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Drug-Induced Models of Cholestasis and Lysosomes

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1. Introduction

Cholestasis, caused by the interrupted excretion of bile, resulting in an accumulation of bile products in the body fluids is characteristic of many human liver diseases (Sherlock & Dooley, 1997). Experimental animal models of cholestasis allow the understanding of pathophysiological mechanisms involved and their clinical correlates (Chang et al., 2005; Chandra & Brower, 2004). The most common experimental models of intrahepatic cholestasis are estrogen-induced, endotoxin-induced and drug-induced cholestasis (Rodriguez-Garay, 2003). Drug-induced cholestasis was described during treatment by different drugs in medical clinic and in experimental research. In experimental medicine, α -naphthylisothiocyanate (ANIT) treatment has been extensively used, permitting to describe not only cholestatic alterations but also compensatory mechanisms. The animal model and transport protein studies are necessary for the progressive understanding of congenital and acquired human cholestasis, and regulatory mechanisms which operate on liver cells. Continuous bile formation is an important function of the liver, and bile is used as a vehicle for the secretion of bile acids and the excretion of lipophilic endo- and xenobiotics (Meier and Stieger, 2000; Hsien et al., 2006). Molecular and cellular mechanisms of intrahepatic cholestasis development are important for understanding of role of different factors in this process and effective therapy. Lysosomes are connected with bile secretion, however their role in cholestasis development is still not clear. Human bile revealed high activity of lysosomal enzymes (β -galactosidase, β -N-acetylglucosaminidase, acid phosphatase) which are suggested to be secreted from lysosomes localized in peribiliar zone of hepatocytes (Korolenko et al., 2007). We tested the hypothesis that impaired lysosomal secretion is related to cholestasis development. Earlier in some works it was shown that in mice and rats increased activity of lysosomal enzymes in bile was connected with their increased secretion.

The aim: to study the mechanism of intrahepatic cholestasis development and the role of lysosomes in bile secretion and cholestasis development. The following models of experimental cholestasis have been used and analyzed in our study: known model of intrahepatic cholestasis induced by α -naphthylisothiocyanate (ANIT) and lysosomotropic agent Triton WR 1339.

2. Drugs induced cholestasis and lysosomotropic agents

In inflammatory disorders such as sepsis, bacterial infections, viral hepatitis as well as toxic or drug-induced hepatitis, inflammatory cytokines can impair bile secretion (Jansen & Sturm, 2003; Paumgartner, 2006). Intrahepatic cholestasis of different pathomechanisms was shown to develop during treatment by several medical drugs (Krell et al., 1987), some of them possessed by lysosomotropic action. According to concept of de Duve et al. (1974) lysosomotropic agents are selectively taken up into lysosomes following their administration to man and animals (Schneider et al., 1997). The effects of lysosomotropic drugs studied *in vivo* and *in vitro* can be used as models of lysosomal storage diseases. These agents include many drugs still used in clinical medicine: aminoglycoside antibiotics, fluoroquin antimicrobial agents (ciprofloxacin), amoxicillin/clavulanic acid, phenothiazine derivatives, antiparasitic and anti-inflammatory drugs (chloroquine and suramin, gold sodium thiomalate) and cardiotoxic drugs like sulmazol (Schneider et al., 1997). Side-effects to these drugs can be caused partially by their lysosomotropic properties. In addition to drugs, other compounds to which man and animals are exposed (e.g., heavy metals, iron compounds, lanthan and gadolinium salts, some cytostatics) are also lysosomotropic. Liver cells, especially Kupffer cells, are known to accumulate lysosomotropic agents. We present our studies which evaluate lysosomal changes in the liver following administration of lysosomotropic agents in experimental animals, and relate them to toxic side-effects or pharmacological action, as was suggested earlier (de Duve et al., 1974). Common features of lysosomal changes include the overload of liver lysosomes by non-digestible material; increased size and number of liver lysosomes; inhibition of several lysosomal enzymes; secondary increase in the activity of some lysosomal enzymes; increased autophagy, and fusion disturbances.

2.1 Biochemical component of bile of intact CBA/C57BL mice

2.1.1 Experimental animals and methods used

All animal procedures were carried out in accordance to approved protocol and recommendations for proper use and care of laboratory animals (European Communities Council Directive 86/609/CEE). Experiments were performed on male CBA/C57BL/6 mice weighting 25-30 g (Institute of Physiology, Siberian Branch of Russian Academy of Medical Sciences, Novosibirsk). To reproduce the model of intrahepatic cholestasis corn oil solution of ANIT was injected intraperitoneally in a single dose of 200 mg/kg (0.2 ml per mouse) (Kodali et al., 2006). The animals were euthanized 24 h after ANIT injection. Triton WR 1339 (Ruger Chemical Co, USA) was dissolved in physiological saline solution and injected intraperitoneally in a single dose of 500 and 1000 mg/kg. The mice were decapitated 24 and 72 h after Triton WR 1339 administration (when significant accumulation of detergent occurred inside of lysosomes). Oil solution of hepatotoxin carbon tetrachloride (CCl₄, 50 mg/kg) was administered intraperitoneally, as a single injection. The mice were used in experiment 24, 48 and 72 h after CCl₄ intoxication. The separate group of animals received Triton WR 1339 two hours before administration of CCl₄ in a dose indicated (combined treatment). The mice were deprived of food (15 h before decapitation), but received water *ad libidum*. Blood serum was obtained by centrifugation of samples at 3000 g, +4° C for 20 minutes, using Eppendorf 5415 R centrifuge (Germany). The bile was taken from gall bladder using a microsyringe; the bile samples from 5 mice were combined to measure the lysosomal enzyme activity.

Serum and bile alanine transaminase (ALT) activity (serum marker of hepatocyte cytolysis) was measured with help of commercial Lachema Diagnostica kits (Czech Republic). Activity of alkaline phosphatase and γ -glutamyltransferase (GGTP) were measured using Vital Diagnostics kits (Saint Petersburg, Russia). The increase of serum activity of alkaline phosphatase and GGTP reflects the development of intrahepatic cholestasis in mice. Fluorescent methods were used to measure the following lysosomal enzyme activity: β -D-galactosidase (high specific activity in hepatocytes) in the bile and serum using 4-methylumbellipheryl- β -D-galactopyranoside as a substrate (Melford Laboratories Ltd, Suffolk, UK); β -hexosaminidase or β -N-acetylglucosaminidase (4-MUF-2-acetamido-2-deoxy- β -D-glucopyranoside, Melford Laboratories Ltd, Suffolk, UK) and chitotriosidase - 4-MUF- β -D-N,N', N''-triacetylchitotrioside (Sigma). Fluorescence of samples was measured on Perkin Elmer 650-10S spectrofluorometer at the excitation and emission wavelengths of 360 and 445 nm, respectively. The results were expressed in μ mol methylumbelliferone released per liter in min. The data obtained were analyzed statistically, using Student t-test; the differences between the means were significant at $p < 0.05$.

