self-recovery stage can be interpreted as a consequence of the fact that the rate of their formation exceeds the intensity of their use in the

synthesis of glutathione and taurine. In the bile of sick rats in the "Tetracycline + FLP-MD dietary supplement" group, on the contrary, a 17% decrease in the total cysteine/cystine fraction was recorded compared to the control group. This may indicate that, despite correction with milk phospholipids, the cysteine reserve is still actively spent on restoring glutathione content and conjugating toxic forms of bile acids that accumulate during the modelling of tetracycline-induced hepatosis. Recent experimental work by J. Liu *et al.* (2025) has shown that an excess of hydrophobic bile acids, by activating FXR/SHP (small heterodimer partner), can inhibit the glutathione synthetase pathway, increasing the consumption of cysteine for antioxidant protection. Thus, lower values of the total cysteine/cystine fraction in bile are consistent with the idea of a continuing load on sulphur-containing amino acids even under therapeutic conditions. In clinically healthy animals in the "Preparation" group, which received only milk phospholipids, the concentration of the cysteine/cystine fraction in bile was 35% higher than in the control group. This may reflect mild stimulation of sulphur-containing metabolism and enhanced conjugation of bile acids against the background of improved membrane transport under the action of phospholipids.

In the bile of sick rats in the "Self-rehabilitation" group, the content of the total arginine/ornithine/lysine fraction was 79% higher than the corresponding values in the control group. This increase in values may be associated with the activation of the ornithine cycle and enhanced detoxification of nitrogen. Ornithine and arginine are key metabolites of this pathway, while lysine reflects the overall increase in protein catabolism. A similar dysregulation of the arginine-ornithine pathway in acute liver injury was described by M. Holeček *et al*. (1996), who recorded a decrease in arginine concentration against a background of increased ornithine content in blood plasma in four models of experimental liver injury in rats, which correlated with the severity of hepatocellular injury. Thus, the levels of these amino acids are considered to be sensitive biomarkers of acute hepatic injury. In the group of animals with hepatosis that received milk phospholipids (the "Tetracycline + "FLP-MD" dietary supplement" group), the total level of these amino acids was also significantly higher than the control by 26%, but lower than during self-rehabilitation. This can be interpreted as a partial normalisation of nitrogen metabolism against the background of the correction: milk phospholipids improved the structural state of hepatocytes, which contributed to the restoration of ornithine cycle enzyme activity. This reduced the need for "excessive" excretion of essential amino acids into bile. These conclusions are consistent with the data of S. Colosimo *et al*. (2023) on the reduction of hyperammonaemia and correction of nitrogen metabolism disorders using amino acid mixtures/modulating drugs in experimental models of metabolic hepatopathology.

In the bile of rats in the "Preparation" group, which received only milk phospholipids, a 42% increase in the concentration of the total arginine/ornithine/lysine fraction was also recorded relative to the control. This shift can be interpreted as moderate stimulation of protein and nitrogen metabolism and a certain adjustment of the ornithine cycle even in the absence of pronounced liver injury. The additional involvement of essential amino acids created better conditions for ammonia utilisation and maintenance of nitrogen homeostasis. This is consistent with data on the potential hepatoprotective role of L-arginine in experimental models of cholestatic liver injury. Y. Ozsoy *et al*. (2011) demonstrated that the use of L-arginine reduced the severity of biochemical and morphological signs of cholestatic injury, which is consistent with the idea of the anabolic

and hepatoprotective potential of additional amino acid administration.

In the bile of sick rats undergoing self-rehabilitation, the histidine/taurine/asparagine fraction was 37% higher than in the control group. Taurine is the end product of sulphur-containing amino acid metabolism and the main substrate for conjugation with bile acids, while histidine and asparagine reflect participation in buffering and anti-inflammatory reactions (via carnosine, histamine, amidation). An increase in their content in rat bile during the self-recovery phase may indicate the activation of bile acid conjugation reactions and the antioxidant and anti-inflammatory potential of hepatocytes. In line with this, a review by M. Stofan & G.L. Guo (2020) noted that FXR receptor activation is a key link in the regulation of bile acid synthesis, conjugation and detoxification and was considered an important mechanism for protecting the liver from metabolic and cholestatic stress. In the bile of sick rats in the "Tetracycline + "FLP-MD" dietary supplement" group, the total content of histidine/taurine/asparagine, on the contrary, was 35% lower than in the control group. This indicates that even with correction by milk phospholipids, the supply of taurine and related amino acids is still insufficiently restored and is likely to be intensively used for the conjugation of toxic forms of bile acids and the neutralisation of oxidative stress products. A. Eguchi *et al*. (2025) showed that in humans with chronic liver disease, a decrease in the glycine/taurine ratio in the serum conjugated bile acid fraction was closely associated with more pronounced deterioration of liver function, higher markers of fibrosis, and a less favourable prognosis for patient recovery. The bile of animals in the "Preparation" group, which received only milk phospholipids, showed the highest concentration of this cluster, which differed by 53% from the control. This can be interpreted as preventive "saturation" with taurine and related amino acids, which created favourable conditions for bile acid conjugation, maintenance of bile secretion and increased liver resistance to metabolic stress. Similarly, A. Gregor *et al.* (2021) showed in a calorie-restricted mouse model that this adaptation was accompanied by an increase in the level of free taurine and taurine-conjugated bile acids in the intestinal wall and an increase in the content of bile acids in the liver and blood plasma. The established patterns were considered an important component of the protective restructuring of bile acid and antioxidant homeostasis.

