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Bilirubin derivatives in bile, blood, and liver of rats upon correction of experimental fatty hepatosis

Josef Illek*
Doctor of Veterinary Sciences, Professor
University of Veterinary Sciences Brno
61242, 1946/1 Palackého Str., Brno, Czech Republic
https://orcid.org/0000-0002-1374-7918

Stanislav Veselskyi
Doctor of Biological Sciences, Senior Researcher
Taras Shevchenko National University of Kyiv
01601, 64/13 Volodymyrska Str., Kyiv, Ukraine
https://orcid.org/0000-0001-9971-0333

Andrii Pototskyi
Postgraduate Student
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0003-2500-6647

Viktoriia Gryshchenko
Doctor of Veterinary Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0003-0622-6345

Yevdokiia Reshetnik
PhD in Biological Sciences
Educational and Scientific Center "Institute of Biology and Medicine"
Taras Shevchenko National University of Kyiv
01601, 64/13 Volodymyrska Str., Kyiv, Ukraine
https://orcid.org/0000-0003-1084-842X

Abstract. The relevance of this study lies in the lack of reliable information on the key aspects of the molecular mechanisms of fatty hepatosis, a common mammalian disease, and its dangerous

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*Corresponding author

complications, such as fibrosis, cirrhosis, and hepatocellular carcinoma. These circumstances substantially complicate the prompt diagnosis of this hepatopathology and reduce the effectiveness of treatment. The metabolism of the heme derivative bilirubin is unique and inherent in the liver, which undergoes transformation due to hepatocyte enzyme systems to form neutral compounds, such as glucurono-conjugates and sulphoconjugates, which undergoes changes and requires analysis in fatty hepatosis in animals. The aim of the study was to determine the characteristic changes in the content of unconjugated bilirubin and its sulphoconjugate, glucuronide, monoglucuronide, monoglucoside, and diglucuronide in the blood, bile, and liver of rats under the modelling of fatty hepatosis and the administration of rehabilitation therapy. For this, the thin-layer chromatography method was used. It was found that under tetracycline-induced fatty hepatosis in laboratory rats, the concentration of unconjugated bilirubin in the blood decreased by 39.3%, bilirubin glucuronide by 44.4%, and the total fraction of bilirubin monoglucuronide and monoglycoside by 78.9%. Oral administration of the milk phospholipid complex in the composition of the BAS "FLP-MD" to sick animals ensured the maintenance of these indicators at the control level. The content of bilirubin glucuronide and bilirubin diglucuronide decreased in the liver tissue of the treated rats. When these animals were administered a phospholipid-containing bioadditives, the level of unconjugated bilirubin in the liver tissue increased by 22.2%, but the content of its conjugated forms decreased. The content of all the studied bilirubin fractions decreased in the bile of sick rats during self-rehabilitation. The components of the BAS "FLP-MD" in fatty hepatosis in rats did not affect the reduction of unconjugated bilirubin in bile but eliminated the inhibitory effect of the modelled pathology on the content of all conjugated forms of bilirubin. The findings can be used as biochemical markers to monitor the pigment metabolism in the development of fatty hepatosis in mammals

Keywords: bile pigments; milk phospholipids; tetracycline hydrochloride; thin-layer chromatography; corrective therapy; fatty degeneration

Introduction

By the definition given in H. Tilg & M. Effenberger (2020) and Y. Liu *et al*. (2024), metabolic dysfunction-associated fatty liver disease (MAFLD) is a chronic progressive disease with a variable spectrum of clinical symptoms and varying severity of course: from steatosis to end-stage liver disease. Recent evidence suggests that total serum bilirubin levels are inversely correlated with the prevalence of metabolic syndrome, including metabolically associated fatty liver disease.

K. Moss *et al*. (2023) investigated the effect of bilirubin levels in patients on the complexity of the course and mortality stratified by the severity of fatty liver disease. As a result, the researchers noted that higher levels of bilirubin in the blood of patients with fatty liver disease, which was diagnosed in patients in combination with other pathologies, were associated with a lower risk of mortality from cardiovascular disease and malignant tumours.

The data presented by H. Han *et al*. (2024) confirm that a moderate increase in total bilirubin has antioxidant and anti-inflammatory properties in many metabolic diseases, including fatty liver disease, and reduces the risk of all-cause mortality. It turns out that a high concentration of total bilirubin in the blood within the physiological range is associated

with a reduced risk of severe fatty liver disease, a reduced probability of its transformation into more severe forms of liver damage and the resulting mortality. These observations prompt the investigation of possible mechanisms of changes in pigment metabolism in metabolically associated fatty liver disease. Attention is also drawn to the question of whether metabolically related fatty liver disease is always accompanied by some increase in the level of total, unconjugated and conjugated bilirubin in the blood and other biological fluids and tissues. The answers to these questions can only be obtained by conducting comprehensive studies with analysis of clinical statistics and experimental modelling of fatty liver.

T. Yamamoto *et al*. (2021) suggest that a risk factor for the development of fatty liver disease is the overuse of antibiotics. The active use of antibiotics in animals has led to the detection of their residues in food derivatives such as milk and dairy products. G. Tarantino & V. Citro (2024) argue that antibiotic residues in the environment affect the intestinal flora of animals and humans, leading to a variety of metabolic changes that may ultimately contribute to the onset and progression of fatty liver. Therefore, the use of models of artificial reproduction of fatty liver in laboratory animals by using high-dose antibiotics in experimental veterinary medicine is of great practical significance. There are currently no proven and highly effective pharmacological treatments for fatty liver disease. The lack of properly validated preclinical studies using successful and relevant experimental disease models is a major obstacle to the development of clinical treatments. D.G. Buyco *et al*. (2021) and H. Wang *et al*. (2023) suggest that to find therapeutic approaches to fatty liver, the full range of metabolic disorders should be identified in model experiments and, based on the findings obtained, promising drugs of various origins should be tested.

A.J. McGlinchey *et al*. (2022) emphasise that the danger of fatty liver disease is that it is not amenable to drug therapy, and in many cases, it progresses to steatohepatitis with further progression to cirrhosis. M. Yin *et al*. (2021) prove the effectiveness and feasibility of using drugs of natural origin with minimal side effects on the body in fatty liver of various aetiologies. This is how phospholipid-containing medicines and phospholipid-containing food bioadditives have proven themselves. D. Wupperfeld *et al*. (2022) explain the effectiveness of phospholipid-containing agents by their hepatoprotective effect. M. Mitrovic *et al*. (2022) note that different sources of phospholipids (oilseeds, seafood, egg yolk, etc.) are used to produce phospholipid-containing agents with hepatoprotective properties, which leads to different properties and the corresponding effectiveness of the use of drugs derived from them.