Electron microscopic study of liver cells was provided according to method described by Trout and Viles (1979).

2.1.2 Composition of bile and serum of intact mice

In bile of intact CBA/C57BL mice comparatively to serum the total protein was significantly decreased (more than 60-times), as well as albumin concentration (Table 1); alkaline phosphatase activity in bile is significantly lower as in serum, whereas similar level of ALT activity was noted; AST activity in bile was reduced about twice comparatively to serum of the same animals (Table 1). Activity of lysosomal enzyme β -D-galactosidase was similar in serum and bile of mice (Table 1). One can conclude that murine bile possessed relatively high activity of lysosomal enzyme as well as ALT and AST activity.

Index	Serum	Bile
Total protein, g/L	65.8 \pm 2.96	1.140 \pm 0.006
Albumin, g/L	30.4 \pm 0.70	0.530 \pm 0.005
ALT activity, U/L	57.2 \pm 1.67	45.4 \pm 1.00
AST activity, U/L	201.0 \pm 10.60	113.6 \pm 14.80
Alkaline phosphatase activity, U/L	268.0 \pm 8.16	48.0 \pm 6.00
β -D-galactosidase activity, μ mol MUF/L per h	12.30 \pm 1.33	15.6 \pm 0.36

Table 1. Concentration of total protein, albumin and activity of enzymes in serum and bile of Intact CBA/C57BL/6 mice

2.2 ANIT as a model of intrahepatic cholestasis

Experimental models of cholestasis in laboratory animals allow us to evaluate the pathophysiological and molecular mechanisms of this disorder. Some similarities were revealed between experimental cholestasis in animals and cholestasis in patients (Moritoki et al., 2006; Dold et al., 2009). Experimental cholestasis in rats and more rarely in mice can be induced by not only common bile duct ligation, but also by treatment with estrogens, endotoxins, and various medical products. Intrahepatic cholestasis induced by ANIT is a convenient model for studies of hepatocyte injury and compensatory mechanisms in

spontaneous recovery of liver function. Changes in hepatocyte transport proteins and dysregulation of bile secretion were revealed in mice with experimental cholestasis (Hsien et al., 2006). Cholestasis in mice and rats was observed in the early period after ANIT treatment (4-8 h), reaching maximum 24 and 48 h after ANIT administration. The severity of cholestasis depended on the dose of ANIT, route and regimen of treatment, and basal level of cytokines (tumor necrosis factor- α , TNF- α). Repeated administration of ANIT to mice is accompanied by the development of biliary cirrhosis; the mechanism of this phenomenon remains unclear (Ferreira et al, 2003).

ANIT is a known hepatotoxic agent that causes acute cholestatic hepatitis with infiltration of neutrophils around bile ducts and necrotic hepatocytes (Kodali et al., 2006; Luyendyk et al., 2011). In this work lysosomal enzyme activity in the bile and blood serum was compared in mice with known experimental intrahepatic cholestasis model induced by ANIT and by lysosomotropic agent Triton WR 1339. ANIT (Aldrich, USA) was administered to mice as an oil solution i.p., single, in a dose of 200 mg/kg. The increase in the concentration of total bilirubin (86.2 ± 8.6 versus 9.9 ± 0.8 mmol/L in the control, $p < 0.001$) and conjugated bilirubin (45.6 ± 4.3 versus 6.10 ± 0.39 mmol/L in the control, $p < 0.001$) in blood serum reflected the development of cholestasis in mice. The development of intrahepatic cholestasis was confirmed by the increase in activity of alkaline phosphatase and γ -glutamyltransferase (GGTP) in blood serum. β -Galactosidase, β -hexosaminidase and chitotriosidase activity significantly increased in the bile, but decreased in the serum of mice after treatment with ANIT (Table 2). Our results indicate that intrahepatic cholestasis is manifested in increased secretion of lysosomal glycosidases into the bile. It was suggested that bile components can aggravate damage to liver cells by affecting the processes of hepatocyte apoptosis and necrosis. ANIT dramatically increased serum GGTP activity (Figure 1), indicating severe cholestasis development according to ALT and AST (Figure 3); there was no change of urine creatinine and uroprotein concentrations (Figure 2). Our results indicate that hepatocyte injury and cholestasis are observed 24 h after ANIT treatment. Total cholesterol concentration increased in blood serum (Figure 4A). Triglyceride level increased, while cholesterol concentration decreased in the bile (Figure 4B). Cholesterol serves as a common precursor of bile acids. The ANIT-induced increase in serum cholesterol concentration reflects lipid metabolism disorder, which is manifested in lipid infiltration of the liver and cholestasis.