In the bile of sick rats in the "Self-rehabilitation" group, a sharp increase in the total content of glycine/serine/glutamine/aspartic acid was noted – more than 119% relative to the control. Glycine and glutamine are not only substrates for the synthesis of glutathione and urea, but also key donors in nitrogen detoxification reactions, while aspartic acid is involved in anion transport and the ornithine cycle. Such a pronounced increase in their levels in bile can be interpreted as a manifestation of enhanced protein catabolism and as a result of the activation of nitrogen metabolism during the stage of independent recovery after tetracycline injury. A similar trend towards an increase in the concentration of free amino acids in bile in conditions of hepatobiliary system damage was described by J. Bugajska *et al*. (2023), who found an increase in the content of most amino acids in bile in gallstone disease compared to the control group. On the other hand, M. Holeček (2024) emphasised that glutamine and aspartate are key participants in ammonium detoxification and ornithine cycle functioning in liver injury. Thus, the data obtained on the increase in the total content of the glycine/glutamine/aspartic acid fraction in bile was consistent with the idea of the enhanced participation of these amino acids in ensuring nitrogen homeostasis at the liver level. In sick

animals from the "Tetracycline + "FLP-MD" dietary supplement" group, against the background of milk phospholipid administration, the content of the total glycine/serine/glutamine/aspartic acid fraction in bile was 28% lower than the corresponding values in the control group. This may indicate that the corrective effect of milk phospholipids was manifested instead of the usual elimination of these amino acids in bile to intensive use in intracellular synthesis of proteins, glutathione and energy production necessary to ensure active regeneration of hepatocytes. A similar transition from catabolic to anabolic amino acid metabolism is described in the work of O. Rom *et al*. (2020), where glycine-containing therapy in experimental metabolic dysfunction-associated steatotic liver disease (MASLD)/NAFLD stimulated β-oxidation of fatty acids and glutathione synthesis in the liver, reducing steatosis, oxidative stress and fibrosis.

In the bile of animals in the "Preparation" group, which received only milk phospholipids, the content of the total fraction of glycine/serine/glutamine/aspartic acid was also significantly higher by 60% than in the control group. This probably reflected the physiological enhancement of bile formation under the influence of phospholipids, increased proliferation and restoration of the biliary tract epithelium, for which glycine and serine are key 'building' substrates. In support of this, S. Pan *et al*. (2021) emphasised that serine and glycine, through one-carbon metabolism, provide proliferating cells with nucleotides, proteins and membrane lipids, which is consistent with the interpretation of the results in this study regarding the established growth of this cluster as a marker of active renewal of the biliary tract epithelium.

In the bile of diseased rats in the "Self-rehabilitation" group, the content of the total methionine/glutamic acid fraction exceeded the control by 117%. Methionine is a methyl group donor and precursor of cysteine, and glutamate is a central node between nitrogen metabolism and energy cycles. Their "accumulation" in bile can be explained by impaired intrahepatic utilisation (methylation, glutathione synthesis) and enhanced transamination against the background of oxidative and lipid stress in tetracycline hepatosis. In sick animals from the "Tetracycline + "FLP-MD" dietary supplement" group, against the background of milk phospholipid use, the content of the total methionine/glutamic acid fraction was 18% lower than in the control group. It is likely that these amino acids were actively used in intracellular metabolism, in particular in the methionine-cysteine cycle, glutathione and protein synthesis, rather than being excreted in bile. In the bile of animals in the "Preparation" group, which received only milk phospholipids, a moderate but statistically significant 18% increase in the content of the total methionine/glutamic acid fraction was observed compared to the control. The observed changes can be explained by the activation of the methionine cycle and increased endogenous synthesis of phospholipids in the liver against the background of the intake of exogenous milk phospholipids. A summary of the data by G. Contarini & M. Povolo (2013) also showed that milk phospholipids can stimulate the restoration of the phospholipid composition of the liver and reduce its lipid overload, which is consistent with the proposed assumption about the activation of the methionine cycle against the background of phospholipid correction.