V.A. Gryshchenko *et al.* (2023) point out that milk is a valuable source of phospholipid complexes, the unique phospholipid spectrum of which is inherent in mammals, and therefore is expected to be the most effective for the designing of preparations for the medication of animals. Researchers have confirmed a substantial positive effect of the biologically active supplement (BAS) FLP-MD, based on a complex of milk phospholipids, on lipid metabolism in animals with calf dyspepsia and toxic hepatitis.

Therefore, the purpose of this study was to determine the content of unconjugated bilirubin and its conjugated forms in blood, bile, and liver tissue in laboratory rats under artificial reproduction of fatty hepatosis, and in the case of corrective therapy based on a complex of milk phospholipids in the composition of the BAS "FLP-MD".

Literature Review

According to A. De & A. Duseja (2022), fatty liver is characterised by the accumulation of lipid

vesicles in hepatocytes without pronounced changes resulting from inflammatory processes in the liver tissue – steatosis (fatty liver) or with lobular inflammation – steatohepatitis. Generally, fatty liver constitutes a wide range of structural and functional disorders, the pathogenesis of which does not involve hepatotropic viral infections, autoimmune processes, or clearly defined genetic risk factors.

According to the data presented in X. Shu *et al*. (2022), fatty degeneration of liver tissue is a fairly common hepatopathology in the world. It is believed that up to 25% of the world's population suffers from it, which is accompanied by hyperlipidemia, obesity, diabetes mellitus, and hypertension. At the same time, according to G. Tarantino & V. Citro (2024), all these disorders (obesity, insulin resistance, metabolic syndrome) are among the factors that provoke the development of fatty liver. N. Chalasani *et al*. (2018) associate the occurrence of this disease primarily with the development of metabolic syndrome and disorders of not only lipid metabolism but also closely related carbohydrate metabolism. H. Tilg & M. Effenberger (2020) and Y. Liu *et al*. (2024) point out the heterogeneous pathogenesis of fatty liver disease, in which metabolic disorder is the leading factor. Since fatty liver is associated with metabolic dysfunction, researchers have proposed to focus on identifying changes in various parts of metabolism in this pathology and to accelerate the search for new effective drugs and treatments.

Metabolic steatohepatitis is one of the hepatocellular diseases that can cause pigment metabolism disorders. T. Yamamoto *et al*. (2021) reported that in the case of severe hepatocellular necrosis, an increase in transaminase activity was observed, and hyperbilirubinaemia developed. In fatty liver disease, no jaundice was observed in the absence of inflammatory processes and significant disorders of bilirubin transport and biotransformation. As summarised by K. Moss *et al*. (2023), an increase in blood bilirubin levels and a decrease in its content in bodily fluids may result from changes in various stages of its metabolism. Specifically, due to excessive production of bilirubin (pathological haemolysis), there is a disturbance in the liver's absorption of bilirubin from the blood and an increase in the level of unconjugated bilirubin, followed by a disturbance in its conjugation caused by a defect in UDP-glucuronosyltransferase, and bile clearance disorders with increased levels of direct bilirubin due to defects in the transport proteins of the channel (apical) domain of the hepatocyte plasma membrane or the inability of bile to reach the small intestine in case of bile duct obstruction. Liver damage caused by any cause and with varying degrees of severity reduces the number of functionally fit hepatocytes, which can impair the absorption of unconjugated bilirubin from the blood plasma and reduce bilirubin clearance through the bile ducts. Therefore, deviations from the normal content of bilirubin and its derivatives can be detected in blood and bile tests even in the absence of jaundice accompanying metabolically determined liver diseases. Over three decades ago, H.S. Kaufman *et al*. (1991) also reported that elevated bilirubin levels in bile may be another pathogenic factor in the formation of gallstones, even in the absence of cholesterol supersaturation of the hepatic secretion. In some models of experimental fatty liver, an increase in blood bilirubin levels was also detected.

M.P. Boychak *et al*. (2022) concluded that an increase in the concentration of conjugated bilirubin in the blood is associated with hepatocellular lesions, not just cholestasis. Whereas changes in unconjugated bilirubin are more indicative of the ability to synthesise less toxic forms of bile pigment metabolites even in critical conditions. Lipid peroxidation and oxidative stress are considered the leading factors in the

development of fatty hepatosis of various aetiologies. N. Sadasivam *et al*. (2022) claim that cytochrome P450 2E1 (CYP2E1), which also produces reactive oxygen species (H_2O_2 and O_2^-), is a provoking factor in the development of fatty liver disease, as inhibition of CYP2E1 has been proven to protect liver cells in disorders regulation of lipid metabolism and oxidative stress. According to the researchers, serum bilirubin can also affect the manifestation of cytoprotective and antioxidant phenomena in the absence of hepatopathology. Serum bilirubin levels are inversely correlated with insulin resistance and the development of diabetes mellitus and cardiovascular disease in patients with fatty hepatitis. At the same time, the correlation between serum bilirubin levels and fatty liver is still unclear. It was suggested that elevated levels of bilirubin and its metabolites in the blood serum can reduce oxidative stress, inflammation, and inhibit the development of fatty liver. However, as noted by X. Shu *et al*. (2022), there was no independent association between serum bilirubin and fatty liver infiltration in non-obese patients. Thus, there is an urgent need to determine the causal relationship between bilirubin metabolism and excretion and fatty hepatosis in randomised controlled trials.

According to D.O. Melnychuk *et al.* (2014), the effectiveness of the use of milk phospholipids in the composition of the BAS "FLP-MD" for the correction of lipid metabolism disorders in toxic hepatitis by eliminating the structural and functional destabilisation of hepatocytes, especially their membrane systems, was established. The established effectiveness of a bioadditive containing milk phospholipids (FLP-MD) is promising for veterinary medicine, including in the development of drug-induced hepatosis. As discussions continue on the involvement of pigment metabolism and fluctuations in the content of bilirubin and its derivatives in the mammalian body in the pathogenesis of fatty liver disease, the question arises as to the specific features of changes in this metabolism when using milk phospholipids in the composition of the BAS "FLP-MD" in conditions of modelled fatty hepatosis.