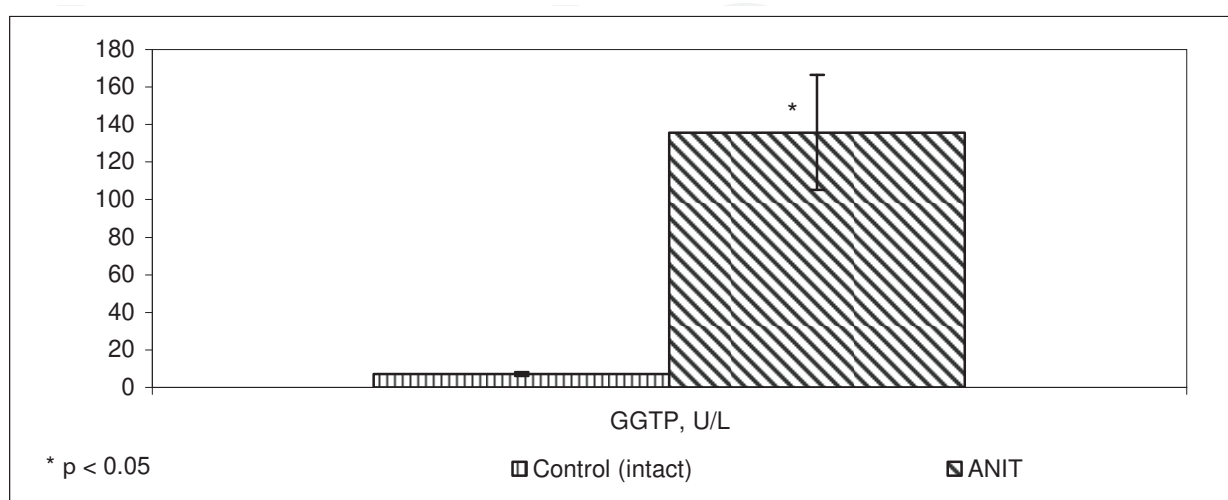


Fig. 1. Effect of ANIT on serum GGTP activity in mice

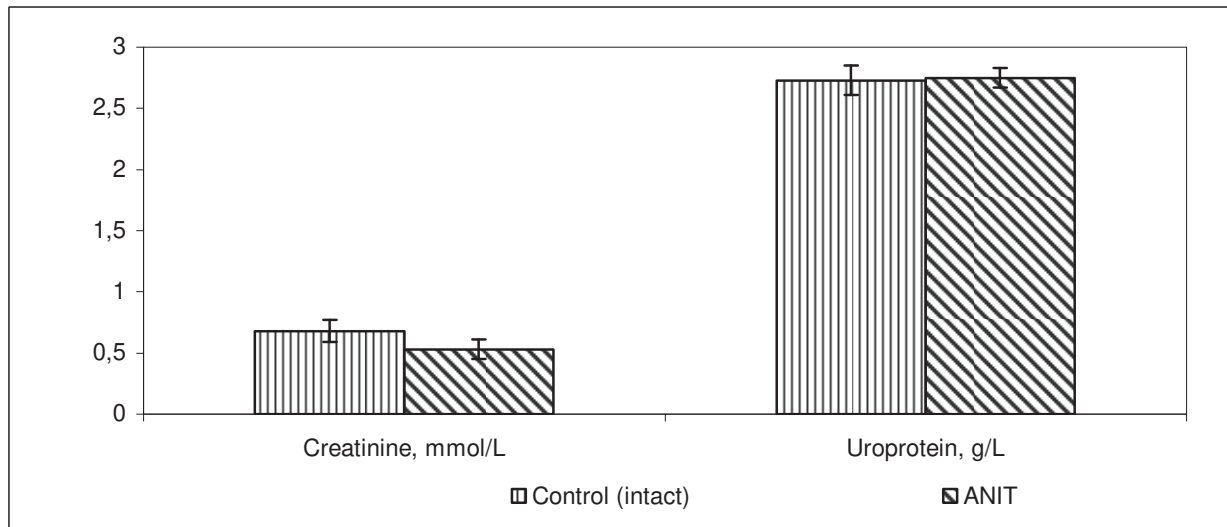


Fig. 2. Effect of ANIT on concentration of creatinine and uroprotein in urine of mice

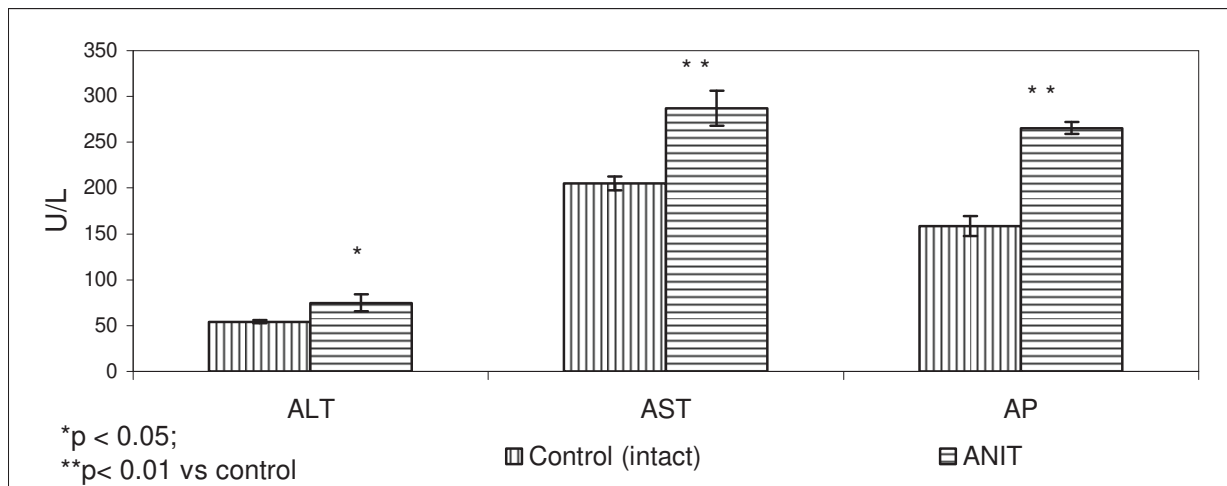


Fig. 3. Effect of ANIT administration in mice on serum ALT, AST and alkaline phosphatase activity

2.2.1 Electron microscopic study of liver (ANIT)

In situ, the increase of gall bladder size of mice was shown (10 μ L bile versus 5 μ L in intact animals); the color of bile was bright yellow. In general, significant ultrastructural changes in liver cells and microcirculatory region were noted. There was heterogeneity among hepatocyte injury: cells with normal ultrastructure and hepatocytes revealing dystrophic changes were observed. Sinusoids were enlarged, the sinusoidal surface of cells was smooth and revealed only small amount of microvilli. The secretory cell function connected with bile formation was suppressed. The significant dilatation of bile capillaries (Figure 5, 6) was observed, the bile duct had increased oval shape; numerous membranous material was often noted inside of bile ducts. These electron microscopic data confirmed intrahepatic cholestasis development. In general, liver cell damage included the enlargement of sinusoids and intercellular spaces; enlargement of bile ducts; suppression of protein synthesis in hepatocytes and their dystrophic changes.