In the bile of sick rats in the "Self-rehabilitation" group, the content of the total fraction of alanine/tyrosine/threonine significantly increased by 40% relative to its values in the control group, and in rats from the "Tetracycline + "FLP-MD" dietary supplement" group against the background of milk phospholipid use, it increased by 43%. Alanine is a key substrate for gluconeogenesis and transamination, tyrosine

is an aromatic amino acid sensitive to the intensity of total proteolysis, while threonine is an essential component of glycoprotein and structural protein synthesis. Thus, a statistically significant increase in the total content of these fractions in both groups can be interpreted as a consequence of the enhanced mobilisation of these amino acids in hepatocytes in response to the development of experimental hepatopathology. In the bile of animals in the "Preparation" group, which received only milk phospholipids, the content of this total amino acid fraction remained unchanged.

At the same time, in the bile of sick rats in the "Self-rehabilitation" group, the content of the total fraction of valine/norvaline/tryptophan increased by 56% relative to the control. Valine belongs to branched-chain amino acids (BCAA), and tryptophan belongs to aromatic amino acids (AAA), so an increase in their total concentration in the secretion can be considered a manifestation of a shift in the BCAA/AAA balance during the recovery phase due to tetracycline-induced liver injury. K. Tajiri & Y. Shimizu (2018) emphasised that in patients with chronic diffuse liver injury and cirrhosis, there was a decrease in plasma concentrations of branched-chain amino acids against a background of an increase in aromatic amino acids, which led to a drop in the Fisher index (BCAA/AAA) index and the BCAA/tyrosine ratio. Such amino acid dysregulation is considered a typical sign of cirrhosis progression and a prognostic marker of its course. Against this background, the detected increase in the total content of valine/norvaline/tryptophan in the bile of rats during self-rehabilitation may indicate their compensatory elimination from the intracellular reserve due to the processes of bile formation and bile secretion. In the bile of rats in the "Tetracycline + "FLP-MD" dietary supplement" group, against the background of the use of milk phospholipids, the content of the total valine/norvaline/tryptophan fraction decreased by 21% compared to the control. This shift can be interpreted as meaning that, under the conditions of the correction applied, these amino acids are used more intensively by hepatocytes for protein synthesis and regeneration processes, compared to their losses in bile. M.C. Trillos-Almanza *et al*. (2024) noted that in patients with end-stage liver disease, BCAA levels were reduced, and their additional administration was considered as an approach to alleviating fibrosis and stimulating regeneration. In their work, the authors demonstrated that BCAAs and their metabolites reduce the activation of stellate cells in the livers of humans and rats. Against this background, the observed decrease in the total valine/norvaline/tryptophan fraction in bile during phospholipid therapy can be interpreted as a manifestation of more "economical" use of BCAAs in the liver to ensure recovery processes, while the absence of significant changes in these amino acids in rats in the "Preparation" group confirms that the nature of the redistribution of BCAA/AAA in bile is caused precisely by liver injury.

Only in sick rats in the "Self-rehabilitation" group did the content of the total leucine/phenylalanine fraction in bile differ significantly higher by 86% compared to the control, while in animals in the "Tetracycline + "FLP-MD" dietary supplement" and "Preparation" groups, these indicators remained unchanged. Leucine is a key branched-chain amino acid, and phenylalanine is a typical aromatic amino acid, so their joint increase in bile can be considered a manifestation of a pronounced amino acid imbalance at the stage of uncorrected recovery after tetracycline injury. Y. Zhang *et al*. (2024) noted that in chronic liver diseases, the BCAA/AAA ratio is a sensitive indicator of the degree of decompensation and prognosis, since a decrease in BCAA and a relative increase in AAA are closely related to the severity of liver injury.

Against this background, the fact that in the group with phospholipid correction, the total leucine/phenylalanine content did not differ from the control level, and in the animals of the "Preparation" group, only a tendency to increase was noted, further confirms the effectiveness of the corrective effect of milk phospholipids on the amino acid balance.

For a comprehensive characterisation of intermediate amino acid metabolism, an analysis of the intrahepatic composition of free amino acids was also performed, which directly reflected the metabolic state of hepatocytes in tetracycline-induced hepatosis against the background of correction with milk phospholipids. Table 2 presents the summarised results of individual total amino acid fractions in the liver of animals from different groups, which, in combination with the corresponding indicators in bile samples, allows to track changes in key links of nitrogen-excreting and bile-forming metabolism in the area of primary injury by toxic doses of tetracycline and, as a result, the development of fatty hepatosis.