Materials and Methods

A series of experimental studies was conducted at the scientific laboratories of the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine and the Educational and Research Centre "Institute of High Technologies" of Taras Shevchenko Kyiv National University in 2022-2023. To determine the effects of milk phospholipids in the BAS "FLP-MD" under conditions of simulated fatty hepatosis on the content of various forms of bilirubin: unconjugated bilirubin, bilirubin sulphate, bilirubin monoglucuronide, total bilirubin monoglucuronide and monoglucoside fractions, bilirubin diglucuronide in liver tissue, blood, and bile, an experimental study was conducted using male *Wistar* rats (32 animals) weighing 200 ± 50 g. Only males were used for the experimental studies because of the known sexual dimorphism of fatty liver disease in mammals (Martin-Grau & Monleon, 2023) and differences in clinical manifestation and prognosis in animals of different sexes. To weigh rats during the experiment, electronic scales ORION OS-0K22 (ORION ELECTRONICS LTD, Europe) were used.

The laboratory animals were maintained in a vivarium under standard state, with an indoor temperature of 22-24°C, 14-hour daylight hours, a standardised nutritious diet and free access to water. The experimental study was conducted according to the European convention for the protection of vertebrate animals used for research and other scientific purposes (1986) and the Law of Ukraine No. 3447-IV (2006). All surgical interventions required by the study plan in acute experiments were

performed according to the ARRIVE guidelines, following the guidelines of Council Directive 2010/63/EU (2010) on the protection of animals used in experiments.

The author's methodology (Gryshchenko *et al.*, 2019) was used to model fatty hepatosis in laboratory rats. For this, applying a soft silicone tubes, the animals were administered a 4% solution of tetracycline hydrochloride in the high dose: 250 mg/kg body weight daily intragastrically for 7 days. As a result, rats developed fatty hepatosis induced by high-dose tetracycline. Rats that were treated with intragastric administration of tetracycline hydrochloride were included in the "Self-rehabilitation" group (n = 8). Animals of the "Correction" group (n = 8) received an intragastric 1% solution of the BAS "FLP-MD", the main active ingredient of which is milk phospholipids, for 7 days before oral administration of the antibiotic and additionally for the next two days after its completion. The daily single dose of the BAS "FLP-MD" was 13.5 mg/kg body weight (Pat. 86516 UA, 2009). Animals that were administered intragastrically with the same volume of distilled water to the volume of antibiotic and bioadditive were in the "Control" group (n = 8). A separate group of animals stayed "intact" in terms of antibiotic use but received milk phospholipids as part of the BAS "FLP-MD" (n = 8). An essential component of the study was regular determination of the animals' body weight and recalculation of the dose of preparations according to its change during the experiment. In acute experiments on rats, bile samples were collected with a cannulated bile duct. The duration of the acute experiment was two hours. The day before the surgery experiment (acute experiment), we weighed the rats and deprived them of access to food. During the day before the acute experiment, animals of all experimental groups had free access to drinking water. The absence of feeding during the day before the acute experiment was conditioned by the need to avoid possible reflex regulatory effects of feed intake on biliary function and blood chemistry.

To perform the surgical intervention in the acute experiment, rats were intraperitoneally injected with sodium thiopental at 7 μg/100 g of body weight, which ensured immobilisation and anaesthesia of the animals. The anaesthetic effect occurred 3-5 minutes after the drug injection. Subsequently, the abdominal wall of the experimental animal was cut along the white line of the abdomen. After that, we cannulated the animal's bile ducts using a special plastic tubes (cannulas) to which a micropipettes were attached. The cannula was fixed in the bile duct using ligatures. Bile samples were distributed into separate plastic epidurals every 30 minutes during the acute experiment. As a result of the one-and-a-half-hour acute experiment, three individual half-hour bile samples were taken from each rat at the end of the acute experiment, blood and liver tissue samples were taken for further determination of the content of bilirubin and its derivatives. In samples of liver tissue, bile and whole heparin stabilising blood, after extraction according to the method Pat. 99031324 UA, MPK A61B5/14, the concentration of free and conjugated forms of bilirubin was identified and determined according to the method under Utility model patent 41602 Ukraine, IPC G01N 33/52, G01N 33/72 (Garnyk, 2009). For this, 50 μL of stabilising aqueous solution was added to the liver, bile, or blood samples obtained in the acute experiment (50 μL each). The stabilising solution contained 5.0% urea and 0.5% ascorbic acid. Subsequently, butanol and acetone were added to the mixture of the biological sample and stabilising solution in the following volume ratio: 2:2:7. The mixture was intensively mixed and centrifuged for 10 minutes at 3,000 rpm. A laboratory centrifuge OPN-8 (Ukraine) was used to centrifuge the resulting mixture. Acetone and butanol

were passively evaporated from the supernatant. Subsequently, 5 µL of the aqueous component from the biological sample extract was applied 2-4 times to pre-marked Silufol chromatographic plates (Czech Republic). The chromatographic separation of pigments extracted according to the method described above is also possible on FN-16 chromatographic paper from Filtrak (Czech Republic). The chromatographic separation of pigments from the processed liver, bile, or blood samples was performed in the chromatography chamber. For this, the side walls of the chromatography chamber were preliminarily lined with filter paper and a mixture of solvents was poured into the chamber: amyl acetic acid ester, concentrated acetic acid, propanol, water, ethylene glycol in a volume ratio of 21:10:5:5:3. The chromatography plates or chromatography paper with the applied samples were placed in the chromatography chamber. After the separation into fractions was completed, solvent was extracted from the chromatograms in a fume hood. The finished chromatograms were previewed in the visible and ultraviolet regions of the spectrum using special light sources (chromatoscope and lamp type A-FC-301) to activate the fluorescence of pyrrole groups in bilirubin and its derivatives. For quantification, the chromatograms were stained using a glass laboratory sprayer to apply the modified diazo reagent: 10 mL of diazo solution No. 1 and 0.25 mL of diazo solution No. 2 with 1.0 mL of formic aldehyde added to the mixture. Densitometry of 5 separate fractions of different forms of bilirubin metabolites was performed in the ultraviolet and visible spectral range using a densitometer DO-1M (Ukraine). The methodology used for the identification and distribution of pigments in bile and blood helped to determine unconjugated bilirubin, bilirubin sulphate, bilirubin monoglucuronide, the total fraction of bilirubin monoglucuronide and monoglucoside, and bilirubin diglucuronide in both of these biological fluids.