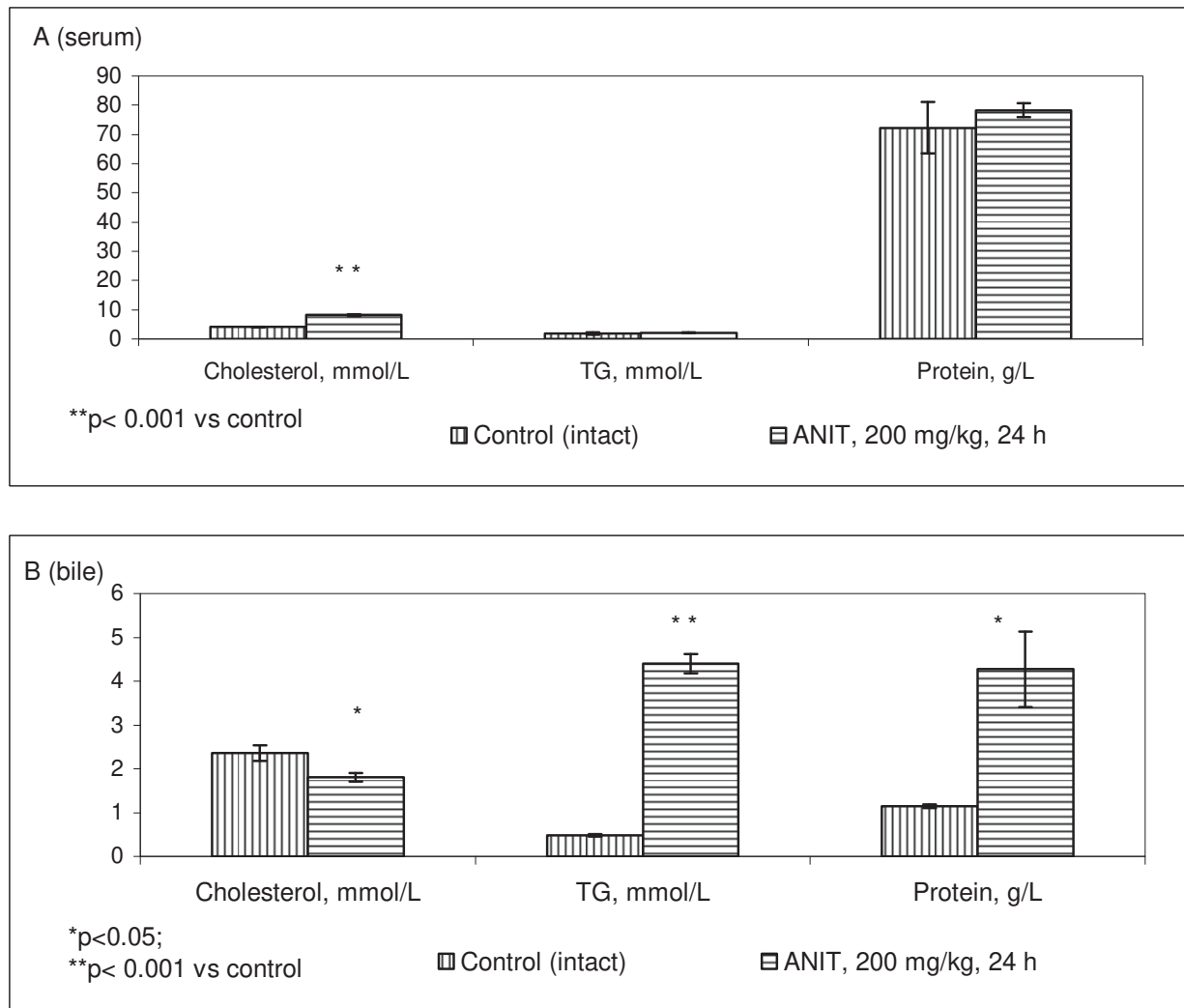


Fig. 4. Concentration of cholesterol, TG and total protein in serum (A) and bile (B) of mice with ANIT administration

2.3 Triton WR 1339t and cholestasis

2.3.1 Triton WR 1339 as a lysosomotropic drug

Triton WR 1339 is non-ionic detergent widely used for isolation of lysosomes (triton-filled lysosomes, tritosomes) in cellular biochemistry (de Duve et al., 1974; Trout and Viles, 1979). Earlier Triton WR 1339 (Tyloxapol) shortly used as a medical drug for treatment of bronchitis. *In vivo* in the doses of 300-1000 mg/kg Triton WR 1339 is taken up by liver cells (mainly by macrophages) and accumulated inside of lysosomes during long period, up to 60 days after the single administration to rats and mice (Schneider et al., 1997). Simultaneously, Triton WR 1339 was shown to induce significant hypercholesterolemia and, especially, hypertriglyceridemia in rats and mice and other laboratory animals, in a dose-dependent manner (Abe et al., 2007; Korolenko et al., 2011). Triton WR 1339 administration to mice (500 mg/kg) was shown to induce significant lipemia sharply increasing both the total lipoprotein-cholesterol and total lipoprotein-triglyceride concentrations in serum of mice. However, the increase in total lipoprotein-triglycerides was much more dramatic (about

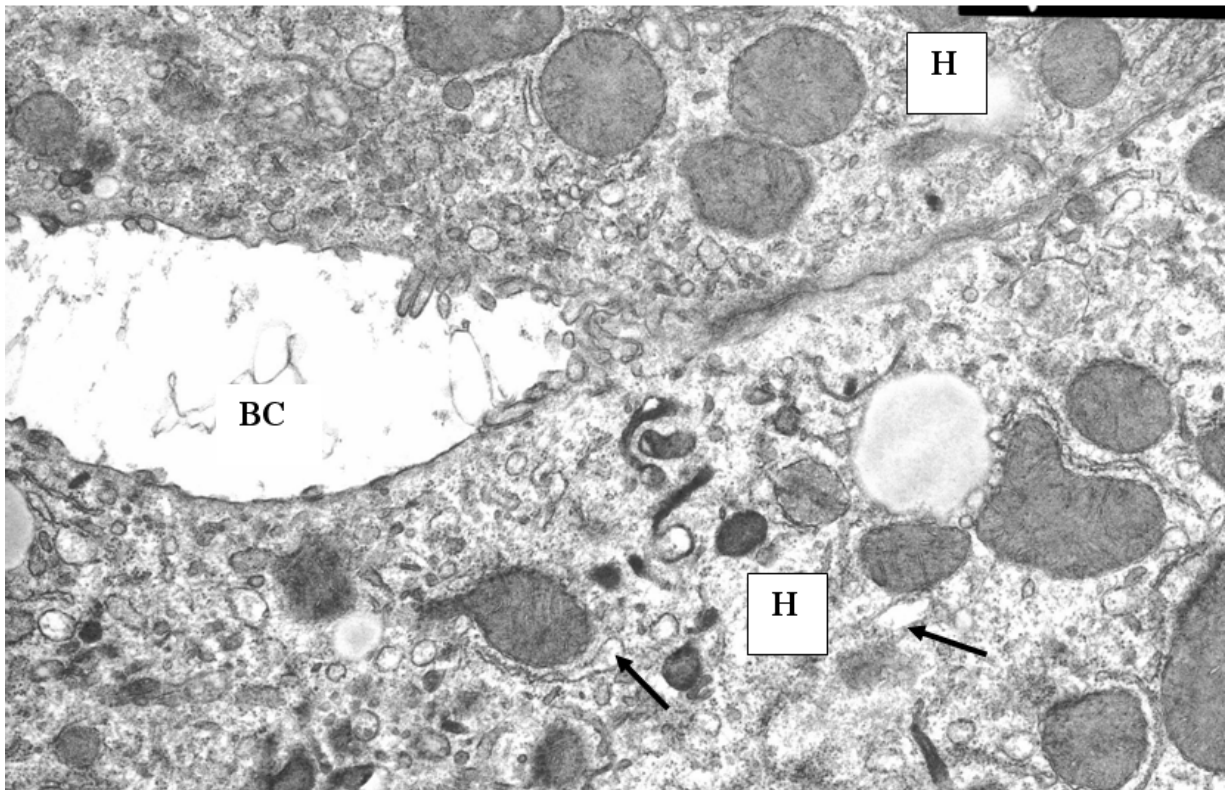


Fig. 5. Hepatocytes of mice with ANIT administration. Enlarged bile capillary (BC), hepatocytes (H) contains enlarged cisterns of endoplasmic reticulum (arrows). X 20 000.