Table 2. Content of free amino acids in liver samples of rats with tetracycline-induced fatty hepatosis and correction with milk phospholipids, mg% (M ± m, n = 6)

Total amino acid fraction	Clinically healthy rats, "Control" group	Rats with tetracycline-induced hepatosis, "Self-rehabilitation" group	Rats with tetracycline-induced hepatosis treated with the dietary supplement "FLP-MD", "Tetracycline + "FLP-MD" supplement" group	Clinically healthy rats treated with the dietary supplement "FLP-MD", "Preparation" group
Cysteine/Cystine	0.30 ± 0.03	0.41 ± 0.04*	0.17 ± 0.02*	0.44 ± 0.05*
Arginine/ Ornithine/Lysine	1.48 ± 0.14	2.74 ± 0.32*	0.86 ± 0.07*	2.10 ± 0.24
Histidine/ Taurine/ Asparagine	0.87 ± 0.07	2.02 ± 0.22*	0.57 ± 0.05*	1.57 ± 0.16*
Glycine/Serine/ Glutamine/ Aspartic acid	2.24 ± 0.20	3.73 ± 0.38*	1.33 ± 0.23*	4.06 ± 0.39*
Methionine/ Glutamic acid	2.36 ± 0.14	3.07 ± 0.24*	1.21 ± 0.19*	3.04 ± 0.23*
Alanine/ Tyrosine/ Threonine	1.20 ± 0.10	1.99 ± 0.09*	1.80 ± 0.17*	2.35 ± 0.20*
Valine/ Norvaline/ Tryptophan	0.82 ± 0.07	1.58 ± 0.14*	0.78 ± 0.04	1.79 ± 0.13*
Leucine/ Norleucine/ Phenylalanine	1.13 ± 0.08	1.82 ± 0.10*	2.10 ± 0.08*	2.70 ± 0.18*

***Note**: *P < 0.05 compared to the control group*
***Source**: authors' own work*

The amino acid profile in the liver of diseased rats with tetracycline-induced hepatosis (the "Self-rehabilitation" group) showed significant alterations. In particular, the content of the

total cysteine/cystine fraction in the liver of rats in this group increased by 37% compared with the control values, whereas in animals from the "Tetracycline + "FLP-MD" dietary supplement" group it decreased by 43%. In clinically healthy rats receiving milk phospholipids (the "Preparation" group), the cysteine/cystine content increased by 47% relative to the control. Thus, the development of tetracycline-induced fatty hepatosis was associated with depletion of the hepatic "thiol" profile, whereas administration of milk phospholipids to diseased animals shifted the balance toward accumulation of reduced sulphur-containing amino acids in hepatocytes. This pattern is probably due to the fact that toxic liver injury led to massive use of cysteine for glutathione synthesis and detoxification of active oxygen species, as well as its entry into the blood, which led to a decrease in the intrahepatic reserve. In contrast, the use of milk phospholipids in sick animals probably enhanced the synthesis and resynthesis of glutathione, partially restoring cysteine reserves in hepatocytes. In a review by M.T. Nguyen *et al.* (2025) on NAFLD therapy with glutathione, it was emphasised that the restoration of intracellular glutathione reserves is accompanied by a decrease in oxidative stress and liver enzyme activity, which indirectly confirms the key role of adequate cysteine supply in maintaining the redox homeostasis of hepatocytes. This is consistent with the results regarding an increase in the cysteine/cystine ratio when using phospholipid correction.

For the total fraction of arginine/ornithine/lysine in the liver of the experimental rats, two opposite trends were observed relative to the control. In the self-rehabilitation group of rats, the total content of the components of this heterogeneous fraction increased by 85% from the control level, while in the tetracycline-induced hepatosis and milk phospholipid administration group, it decreased by 42%. In clinically healthy animals, against the background of milk phospholipid administration, only a tendency to increase this indicator (by 42%) compared to the control was recorded. Thus, the tetracycline load on the liver in the experimental rats against the background of milk phospholipid administration was accompanied by depletion of arginine, ornithine and lysine depots, while in the case of spontaneous recovery, their compensatory increase was observed in the studied samples (the phenomenon of adaptive compensation). At the same time, intragastric administration of milk phospholipids to clinically healthy animals provoked only a tendency towards a further increase in the values of this fraction. At the molecular level, arginine and ornithine are key participants in the ornithine cycle, which detoxifies ammonia and protects the body, while lysine is the main essential amino acid sensitive to changes in total proteolysis and protein synthesis. The decrease in their total content in the liver during tetracycline exposure in rats is likely to be the result of changes in the ornithine cycle and arginase activity, accompanied by intense depletion of intracellular arginine.

In a review by S. Thakur *et al*. (2024) emphasised that metabolites of the ornithine cycle, including arginine and ornithine, can be considered promising biomarkers of hepatotoxicity, as their imbalance reflects the stress of ammonia detoxification and hepatocyte damage. In contrast, a sharp increase in the arginine/ornithine/lysine cluster in the liver during the self-rehabilitation phase can be interpreted as the activation of ureagenesis and anabolic processes aimed at restoring protein and nitrogen balance. This assumption was indirectly supported by experimental data from S. Al-Dalaen *et al*. (2016), which showed that the additional administration of L-arginine in CCl_4-induced hepatitis in rats enhanced the antioxidant protection of the liver and improved the morphological condition of hepatocytes,

indicating the hepatoprotective potential of arginine-dependent pathways. In a model of tetracycline-induced liver injury in rats, the increase in the total arginine/ornithine/lysine fraction in the case of self-rehabilitation and the tendency for its increase in clinically healthy animals against the background of milk phospholipid administration are also consistent with the idea that the restoration and maintenance of the optimal composition of amino acids in the ornithine cycle is an important link in the repair of toxic liver injury.