The results of the study were statistically processed using Statistica 5.0 software (StatSoft Inc., USA). According to the recommendations of N.B. Filimonova *et al*. (2005) used the t-Student's test to determine the statistical reliability of differences between the values of indicators of pigment metabolism in the studied groups. To test the nature of the distribution for normality, the study employed the Shapiro-Wilk test offered by Statistica 5.0 software. It was found that the concentrations of all five fractions of bilirubin derivatives obtained in the experiment were normally distributed. Differences between two comparable indicators from two different samples were defined as statistically reliable at $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Results and Discussion

To investigate the mechanisms of influence of the components of the BAS "FLP-MD" on the metabolism of bile pigments in the body of laboratory rats with artificial reproduction of fatty hepatosis, the content of bilirubin metabolites was determined in samples of blood, bile, and liver tissue. A study of the levels of various bilirubin metabolites in liver tissue, bile, and blood obtained from rats with artificially reproduced fatty hepatosis showed that both in the modelling of this hepatopathology and in case of using milk phospholipids in the composition of the "FLP-MD" bioadditive, the fractions of free and conjugated bilirubin underwent substantial quantitative changes.

The concentration of unconjugated bilirubin in the blood of animals with tetracycline-induced liver damage was 39.3% lower than the control value (Table 1). Oral administration of the milk phospholipid complex in the BAS "FLP-MD" to rats both under conditions of modelled pathology and in intact animals did not affect the concentration of unconjugated bilirubin in the blood, and therefore it did not differ from the control values.

Table 1. The content (mg%) of bilirubin derivatives in the blood of experimental rats under experimental conditions (M ± m, n = 8)

Bile pigment fraction of blood	Animal group			
	"Control"	"Self-rehabilitation"	"Correction"	"Healthy + BAS "FLP-MD""
Bilirubin unconjugated	0.56 ± 0.16	0.34 ± 0.10*#	0.55 ± 0.06	0.64 ± 0.09
Bilirubin sulphate	0.0087 ± 0.0045	0.0042 ± 0.0027	0.0085 ± 0.0021	0.0105 ± 0.0024
Bilirubin glucuronide	0.054 ± 0.016	0.030 ± 0.010*###	0.066 ± 0.007	0.057 ± 0.020
Bilirubin monoglucuronide and monoglucoside	0.019 ± 0.009	0.004 ± 0.002**###	0.020 ± 0.006	0.019 ± 0.006
Bilirubin diglucuronide	0.067 ± 0.02	0.047 ± 0.02	0.060 ± 0.01	0.063 ± 0.01

Notes: **P < 0.05, **P < 0.01, statistically significant differences between the indicators of the experimental groups ("Self-rehabilitation", "Correction", "Healthy + BAS "FLP-MD"" groups) and the control indicators ("Control" group); #P < 0.05, ###P < 0.001, the difference is significant between the values of indicators in the "Correction" and "Self-rehabilitation" groups*

Source*: developed by the author of this study*

Thus, the administration of the phospholipid complex in the form of the BAS "FLP-MD" to rats did not cause significant changes in the concentration of unconjugated bilirubin in the blood and slightly reduced it in the bile in intact animals. In rats with artificially reproduced fatty hepatosis, the administration of the BAS "FLP-MD" had an effective effect in terms of complete recovery to the control values of unconjugated bilirubin concentration in the blood and partial recovery in bile. Therewith, the concentration of unconjugated bilirubin in the blood of animals with modelled fatty hepatosis was substantially lower (by 39.3%) than the control value.

In most cases of liver pathology, researchers observed hyperbilirubinaemia due to an increase in the level of its unconjugated form in the blood. As pointed out by S. Gazzin *et al*. (2017), unconjugated hyperbilirubinaemia is associated with increased bilirubin production, impaired delivery from the blood to hepatocytes, and improper functioning of enzyme systems that ensure pigment conjugation in hepatocytes. Generally, unconjugated bilirubin can bind to biomembranes, which changes their fluidity, affects mitochondrial respiration, and the production of reactive oxygen species. Thus, this form of pigment acts on its own transport and enzyme systems, changing metabolism. The researchers emphasised that unconjugated hyperbilirubinaemia is not typical for fatty hepatosis, and its manifestations are recorded in the transition of steatosis to steatohepatitis with further progression of liver fibrosis. The experiment revealed that the level of unconjugated bilirubin in the blood of rats of the "Self-rehabilitation" group was characterised by a decrease compared to the control and the value of this indicator in the "Correction" group. Situations in which blood bilirubin concentrations are substantially reduced, as reported by Y. Aoki *et al*. (2022), also correlate with impaired renal function and pancreatic damage. In this regard, the authors of the publication emphasise the need for constant monitoring of the kidneys in case of low bilirubin levels. According to Y. Aoki *et al*. (2022), the search for therapeutic measures to normalise pathologically reduced levels of unconjugated bilirubin is a vital area of research to prevent hypobilirubinaemia-related disorders of body functions.

C.-C. Zhao *et al*. (2023) reported normal and slightly elevated levels of both unconjugated and conjugated bilirubin fractions in blood

plasma, which may suggest the anti-inflammatory and protective effects of unconjugated bilirubin and is an angioprotective factor. L. Vitek & C. Tiribelli (2021) noted that unconjugated bilirubin can be considered as a biologically active factor with antioxidant properties that reduces the risks of cardiovascular disease and atherosclerosis, metabolic syndrome and diabetes mellitus, autoimmune and neurodegenerative diseases. Bilirubin should be considered not only as a potentially dangerous end product of heme metabolism, but also as a kind of "yellow hormone" that affects metabolism, which requires thorough investigation. Conditions in which the level of bilirubin in the blood decreases can be dangerous for the course of many common diseases and increase the risk of complications. D.O. Melnychuk *et al*. (2014) observed a decrease in the content of unconjugated bilirubin (by 20%) in the whole blood of rats with simulated cadmium chloride injury at a dose corresponding to 1⁄50 LD_{50}.

We assume that the 39.3% decrease in the concentration of unconjugated bilirubin in the blood of diseased rats may be explained by the inhibition of albumin biosynthesis in hepatocyte damage and reduced haemoglobin production due to inhibition of the hematopoietic function of the red bone marrow under conditions of artificial reproduction of fatty hepatosis, as demonstrated in a previous study (Gryshchenko *et al.*, 2019).