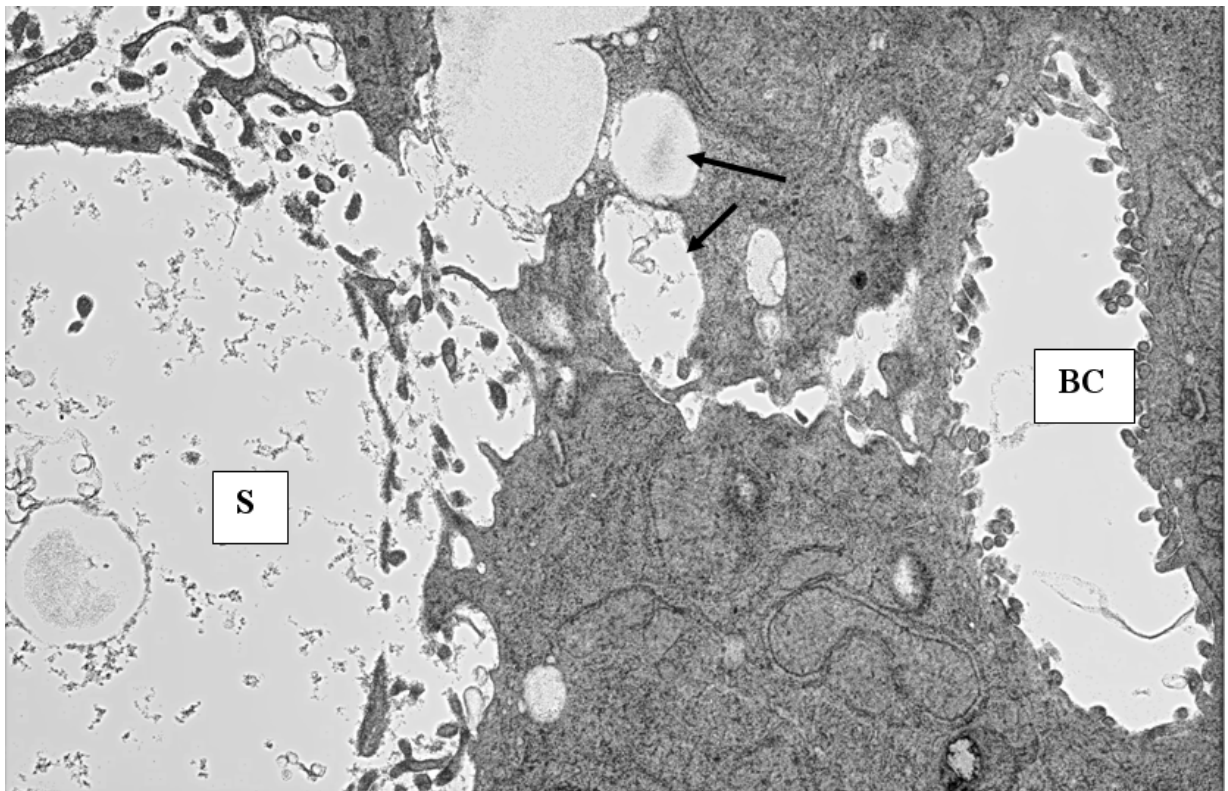


Fig. 6. Electronogram of liver of mice with ANIT administration. Enlarged sinusoid (S) and enlarged bile capillary (BC), hepatocytes contains large vacuoles (arrows). X 20 000.

10-times) than the elevation of the total lipoprotein-cholesterol (about 5-times), relative to control (intact) mice (Korolenko et al., 2011). The Triton WR 1339 hyperlipidemic mouse model is a widely used model for inhibition of lysosomal lipolysis and evaluation of different hypolipidemic drugs (Abe et al., 2007). It was shown that accumulation of Triton WR 1339 occurred simultaneously with some serum lipoproteins inside of liver lysosomes. Triton WR 1339 administration to mice and rats was shown to increase cholesterol synthesis (fatty acids precursor) in liver cells. Possibly, significant hypercholesterolemia induced by Triton WR 1339 administration to mice was related to cholestasis development (presented below), which is not known enough in literature.

2.3.2 Biochemical characteristic of Triton WR 1339-induced cholestasis in mice

Triton WR 1339 (Ruger Chemical Co., USA) was administered in mice as a single injection, i.p., in a dose of 100 mg/100 g, the appropriate animals received the same volume of saline solution. Mice were decapitated 24, 48, 72 h after the single injection. CBA/C57BL/6 male mice of weight 26.8 ± 0.40 g were used. Single administration of Triton WR 1339 (500 mg/kg) in mice was followed by significant increase in concentrations of total cholesterol, TG in serum without changes of the total protein concentration (Figure 7 A). In bile, on the contrary there were no changes of cholesterol and TG level (Figure 7 B); the increase of the total protein concentration was noted in bile.

In mice treated by Triton WR 1339 (500 mg/kg) intrahepatic cholestasis development was confirmed by increased level of serum alkaline phosphatase and GGTP activity (Figure 8). The size of gall-bladder *in situ* was increased, the bile had yellow color. According to electron microscopy study of liver cells numerous triton-filled lysosomes were observed in non-parenchymal (especially in Kupffer cells) and parenchymal cells (Figure 9, 10); enlargement of bile ducts was noted as well as other morphological signs of intrahepatic cholestasis. However, in general the enlargement of bile ducts was shown in a less degree as compare to ANIT-induced model of cholestasis.