Quantitative characteristics of the total fraction of histidine/taurine/asparagine in rat liver samples (Table 2) showed opposite changes relative to the control. In animals in the "Self-rehabilitation" group, the total content of these amino acids increased by 132% from the control level. In animals in the "Tetracycline + "FLP-MD" dietary supplement" group, on the contrary, it decreased by 34%, while in animals in the "Preparation" group, which received milk phospholipids, it increased by 80% from the control. Thus, tetracycline-induced changes in the liver under conditions of milk phospholipid administration were accompanied by a marked depletion of histidine, taurine and asparagine, while in animals undergoing self-recovery and separately against the background of the administration of milk phospholipids to clinically healthy rats, this total content accumulated significantly.

This pattern is consistent with the functions of these amino acids. Histidine is a precursor of carnosine and histamine and acts as a buffer and antioxidant; taurine ensures bile acid conjugation, osmoregulation and has a pronounced antioxidant and anti-inflammatory effect; asparagine, with the participation of asparaginase, forms a "stress response" of hepatocytes, accumulating nitrogen and maintaining energy balance. A sharp decrease in the content of amino acids of this heterogeneous fraction in the liver of rats with tetracycline injury is associated with their intensive use in antioxidant and detoxification processes. On the other hand, a significant increase in the total content of histidine/taurine/asparagine during self-rehabilitation and in clinically healthy animals under the influence of milk phospholipids indicates the activation of recovery processes and the accumulation of substrates for antioxidant protection and membrane repair.

This interpretation is supported by experimental data from other researchers. Thus, W. Liu *et al*. (2008) showed that the addition of histidine and carnosine in chronic alcoholic liver injury in mice significantly reduced the activity of transaminases and markers of oxidative and inflammatory stress, which is consistent with the role of histidine as a component of the anti-inflammatory and antioxidant response. Against this background, a marked increase in the total fraction of histidine/taurine/asparagine in the liver under conditions of self-rehabilitation and against the background of the use of milk phospholipids was considered as a marker of the activation of hepatoprotective, antioxidant and reparative mechanisms, while its decrease in animals of the "Tetracycline + "FLP-MD" dietary supplement" group was considered an indicator of intensive involvement of these reserves.

The content of the total fraction of glycine/serine/glutamine/aspartic acid in rat liver samples underwent significant and mutually opposite shifts. In particular, in rats in the "Self-rehabilitation" group, the content of this total fraction increased by 67% from the control level, while in animals with tetracycline-induced liver injury in the "Tetracycline + "FLP-MD" dietary supplement" group, on the contrary, a decrease in its level by 41% from the control was noted. In clinically healthy animals in the "Preparation" group, against the background of the introduction of milk phospholipids, the content of this fraction in the liver reached its

highest values, increasing by 81% relative to the control group parameters. Thus, the use of tetracycline in toxic doses led to the intensive use of components of the heterogeneous fraction of glycine/serine/glutamine/aspartic acid, accompanied by a decrease in their content in liver samples, while in the case of self-recovery and when milk phospholipids were administered to clinically healthy animals, their content increased significantly. These amino acids are at the intersection of one-carbon metabolism, glutathione synthesis and ammonia detoxification. Glycine and serine are key donors of one-carbon groups and precursors of glutathione; glutamine and aspartic acid act as "reservoirs" for ammonium and replenish the Citric acid cycle. A sharp decrease in the levels of this fraction in the liver during tetracycline loading may be associated with the quantitative redistribution of these amino acids in the bodies of sick rats, which are also actively consumed in the neutralisation of toxic products of nitrogen metabolism and in the intensification of oxidative processes. Conversely, during self-rehabilitation and especially in clinically healthy rats that were administered milk phospholipids, an increase in the content of amino acids of this fraction in the liver parenchyma may reflect the activation of one-carbon metabolism and the preparation of the substrate base for glutathione synthesis and the restoration of energy and nitrogen homeostasis.

Thus, milk phospholipids probably contribute to the rebalancing of amino acids in the liver of animals from a catabolic, stressful state to an anabolic and cytoprotective one. This is consistent with the metabolic studies by R.P. da Silva *et al*. (2020), who emphasised that disturbances in the one-carbon metabolism of glycine/serine and glutamine content are a key link in the progression of fatty liver disease. Overall, these data are consistent with the results obtained regarding the intensive use of the hepatic fraction of glycine/serine/glutamine/ aspartic acid at the peak of tetracycline injury, which may be the result of switching between the acute stress phase and the repair phase and the enhancement of cytoprotective mechanisms, and its insufficient involvement in the recovery processes in animals during self-rehabilitation. At the same time, in clinically healthy rats that were administered milk phospholipids, their excessive mobilisation was established, which may have a positive effect on synthetic processes in hepatocytes.