The unconjugated bilirubin content stayed unchanged in the liver tissue of the "Self-rehabilitation" group rats (Table 2). On the contrary, the administration of the milk phospholipid complex in the BAS "FLP-MD" in intact animals caused a 27.6% decrease in the content of unconjugated bilirubin in liver tissue compared to the control. However, when using the BAS "FLP-MD"in animals with experimental fatty hepatosis, the concentration of unconjugated bilirubin in liver tissue was 22.2% higher than in the control.

Table 2. Content (mg%) of bile pigments in the liver tissue of experimental rats under experimental conditions (M ± m, n = 8)

Bile pigment fraction	Animal group			
	"Control"	"Self-rehabilitation"	"Correction"	"Healthy + BAS "FLP-MD""
Bilirubinunconjugated	3.69 ± 0.82	3.11 ± 0.51$^{\#\#\#}$	4.51 ± 0.65*	2.67 ± 0.56$^{*\#\#\#}$
Bilirubin sulphate	1.56 ± 0.44	1.78 ± 0.27$^{\#\#\#}$	0.95 ± 0.16**	1.77 ± 0.34$^{\#\#\#}$
Bilirubin glucuronide	13.24 ± 1.93	10.50 ± 1.77$^{*\#}$	9.16 ± 0.74***	13.16 ± 2.11$^{\#\#\#}$
Bilirubin monoglucuronide and monoglucoside	1.79 ± 0.33	1.51 ± 0.21$^{\#\#\#}$	0.99 ± 0.16***	1.84 ± 0.29$^{\#\#\#}$
Bilirubin diglucuronide	31.31 ± 4.76	25.09 ± 2.22$^{*\#\#\#}$	19.74 ± 2.06***	27.81 ± 3.06

Notes: **P<0.05, **P<0.01, ***P<0.001, statistically significant differences between the indicators of the experimental groups ("Self-rehabilitation", "Correction", "Healthy + BAS "FLP-MD"" groups) and the control indicators ("Control" group); # P < 0.05, ### P < 0.001, the difference is significant between the values of indicators in the rats of the "Correction" and "Self-rehabilitation" groups or the "Healthy + BAS "FLP-MD"" group*
Source*: developed by the author of this study*

The detected accumulation of unconjugated bilirubin in the liver tissue of the diseased animals of the "Correction" group may be associated with the intensification of pigment transport from the blood to hepatocytes under the influence of milk phospholipids in the BAS "FLP-MD" due to the stimulation of albumin synthesis. We also assume that the components

of the BAS "FLP-MD" can stimulate the uptake of unconjugated bilirubin by hepatocytes in conditions of simulated fatty hepatosis, which is a significant therapeutic property of the phospholipid bioadditive under study and its ability to accelerate repair processes in cell membranes.

Unconjugated bilirubin was found in all three bile samples obtained from rats with modelled pathology at a concentration lower than in the first, second, and third control samples by 29.5%, 26.0% and 29.9%, respectively (Table 3).

Table 3. The content (mg%) of unconjugated bilirubin in the bile of experimental rats under experimental conditions (M ± m, n = 8)

Experiment time, min	Animal group		
	"Control"	"Self-rehabilitation"	"Correction"
30	2.37 ± 0.33	1.67 ± 0.23***	1.90 ± 0.25**
60	2.30 ± 0.26	1.71 ± 0.27***	1.90 ± 0.26**
90	2.21 ± .27	1.55 ± 0.26***	1.81 ± 0.23*#

Notes: **P<0.05, **P<0.01, ***P<0.001, statistically significant differences between the indicators of the experimental groups ("Self-rehabilitation", "Correction", "Healthy + BAS "FLP-MD"" groups) and the control indicators ("Control" group); # P < 0.05, the difference is significant between the values of the indicator in the "Correction" and "Self-rehabilitation" groups*
Source*: developed by the author of this study*

When using the BAS "FLP-MD" in animals with modelled fatty hepatosis, the concentration of unconjugated bilirubin in all three bile samples remains lower than in control samples by 19.8%, 17.4%, 18.1%, respectively. However, when comparing the results in the "Self-rehabilitation" and "Correction" groups, it was found that the content of unconjugated bilirubin in the third bile sample of animals treated with a biologically active phospholipid supplement was 17% higher than the content of unconjugated bilirubin in the third bile sample of rats with modelled fatty hepatosis (Table 3). Therewith, the concentration of bilirubin sulphate in all three bile samples obtained from rats with experimental hepatopathology was substantially lower compared to the first, second, and third samples in the control, by 20.8%, 22.6% and 36.0%, respectively (Table 4).

Table 4. The content (mg%) of bilirubin sulphate in the bile of experimental rats under experimental conditions (M ± m, n = 8)

Experiment time, min	Group of rats		
	"Control"	"Self-rehabilitation"	"Correction"
30	1.06 ± 0.21	0.84 ± 0.13*	1.17 ± 0.18###
60	1.06 ± 0.18	0.82 ± 0.10**	1.00 ± 0.26###
90	1.00 ± 0.17	0.74 ± 0.11***	1.01 ± 0.23###

Notes: **P<0.05, **P<0.01, ***P<0.01, statistically significant differences between the indicators of the experimental groups ("Self-rehabilitation", "Correction", "Healthy + BAS "FLP-MD"" groups) and the control indicators ("Control" group); ### P < 0.001, the difference is significant between the values of the indicator in the "Correction" and "Self-rehabilitation" groups*
Source*: developed by the author of this study*

In case of administration of BAS "FLP-MD" in animals with experimental fatty hepatosis, in all three bile samples the concentration of bilirubin sulphate did not differ from the control and, respectively, was 39.3%, 36.0%, and 36.5% higher than the values of these indicators in the "Self-rehabilitation" group (Table 4). Therewith, there were no changes in the concentration of bilirubin sulphate in the blood samples of experimental animals with modelled hepatopathology and with the use of BAS "FLP-MD" (Table 1). Consequently, no accumulation of this bilirubin metabolite in the innner environment of the organism was observed.

Therewith, the study of the content of bilirubin sulphate in liver tissue showed that in the case of correction of the conditional liver status in animals with modelled fatty hepatosis, the concentration of this metabolite increased by 39.1% compared with the control (Table 2). As a result, it was found that under the influence of the components of the bioadditive in the conditions of modelling hepatopathology, the processes of formation of bilirubin sulphoconjugates by the enzyme systems of hepatocytes intensified, which positively characterises the neutralising function of the liver. D.O. Melnychuk *et al*. (2014) noted a comparable increment in the concentration of bilirubin sulphate in liver tissue (by 33%) after exposure of rats to toxic doses of cadmium chloride (for 14 days). According to the researchers, growing of the content of bilirubin sulphate in rat liver tissue after xenobiotic exposure may indicate an increase in the role of neutral pair compounds formation in case of compensatory intensification of detoxification processes.