2.3.3 The comparison of serum and bile lysosomal enzymes in Triton WR- and ANIT-induced models of cholestasis

The following lysosomal enzymes were compared in two cholestasis models studied: β -D-galactosidase, β -hexosaminidase and chitotriosidase. In intact mice we observed similar activity of β -D-galactosidase in serum and bile, whereas activity of β -hexosaminidase was significantly higher in serum (about 40-times) as compare to bile, as well as chitotriosidase activity (about 6-times higher in serum comparatively to bile) (Table 2). Both models of cholestasis were characterized by decreased β -D-galactosidase and β -hexosaminidase activity in serum (Table 2). However, serum chitotriosidase activity increased in ANIT model and decreased in Triton WR 1339-induced cholestasis (Table 2). In bile all lysosomal enzymes studied - β -D-galactosidase, β -hexosaminidase and chitotriosidase activity were significantly increased (Table 2), especially β -D-galactosidase (about 10-times higher in bile as compare to serum in Triton WR 1339-treated mice (Table 2). In ANIT model we observed higher chitotriosidase activity in bile as compare to Triton WR 1339.

So, both models studied were characterized by increased level of lysosomal enzymes in bile.

Group/Index	Serum	Bile
Intact (β -D-galactosidase, MUF/L per h)	12.3 \pm 1.33	12.30 \pm 1.63
Triton WR 1339 (β -D-galactosidase, , MUF/L per h)	6.1 \pm 0.60 *	117.3 \pm 1.40*
ANIT (β -D-galactosidase, , MUF/L per h)	4.6 \pm 0.32*	22.2 \pm 0.34*
Intact (β -hexosaminidase, μ mol MUF/L per min)	7.9 \pm 0.95	0.54 \pm 0.07
Triton WR 1339 (β -hexosaminidase, μ mol MUF/L per min)	4.0 \pm 0.47*	2.5 \pm 0.20*
ANIT (β -hexosaminidase, μ mol MUF/L per min)	1.52 \pm 0.05*	1.57 \pm 0.10*
Intact (chitotriosidase, μ mol MUF/L per min)	20.50 \pm 2.79	3.40 \pm 0.28
Triton WR 1339 (chitotriosidase, μ mol MUF/L per min)	8.90 \pm 0.81*	11.85 \pm 0.40*
ANIT (chitotriosidase, μ mol MUF/L per min)	35.0 \pm 2.79*	17.50 \pm 3.56*

Table 2. Effect of Triton WR 1339 and ANIT on activity of lysosomal enzymes in serum and bile of mice

*p < 0.01 vs control (intact mice)

Triton WR 1339, 1000 mg/kg, 72 h. ANIT, 200 mg/kg, 24 h. MUF- methylumbelliferyl.

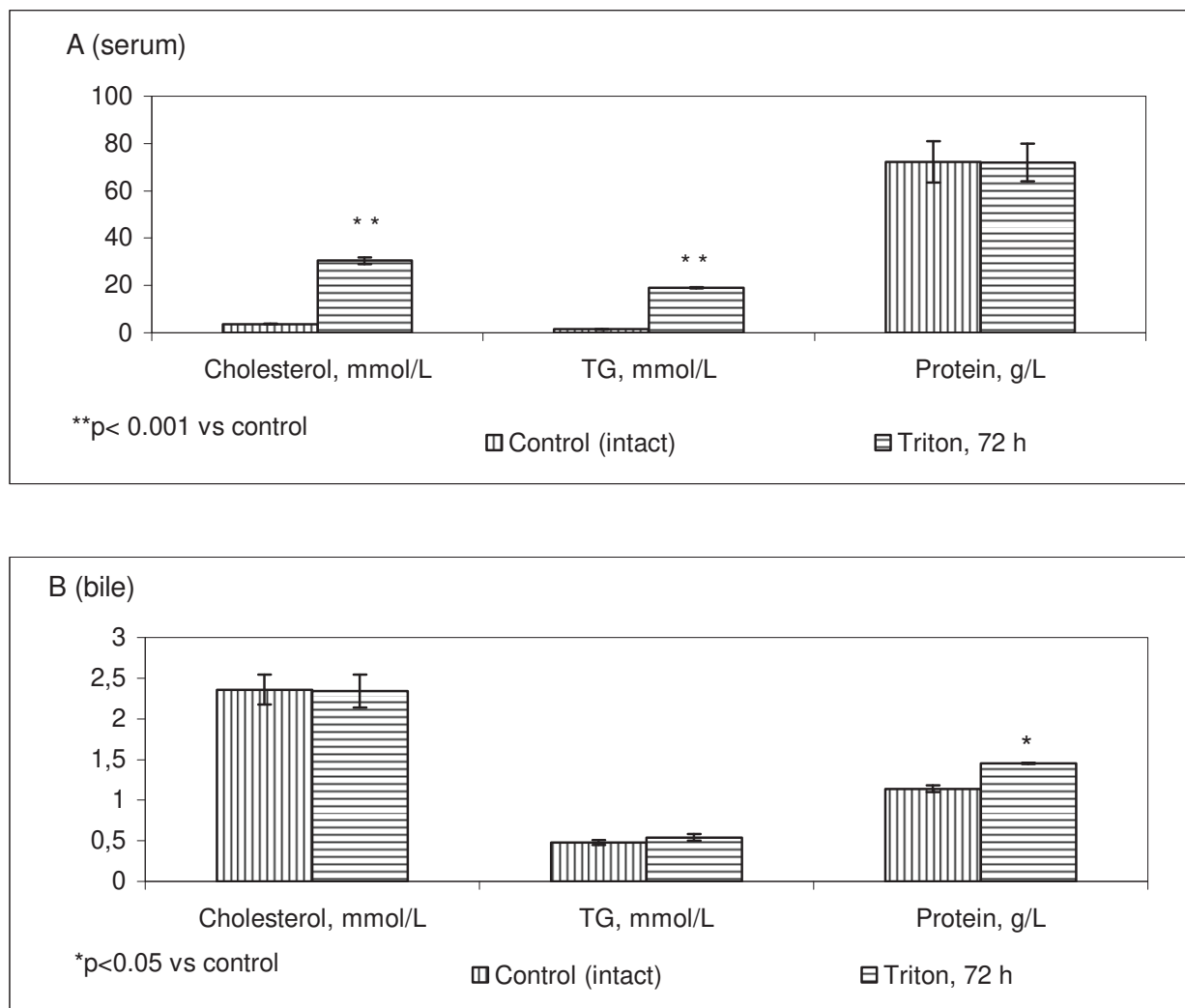


Fig. 7. Effect of Triton WR 1339 administration in mice on serum (A) and bile (B) cholesterol, TG and total protein concentration