In the case of the total fraction of methionine/glutamic acid in rat liver samples (Table 2), there were mixed but significant changes relative to the control. In the "Self-rehabilitation" group of rats, the total content of amino acids in this fraction increased by 30% relative to the control level, and in clinically healthy animals receiving milk phospholipids, it increased by 29%. In contrast, in sick rats in the "Tetracycline + "FLP-MD" dietary supplement" group, the quantitative parameters of this fraction decreased by 49% relative to the control. Thus, the administration of tetracycline hydrochloride in toxic doses to rats was accompanied by increased utilisation of the hepatic pool of methionine and glutamic acid, while in the self-recovery phase and against the background of the use of milk phospholipids in clinically healthy animals, moderate mobilisation of this group of amino acids was observed. This profile is consistent with the fact that methionine and glutamate are at the centre of one-carbon metabolism, S-adenosylmethionine and glutathione synthesis. A marked decrease in their total content in the liver of rats under tetracycline load can be interpreted as a sign of intensification of the methionine cycle and glutamate-dependent pathways in hepatocytes, which is important for maintaining the proper level of methylation and antioxidant protection in pathologically altered hepatocytes. Conversely,

an increase in the total fraction of methionine/glutamic acid during self-rehabilitation and against the background of the administration of milk phospholipids to clinically healthy animals is caused by their insufficient use in the above-mentioned processes.

At the same time, the quantitative parameters of the total alanine/tyrosine/threonine fraction in the liver of rats (Table 2) indicate similar trends in changes in animals from different experimental groups in relation to the control. In particular, in rats of the "Self-rehabilitation" group, the total content of amino acids of this fraction increased by 65% compared to the control, in animals of the "Tetracycline + "FLP-MD" dietary supplement" group – by 50%, and in clinically healthy rats that received milk phospholipids, it increased by 95%. Thus, the artificial administration of toxic doses of tetracycline hydrochloride to rats was accompanied by a marked increase in the content of the alanine/tyrosine/threonine fraction in hepatocytes, both under conditions of self-rehabilitation and in the case of prophylactic administration of milk phospholipids. Alanine is a key gluconeogenic substrate and marker of proteolysis, tyrosine belongs to aromatic amino acids sensitive to impaired liver metabolism, and threonine is involved in the synthesis of glycoproteins and can be converted to glycine/serine. A marked increase in this group of amino acids in tetracycline-induced injury can be interpreted as a consequence of increased breakdown of peripheral tissue proteins and reduced utilisation of these amino acids in liver dysfunction against a background of mitochondrial disorders. At the same time, a significant increase in the content of alanine/tyrosine/threonine in hepatocytes may reflect their mobilisation to ensure regenerative processes in hepatocytes, in particular activation of gluconeogenesis, protein synthesis and cell membrane repair, when these amino acids accumulate as important substrates for the energy and structural restoration of hepatocytes.

The established patterns are consistent with the findings of C. Fotakis *et al*. (2023), who demonstrated in a metabolomic study of blood serum from NAFLD patients that elevated concentrations of alanine and tyrosine are associated with greater disease severity, and that threonine levels in women are inversely related to the progression of steatosis. This emphasises that quantitative shifts in the total alanine/tyrosine/threonine fraction are a sensitive marker of liver metabolism, and their excessive accumulation in the liver against the background of milk phospholipid administration can be considered a reflection of activated but controlled adaptive-anabolic processes, in contrast to uncontrolled dysfunction in tetracycline injury.

Quantitative analysis of the total fraction of valine/norvaline/tryptophan in rat liver samples relative to the control showed a significant increase in the content of its components, especially pronounced in the "Self-rehabilitation" and "Preparation" groups. Thus, in rats of the "Self-rehabilitation" group, their level increased by 93% relative to the control. In animals of the "Preparation" group, which received milk phospholipids, the content of this amino acid fraction increased by 118% compared to the control. In rats from the "Tetracycline + "FLP-MD" dietary supplement" group, this indicator was finally restored. Such quantitative changes in the amino acid spectrum can be interpreted as the transition of the liver from an acute toxic phase to an anabolic-regenerative mode. Valine, as a representative of BCAA, is an important substrate for energy supply and protein synthesis, and tryptophan is important for the kynurenine and serotonin pathways, which modulate inflammation, microcirculation, and tissue regeneration. In tetracycline-induced liver cell injury in animals in the "Self-rehabilitation" group, the

use of these amino acids was more focused on the benefit of extrahepatic tissues and the immune response, so no significant changes were observed in the liver itself. In contrast, in sick animals in the "Tetracycline + "FLP-MD" dietary supplement" group, under conditions of phospholipid correction, there was an improvement in mitochondrial function and membrane integrity, which contributed to more active use of valine and tryptophan by hepatocytes, ensuring intensive protein synthesis, remodelling of the extracellular matrix and restoration of microvessels. A similar idea was supported by Y. Morine *et al.* (2022), who showed that in patients with hepatocellular carcinoma, the levels of essential amino acids, in particular valine and tryptophan, increased in both tumour liver tissue and blood serum before surgery, and decreased to normal values after liver resection. The authors interpreted this as a reflection of metabolic restructuring and a high demand for these amino acids in cells with enhanced growth and remodelling of liver tissue. In the context of the established changes in the model of tetracycline-induced liver injury, this allows to consider the increase in the total valine/norvaline/tryptophan fraction in sick rats in the case of self-rehabilitation as a marker of controlled regenerative-anabolic processes in the liver, and in clinically healthy animals against the background of milk phospholipid administration as a result of their active accumulation.