Back in the 1990s, H.S. Kaufman *et al*. (1991) showed that bilirubin monoglucuronide in bile interacts with bile acids and is incorporated into mixed micellar bile particles. Thus, bilirubin monoglucuronide displaces cholesterol from bile micelles to cholesterol vesicles, which contributes to the formation of its monohydrate crystals, which provokes the development of cholelithiasis. This is not the only evidence of the pathogenetic significance of disorders of bilirubin glucuronide metabolism and excretion. Adverse drug reactions may be caused by changes in bilirubin metabolism and the development of jaundice. As noted by X. Yang *et al*. (2022), the leading mechanism appears to be the inhibition of bilirubin glucuronidation mediated by UDP-glucuronosyltransferase 1A1 (UGT1A1) and the factual competition of many compounds, particularly those containing phenolic hydroxyl groups, with the endogenous metabolite bilirubin. As a result, the activity of UDP-glucuronosyltransferase 1A1 towards bilirubin is inhibited and the formation of bilirubin glucuronide is reduced. According to E. Järvinen *et al*. (2022), glucuronic and sulphate metabolites are mostly unable to freely penetrate the plasma membranes of cells. Therefore, for their entry into hepatocytes and excretion into bile, proper activity of organic anion transporters of the basal domains of cell membranes and multidrug-resistant transporters of the apical domains of plasma membranes of liver cells is required. Therefore, changes in the concentration of bilirubin glucuronide in bile may be caused by the effect of hepatotropic factors on the transport systems of the hepatocyte plasma membrane, which ensure the excretion of this form of bilirubin to the bile ducts.

It was found that in animals with modelled fatty hepatosis, the concentration of bilirubin glucuronide in all three bile samples was significantly lower than in control bile samples. Specifically, in the first bile sample of the "Self-rehabilitation" group, the concentration of bilirubin glucuronide decreased by 34.0%, in the second sample – by 35.8%, and in the third sample – by 36.4% compared to the values of this indicator in the control group (Table 5).

Table 5. Bilirubin glucuronide content (mg%) in bile of experimental rats under experimental conditions (M ± m, n = 8)

Experiment time, min	Group of rats		
	"Control"	"Self-rehabilitation"	"Correction"
30	11.69 ± 1.89	7.72 ± 1.09***	10.96 ± 2.18###
60	11.81 ± 1.81	7.58 ± 1.27***	11.27 ± 2.43###
90	11.06 ± 1.82	7.03 ± .21***	10.52 ± 1.83###

Notes: **P<0.05, **P<0.01, ***P<0.01, statistically significant differences between the indicators of the experimental groups ("Self-rehabilitation", "Correction", "Healthy + BAS "FLP-MD"" groups) and the control indicators ("Control" group); ### P<0.001, the difference is significant between the values of the indicator in the "Correction" and "Self-rehabilitation" groups*
Source*: developed by the author of this study*

When comparing the "Self-rehabilitation" and "Correction" groups, it was found that the content of bilirubin glucuronide in animals administered the bioadditive exceeded that of rats with experimental fatty hepatosis. Thus, in the first sample of bile from the "Correction" group, the content of bilirubin glucuronide was 42.0% higher than in the bile from the "Self-rehabilitation" group, in the second sample – by 48.7%, and in the third sample - by 49.6%. Therewith, no statistically significant differences were observed between the bilirubin glucuronide content in the bile samples of the "Control" and "Correction" groups (Table 5). In the blood of animals with artificially reproduced fatty hepatosis, the concentration of bilirubin glucuronide decreased by 44.4% compared to the control values, and in case of administration of the bioadditive to both intact animals and rats with hepatopathology, this indicator was close to the control values (Table 1). In the liver tissue, the content of bilirubin glucuronide in both fatty hepatosis and the use of the BAS "FLP-MD" was substantially lower than in the control (Table 2). However, we believe that the mechanisms of this decline are different. Namely, in the context of artificial modelling of fatty hepatosis, a reduction in the content of bilirubin glucuronide in liver tissue is associated with a general decrease in bilirubin levels in the body due to the characteristic development of anaemia in this pathology (Gryshchenko *et al.*, 2019). The use of a phospholipid preparation intensifies the transport of bilirubin glucuronide from hepatocytes. Thus, the use of milk phospholipids in the BAS "FLP-MD" in sick rats has a pronounced restorative effect on the processes that determine the formation and flow of bilirubin glucuronide into the bile in animals with experimental fatty hepatosis.

It was found that in patients of the "Self-rehabilitation" group, in all three bile samples, the concentration of the components of the total bilirubin fraction monoglucuronide and monoglucoside was lower than in control samples by 26.5%, 31.2%, and 25.5%, respectively. Whereas oral administration of BAS "FLP-MD" in rats of the "Correction" group eliminated the inhibitory effect on the concentration of the components of the studied total bilirubin fraction monoglucuronide and monoglucoside (Table 6).

Table 6. The content (mg%) of the common fraction of bilirubin monoglucuronide and monoglucoside in the bile of experimental rats under experimental conditions (M ± m, n = 8)

Experiment time, min	Group of rats		
	"Control"	"Self-rehabilitation"	"Correction"
30	1.17 ± 0.15	0.86 ± 0.18***	1.01 ± 0.26
60	1.26 ± 0.24	0.88 ± 0.12***	1.02 ± 0.27

Table 6. Continued

Experiment time, min	Group of rats		
	"Control"	"Self-rehabilitation"	"Correction"
90	1.10 ± 0.14	0.82 ± 0.11***	0.94 ± 0.26

Notes: **P < 0.05, **P < 0.01, ***P < 0.01, statistically significant differences between the indicators of the experimental groups ("Self-rehabilitation", "Correction", "Healthy + BAS "FLP-MD"" groups) and the control indicators ("Control" group); # P < 0.05, the difference is significant between the values of the indicators in the "Correction" and "Self-rehabilitation" groups*
Source*: developed by the author of this study*

Thus, according to the described results of determining the effectiveness of the milk phospholipid complex in the BAS "FLP-MD" on the background of simulated liver pathology, it was noted that the endogenous formation and stimulation of bilirubin monoglucuronide and monoglucoside in bile were enhanced. This effect of the BAS "FLP-MD" was manifested in the fact that the content of bilirubin monoglucuronide and monoglucoside in the bile of these animals approached the level of control values. In the blood of animals with fatty hepatosis, a 78.9% decrease in the concentration of bilirubin monoglucuronide and monoglucoside was observed (Table 1), which is possible with the simultaneous development of anaemia in diseased animals, as described earlier (Gryshchenko *et al*., 2019). This leads to a decrease in the content of heme transformation derivatives in the blood in response to artificial modelling of liver pathology. Administration of the bioadditive to sick animals completely eliminates this depressive effect.