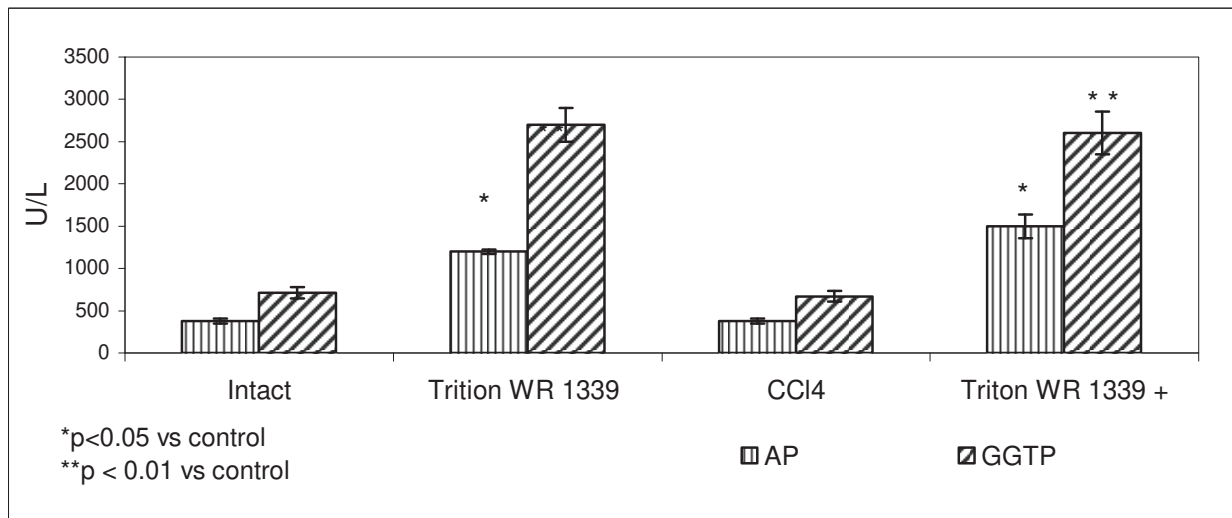


Fig. 8. Effect of Triton WR 1339 on serum alkaline phosphatase (AP) and GGTP activity in intact mice and with acute toxic hepatitis

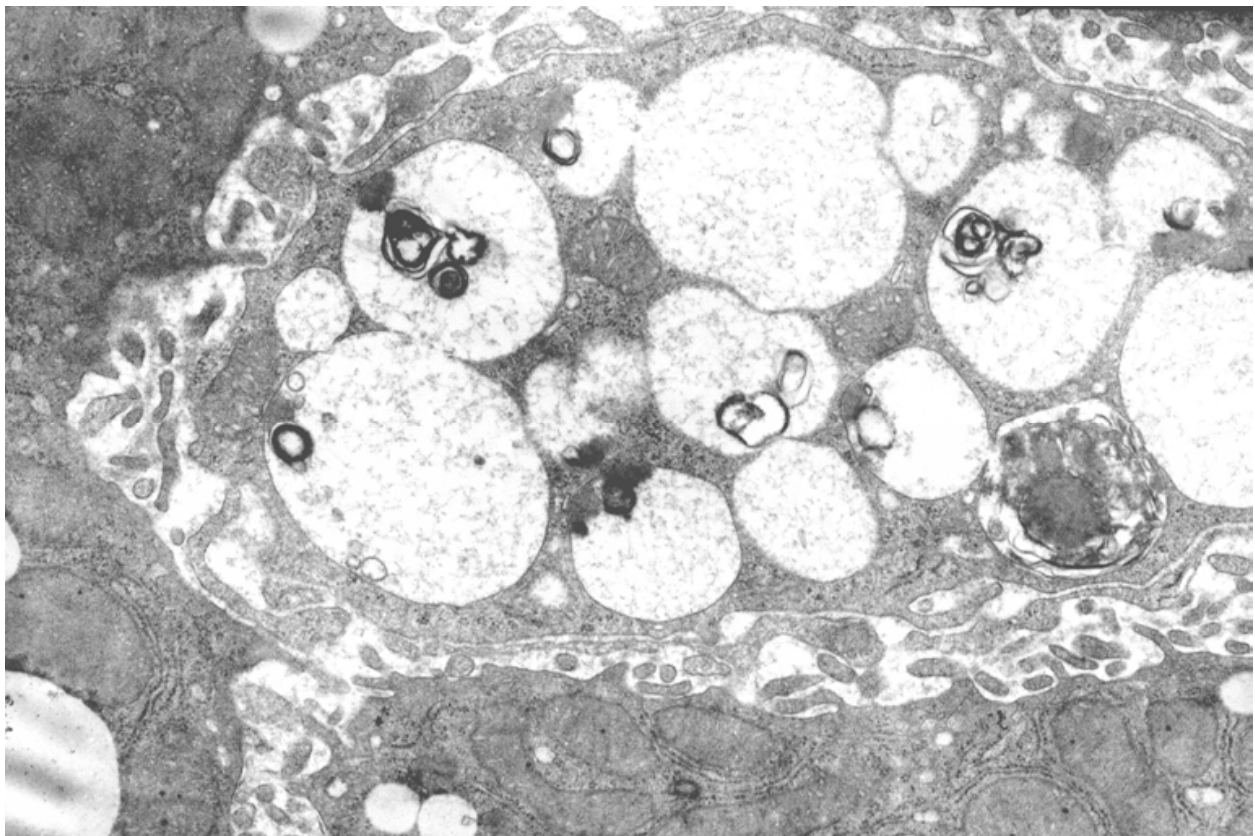


Fig. 9. Electronogram of liver macrophage, triton-filled lysosomes. Triton WR 1339, 1000 mg/kg, 72 h. X 20 000.