In the liver of rats, a consistent and statistically significant increase in the total leucine/norleucine/phenylalanine fraction relative to the control was observed in all experimental groups. In sick animals in the "Self-rehabilitation" group, the total content of these amino acids exceeded the control by 61%, in the "Tetracycline + "FLP-MD" dietary supplement" group by 86%, while in clinically healthy animals receiving milk phospholipids, it increased by 139% from control values. Thus, tetracycline-induced liver injury was accompanied by a pronounced accumulation of intracellular reserves of the amino acid groups leucine/norleucine/phenylalanine in hepatocytes, and with the additional administration of milk phospholipids to clinically healthy animals, these processes occurred more actively.

The established patterns should be interpreted taking into account that leucine, together with norleucine, are representatives of branched-chain amino acids with powerful anabolic and mTOR-activating potential, while phenylalanine belongs to aromatic amino acids that are sensitive to disturbances in liver metabolism. In acute tetracycline-induced hepatosis, the increase in the content of this mixed group of amino acids in the liver reflected a combination of enhanced proteolysis of peripheral tissues (the influx of leucine and phenylalanine from extrahepatic proteins) and limited oxidation of these amino acids by damaged mitochondria. In the self-rehabilitation group, and especially against the background of milk phospholipid administration, the further increase in the leucine/phenylalanine fraction content in the liver parenchyma should be interpreted as a switch to an anabolic-regenerative mode: phospholipids stabilise membranes and mitochondria, which facilitates the uptake of BCAA/AAA by hepatocytes and forms a reserve of substrates for intensive protein synthesis, cytoskeleton remodelling and synthesis of phospholipid components of membranes without signs of profound decompensation.

The classic clinical picture of amino acid imbalance in chronic liver disease was described by H. Enomoto *et al*. (2023), which contrasts to some extent with the results presented in this material. The authors showed that in patients with cirrhosis, blood serum is characterised by decreased levels of BCAAs (valine, leucine, isoleucine) and increased levels of aromatic amino acids, leading to a decrease in the

BCAA/AAA ratio and the BCAA/tyrosine ratio (BTR); low BTR correlates with the progression of fibrosis, sarcopenia, hypoalbuminaemia and a worse prognosis. Against this background, the unidirectional increase in BCAA and phenylalanine in the liver tissue of the experimental rats can be considered as a manifestation of adaptation at an early stage and intensification of regenerative processes in the modelling of acute tetracycline-induced injury and prophylactic phospholipid correction, rather than "classical" plasma amino acid imbalance characteristic of decompensated cirrhosis.

Thus, according to the results of experimental studies, certain patterns have been established regarding the quantitative redistribution in the hepatobiliary system of rats of most of the studied heterogeneous amino acid fractions depending on the functional state of the liver. This indicates significant changes in the intermediate metabolism of amino acids in the case of artificial reproduction of fatty hepatosis in rats, which is closely related to functional disorders of the liver, in particular the protein-synthesising ability of hepatocytes. The use of milk phospholipids in sick rats contributed to better utilisation of amino acids in synthetic and regenerative processes in hepatocytes, and even to the return of certain indicators to physiological limits: in bile – the leucine/norleucine/phenylalanine fraction, and in the liver – the valine/norvaline/tryptophan fraction. Overall, this indicates a positive corrective effect of the milk phospholipid-based dietary supplement, once again proving the close relationship between the structural state of hepatocytes and their metabolic activity.

Conclusions

The results of the experimental study demonstrated significant quantitative changes in heterogeneous amino acid fractions in the hepatobiliary system of rats depending on the functional state of the liver. In particular, in the bile of rats undergoing self-rehabilitation after acute toxic liver injury caused by tetracycline hydrochloride, a significant increase in the content of all studied amino acid fractions was observed. The most pronounced changes occurred in the fractions of arginine/ornithine/lysine (by 79%), glycine/serine/glutamine/aspartic acid (by 119%), methionine/glutamic acid (by 117%), and leucine/norleucine/phenylalanine (by 86%). At the same time, similar patterns were observed in the liver of animals in this group. Significant changes were recorded in the fractions of arginine/ornithine/lysine (by 85%), histidine/taurine/asparagine (by 132%), and valine/norvaline/tryptophan (by 93%). Among these, the arginine/ornithine/lysine fraction proved to be the most sensitive to the development of the acute form of hepatopathy, showing the most pronounced changes both in the bile and in the liver of the diseased animals. These patterns may indicate intensification of catabolic processes and blockage of the further utilisation of these amino acids in maintaining nitrogen homeostasis and an adequate level of antioxidant processes.