Under the conditions of artificial modelling of hepatopathology, due to the introduction of toxic doses of tetracycline hydrochloride ("Self-rehabilitation" group), the concentration of bilirubin diglucuronide in the bile of diseased animals decreased. Thus, in the first sample of bile from the "Self-rehabilitation" group, the content of bilirubin diglucuronide was 24.0% lower than in the control group, in the second sample – by 22.4%, and in the third sample – by 24.0% (Table 7).

Table 7. Bilirubin diglucuronide content (mg%) in bile of experimental rats under experimental conditions (M ± m, n = 8)

Experiment time, min	Group of rats		
	"Control"	"Self-rehabilitation"	"Correction"
30	21.23 ± 2.37	16.13 ± 1.92***###	19.90 ± 2.93
60	21.03 ± 1.86	16.31 ± 1.45***###	19.89 ± 2.74
90	19.81 ± 1.98	15.06 ± 1.52***###	19.44 ± 2.46

Notes: **** P < 0.01 statistically significant differences between the indicators of the experimental groups ("Self-rehabilitation", "Correction", "Healthy + BAS "FLP-MD" groups) and the control indicators ("Control" group); ### P < 0.001, ## P < 0.01, the difference is significant between the values of the indicator in the "Correction" and "Self-rehabilitation" groups*
Source*: developed by the author of this study*

Correction of the condition of animals with experimental fatty hepatosis with the help of BAS "FLP-MD" contributed to the restoration of bilirubin diglucuronide content in rat bile. Notably, both in the modelling of fatty hepatosis and in the group of animals treated with milk phospholipids as part of the BAS "FLP-MD", no significant changes in the concentration of bilirubin diglucuronide in the blood were detected (Table 1). Therewith, a

19.9% decrease in the concentration of bilirubin diglucuronide was recorded in the liver tissue of the diseased rats, which is probably due to the inhibition of hepatocyte enzyme systems of bilirubin glucuronidation. In the liver samples of animals treated with the BAS "FLP-MD" on the background of experimental fatty hepatosis, the content of bilirubin diglucuronide was 37.0% lower than the control values, which may be due to the intensive formation and excretion of this bilirubin metabolite into the bile ducts.

Hyperbilirubinaemia has always been noted as a typical sign of liver damage of various aetiologies and was considered a marker of the relevant severity of hepatopathology. To a large extent, the accumulation of bilirubin and its metabolites is caused by the activation of certain genetic transcription factors. S. Miura & A. Suzuki (2020) noted that bilirubin accumulation in blood and tissues is associated with over-expression of the transcription factor snail, which leads to impaired secretion of primary bile by hepatocytes and is further manifested by the development of steatohepatitis. Lipid accumulation in liver cells is caused by an excess of snail in steatohepatitis. Eventually, this causes pathological changes in the liver tissue, accompanied by a violation of bile acid homeostasis, the development of further mitochondrial dysfunctions and oxidative stress. It is important that snail induces epithelial-mesenchymal degeneration with subsequent transition to hepatocellular carcinoma. Thus, the overactivity of the above-mentioned transcription factor in knockout and mutant mice with the corresponding gene alleles correlates with fatty liver. Therefore, it is important to find all possible factors that would allow controlling the proper activity of snail in hepatocytes.

In a study of the correlations between total blood bilirubin and the severity of steatosis in patients with fatty liver, X. Ma *et al*. (2022) pointed out how excess snail may influence the complex relationship between bilirubin levels in the body's internal environment and the intensity of fatty liver. According to their findings, there is a negative correlation between the level of circulating bilirubin in the blood and the risk of developing fatty liver disease. Patients with fatty liver usually have low levels of direct bilirubin in their blood. Therewith, the level of uridine 5'-diphospho-glucuronosyltransferase (UGT1A1) activity positively correlated with the intensity of hepatocyte lipidosis. X. Ma *et al*. (2022) emphasised the need for further research to identify the relationship between fatty liver disease, circulating total bilirubin and UGT1A1 activity. According to the analysis of X. Shu *et al*. (2022), the leading risk factors for the development of fatty hepatosis include obesity, dyslipidaemia, diabetes mellitus with insulin resistance, and metabolic syndrome. Although the aetiology of fatty hepatosis is insufficiently understood, oxidative stress is considered a component of possible pathogenic mechanisms. X. Shu *et al*. (2022) emphasised that simultaneous changes in both direct and total bilirubin are unlikely to be associated with the risk of initial development of fatty liver. However, the risk of more severe fatty liver is inversely related to the level of bilirubin in the blood due to the inability to exercise its antioxidant properties in case of a decrease in its concentration in the body.

Thus, the decrease in the content of various bilirubin metabolites in tetracycline-induced fatty liver disease found in the present study indicates the possibility of a decrease in the body's adaptive capacity in the conditions of this type of liver damage with intensifying pathological changes in all physiological systems. Therewith, modelling of tetracycline-induced fatty liver in *Wistar* rats revealed characteristic changes in bile pigment metabolism

caused by the direct cytotoxic effect of high doses of the antibiotic on hepatocytes. According to the findings of chromatographic analysis of the content of bilirubin and its derivatives in the blood, liver, and bile of rats under artificial modelling of hepatopathology, substantial disorders of bile pigment metabolism were found, which is primarily associated with the level of structural and functional destabilisation of hepatocytes, specifically, their membrane systems. Experimental reproduction of this hepatopathology in laboratory rats helped to determine the corrective effectiveness of milk phospholipids in the composition of the BAS "FLP-MD", the use of which substantially improved the bile-forming and biliary functions of the liver and accelerated the recovery of pigment metabolism in diseased animals.