When milk phospholipids were administered to the diseased animals, opposite changes in the quantitative parameters of the amino acid profile of the hepatobiliary system were observed. In particular, in the bile of these animals the most pronounced decrease in content was recorded for the histidine/taurine/asparagine fraction (by 35%). At the same time, an increase in levels was detected only for two fractions: arginine/ornithine/lysine (by 26%) and alanine/tyrosine/threonine (by 43%). Restoration of quantitative characteristics was observed only for the leucine/norleucine/phenylalanine fraction. Similar patterns were detected in the liver samples of animals in this group. Under conditions of administration of milk phospholipids to diseased animals, the most sensitive changes

in the biliary system were observed in two amino acid fractions: alanine/tyrosine/threonine and leucine/norleucine/phenylalanine.

At the same time, in the bile of clinically healthy rats the most pronounced increase in concentration was observed for two amino acid fractions: histidine/taurine/asparagine and glycine/serine/glutamine/aspartic acid. Three fractions remained unchanged: alanine/tyrosine/threonine, valine/norvaline/tryptophan, and leucine/norleucine/phenylalanine. In liver samples from these animals, the greatest increases in content were detected in similar fractions: alanine/tyrosine/threonine (by 95%), valine/norvaline/tryptophan (by 118%), and leucine/norleucine/phenylalanine (by 139%). Elevated levels were also recorded for the histidine/taurine/asparagine fraction (by 80%) and the glycine/serine/glutamine/aspartic acid fraction (by 81%). Overall, this indicates specific features of the influence of milk phospholipids on certain links of nitrogen metabolism associated with the hepatobiliary system of mammals.

Thus, a pronounced corrective effect of milk phospholipids on intermediate amino acid metabolism was observed, as well as the possibility of using the most sensitive indicators as markers of the development of hepatopathology and of the effectiveness of therapy. In the future, it is planned to investigate substrates of the tricarboxylic acid cycle in order to clarify further metabolic pathways for the utilisation of free amino acids in the animal organism during the development of the drug-induced form of fatty hepatosis.

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Conflict of Interest

None.

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Амінокислоти біліарної системи в щурів за тетрацикліндукованого гепатозу і застосування фосфоліпідів молока

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Анотація. Актуальність дослідження зумовлена високою частотою медикаментозного ушкодження печінки в ссавців та недостатнім вивченням питання щодо порушень метаболізму амінокислот за гострої форми гепатопатії відповідного генезу. У зв'язку з цим, робота була спрямована на виявлення специфічних змін профілю вільних амінокислот у жовчі та печінці щурів за тетрацикліндукованого жирового гепатозу, а також на визначення коригувального ефекту дії фосфоліпідної фракції молока. Провідним підходом у дослідженні цієї проблеми був експеримент на лабораторних тваринах із моделюванням жирового гепатозу завдяки цитотоксичному ефекту дії тетрацикліну, з подальшим відбором зразків жовчі й печінки, наступним дослідженням спектру вільних амінокислот методом розподільної паперової хроматографії з нінгідриновим проявленням. Встановлено, що загальний патерн змін у гепатобіліарній системі хворих щурів свідчив про порушення процесів кон'югації жовчних кислот і мітохондріальну дисфункцію, а також виражене блокування використання вільних амінокислот у метаболічних процесах. При цьому, застосування щурам за тетрацикліндукованого жирового гепатозу фосфоліпідовмісної терапії активізувало використання внутрішньопечінкового резерву амінокислот, залучених до кон'югації жовчних кислот і антиоксидантного захисту, зокрема сірковмісних представників та сумарної фракції гліцину/серину/глутаміну. Визначено, що у жовчі цих щурів також зменшувався вміст гліцино- і тауриновмісних фракцій амінокислот за одночасного зростання рівня сумарних фракцій аланіну/тирозину/треоніну й аргініну/орнітину/лізину, що підтверджує актуалізацію в печінці процесів детоксикації із залученням вільних амінокислот. Виявлено, що застосування фосфоліпідної фракції молока зменшувало прояви порушення в проміжному обміні амінокислот та сприяло відновленню вмісту лейцино- і валіновмісних фракцій у жовчі й печінці хворих щурів. Встановлені закономірності мають практичну цінність для лабораторної діагностики та доклінічної оцінки гепатопротекторних засобів, оскільки амінокислотний профіль гепатобіліарної системи може бути використаний в якості чутливого індикатору тяжкості ушкодження печінки та як критерій у визначенні ефективності терапії

Ключові слова: хроматографія; фракції амінокислот; печінка; жовч; коригувальна терапія; тетрацикліну гідрохлорид; лабораторна діагностика