Conclusions

In conditions of tetracycline-induced fatty hepatosis in male *Wistar* rats, a significant decrease in blood levels of unconjugated bilirubin by 39.3%, bilirubin glucuronide by 44.4%, and the total fraction of bilirubin monoglucuronide and monoglycoside by 78.9% was observed compared to the corresponding control. The use of the milk phospholipid complex in the BAS "FLP-MD" in sick animals eliminated the above effects of pathological changes in the functioning of liver cells and the level of the studied unconjugated and conjugated forms of bilirubin in the blood. The ability of the BAS "FLP-MD" to stabilise the concentration of bilirubin and its derivatives in the blood at a physiological level in the development of experimental fatty hepatosis is one of the mechanisms of its hepatoprotective effect mediated by the reparative, antioxidant, and choleretic properties of milk phospholipids.

Therewith, in the liver tissue of rats with experimental fatty hepatosis, the content of bilirubin glucuronide decreased by 20.7% and bilirubin diglucuronide by 19.9% compared to the control. The use of phospholipid-containing BAS in rats with experimental fatty hepatosis led to a decrease in the content of bilirubin sulphate, bilirubin glucuronide in the liver tissue, bilirubin monoglucuronide, and the total fraction of monoglucoside and bilirubin diglucuronide, which is associated with the intensive excretion of conjugated forms of bilirubin into the bile ducts, and thus the choleretic effect of milk phospholipids. The accumulation of unconjugated bilirubin in the liver tissue of rats with the use of the BAS "FLP-MD" against the background of fatty hepatosis indicates the intensification of its removal from the blood by the transport systems of hepatocytes under the influence of the active substances of the bioadditive. The ability of the BAS "FLP-MD" to enhance the movement of unconjugated bilirubin from the blood to liver cells under conditions of modelled fatty hepatosis is another mechanism of its therapeutic effect, which prevents the development of intoxication and jaundice.

The revealed unidirectional changes – a decrease in the concentration of unconjugated bilirubin and its conjugated forms in bile both in tetracycline-induced fatty hepatosis and in the conditions of administration of BAS "FLP-MD" to sick rats – are conditioned by different mechanisms. Specifically, under conditions of experimental fatty hepatosis caused by inhibition of protein synthesis processes and associated anaemia, a decrease in the pigment content in bile was observed. We assume the fact that under the action of the BAS "FLP-MD", synthetic and energy-dependent transport and enzymatic processes of bilirubin biotransformation are enhanced, which, together with the simultaneous choleretic effect of the investigated phospholipid bioadditive, contributed to a decrease in the concentration of unconjugated and conjugated bilirubin in bile. The

established regularities allow recommending this bioadditive as a means of corrective therapy for the development of pigment metabolism disorders in animals with fatty hepatosis, as well as for the prevention of cholestasis and bilirubin encephalopathy.

In further experimental studies, it is planned to study the features of protein biosynthesis in the liver tissue of the liver in simulated toxic form of fatty hepatosis in animals.

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None.

Conflict of Interest

None.

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Похідні білірубіну в жовчі, крові та печінці щурів за корекції експериментального жирового гепатозу

Йозеф Іллек
Доктор ветеринарних наук, професор
Університет ветеринарних наук Брно
61242, вул. Палацького, 1946/1, м. Брно, Чеська Республіка
https://orcid.org/0000-0002-1374-7918

Станіслав Весельський
Доктор біологічних наук, старший науковий співробітник
Київський національний університет імені Тараса Шевченка
01601, вул. Володимирська, 64/13, м. Київ, Україна
https://orcid.org/0000-0001-9971-0333

Андрій Потоцький
Аспірант
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0003-2500-6647

Вікторія Грищенко
Доктор ветеринарних наук, професор
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0001-6601-1392

Євдокія Решетнік
Кандидат біологічних наук
Київський національний університет імені Тараса Шевченка
01601, вул. Володимирська, 64/13, м. Київ, Україна
https://orcid.org/0000-0003-1084-842X

Анотація. Актуальність дослідження полягає у відсутності на сьогодні достовірної інформації щодо провідних аспектів молекулярних механізмів виникнення поширеного в ссавців жирового гепатозу та його небезпечних ускладнень – фіброзу, цирозу та гепатоцелюлярної карциноми. Ці обставини істотно ускладнюють своєчасну діагностику зазначеної гепатопатології та знижують ефективність лікування. Унікальним і властивим для печінки є метаболізм похідного гему – білірубіну, який завдяки ензимним системам гепатоцитів зазнає трансформації з утворенням нейтральних сполук – глюкуронокон'югатів та сульфокон'югатів, що зазнає змін і потребує аналізу за жирового гепатозу в тварин. Мета дослідження полягала у визначенні характерних змін вмісту некон'югованого білірубіну та його сульфокон'югату, глюкуроніду, моноглюкуроніду, моноглюкозиду і диглюкуроніду в крові, жовчі та печінці щурів за моделювання жирового гепатозу й призначення відновлювальної терапії. Для цього використовували метод тонкошарової хроматографії. Встановлено, що за тетрациклініндукованого жирового гепатозу в лабораторних щурів відбувалося зменшення в крові концентрації некон'югованого білірубіну на 39,3 %, білірубіну

глюкуроніду на 44,4 %, сумарної фракції білірубіну моноглюкуроніду та моноглікозиду на 78,9 %. Пероральне введення хворим тваринам комплексу фосфоліпідів молока у складі біодобавки «FLP-MD» забезпечувало підтримання вказаних показників на рівні контролю. У тканині печінки хворих щурів знижувався вміст білірубіну глюкуроніду й білірубіну диглюкуроніду. У разі застосування цим тваринам фосфоліпідовмісної біодобавки в тканині печінки зростав рівень некон'югованого білірубіну на 22,2 %, але зменшувався вміст його кон'югованих форм. У жовчі хворих щурів за самореабілітації зменшувався вміст усіх досліджуваних фракцій білірубіну. Компоненти біодобавки «FLP-MD» за жирового гепатозу в щурів не впливали на зменшення вмісту некон'югованого білірубіну в жовчі, але усували інгібуючий ефект змодельованої патології на вміст усіх кон'югованих форм білірубіну. Отримані результати можуть бути використані в якості біохімічних маркерів для контролю за показниками пігментного обміну за розвитку жирового гепатозу в ссавців

Ключові слова: жовчні пігменти; фосфоліпіди молока; тетрацикліну гідрохлорид; тонкошарова хроматографія; коригувальна терапія; жирова дистрофія