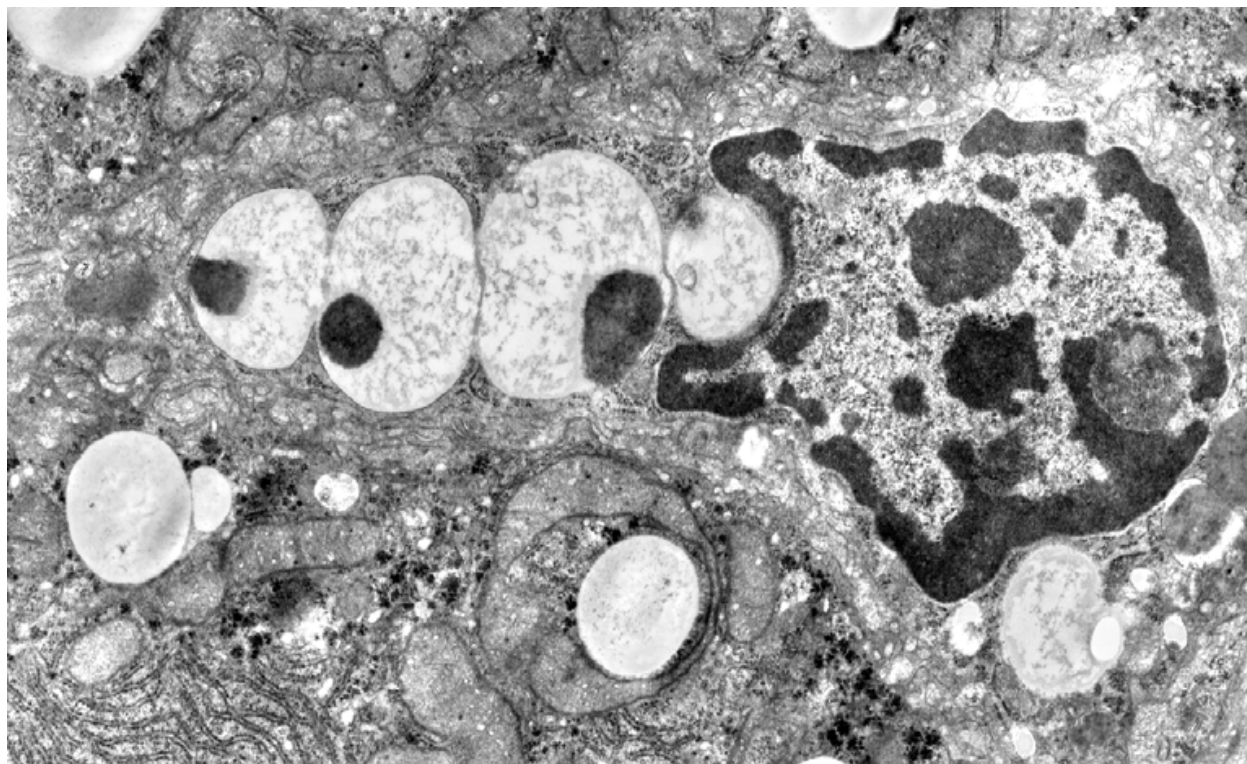


Fig. 10. Electronogram of liver macrophage, Triton WR 1339, 1000 mg/kg, 48 h; enlarged macrophage with numerous triton-filled lysosomes. X 20 000

2.4 Effect of Triton WR 1339 on liver cells ultrastructure

Macroscopically, *in situ*; bile color was bright yellow. The morphological changes of liver cells under Triton WR 1339 administration in rats and mice were described earlier (Trout & Viles, 1979; Schneider et al., 1997), however there was no data collected about the intrahepatic cholestasis development. As was shown before, we observed numerous triton-filled lysosomes in Kupffer cells and also in hepatocytes (Figure 9, 10). The ultrastructural signs of cholestasis induced by Triton WR 1339 in mice included enlargement of bile duct, however these changes were expressed in a lesser degree as compare to ANIT model.

3. The role of liver macrophage in cholestasis development

3.1 Macrophage depression model

Gadolinium chloride is widely used for modeling selective depression of liver macrophages. Selective elimination of the subpopulation of large Kupffer cells, inhibition of receptor-mediated endocytosis and phagocytosis of carbon particles in liver macrophages, increase in the number of mRNA transcripts, and stimulation of TNF- α , IL-1, and IL-6 production were observed 24-48 h after intravenous injection of gadolinium chloride (Ding et al., 2003). The phagocytic function of macrophages recovered and population of liver macrophages was restored after 3 and 4 days, respectively. It was hypothesized that the mechanism of gadolinium accumulation in lysosomes is similar to that for other lysosomotropic agents. This accumulation is accompanied by labilization of lysosomes and leads to cell damage. The consequences of intracellular gadolinium accumulation and its effect on lysosomal

functions remain unclear. We studied the kinetics of gadolinium accumulation in the liver and its effects on liver lysosomes and activity of lysosomal enzymes in blood serum. Among lysosomal enzymes we have chosen β -N-acetylhexosaminidase, a marker enzyme of liver macrophages, β -D-glucuronidase and β -D-galactosidase, easily solubilized matrix lysosomal enzymes, whose activity varies more significantly in comparison with membrane-bound enzymes. The suppression of functional activity of Kupffer cells was also followed by the changes in activity of blood serum chitotriosidase, a novel macrophage enzyme (Korolenko et al, 2008).

We have shown that accumulation of gadolinium (as lysosomotropic agent) occurred during the period of its maximum uptake (the day 1 after gadolinium chloride *in vivo* injection) followed by decrease of its concentration (days 5-37). Accumulation of gadolinium inside of lysosomes of liver cells led to structural and functional changes in liver lysosomes. Macrophage depression of the affected macrophages was accompanied by a decrease in osmotic fragility of lysosomes on the day 2 and increase in their osmotic susceptibility during repopulation of macrophages on days 5 and 8. The hypotonic treatment of liver homogenates (0.125 M sucrose at 0° C during 30 min) revealed increased free activity of acid phosphatase (as a result of the increased number of secondary lysosomes in the whole population of lysosomes).

Macrophage depression induced by gadolinium chloride *in vivo* was used in studies of the role of macrophages in different experimental models. In our study, electron microscopy of liver cells showed that injection of gadolinium chloride (14 mg/kg) in intact mice was followed by a significant decrease in the numerical density (788.0 ± 69.1 vs. 1053.0 ± 60.5 per mm^2 in the control, $p < 0.01$) and diminished size of liver macrophages. The relative volume of primary lysosomes and, especially of secondary lysosomes was significantly lower than in the control (1.70 ± 0.64 versus $7.60 \pm 1.08\%$ in the control, $p < 0.01$). High concentration of gadolinium in the liver was associated mainly with selective intralysosomal accumulation of gadolinium in nonparenchymal liver cells (85% of the administered dose, 1 h after gadolinium chloride administration). Liver function tests (measured according to serum ALT activity) remained unchanged under these conditions. The decrease in the number of liver macrophages and lysosomes in these cells was accompanied by a decrease in specific activity of lysosomal enzymes - cathepsin D and cathepsin B in liver homogenates. No changes were found in activities of cathepsin S, a macrophage-specific cysteine protease (Table 3). In serum the total MMP-2 concentration (proenzyme and active enzyme) was also decreased; TIMP-1 concentration (major endogenous MMP inhibitor) remained unchanged in serum (Table 3). These changes were probably related to interaction between Kupffer cells and stellate cells in the liver, which serve as the main source of MMP and TIMP-1. Our results showed that single injection of gadolinium chloride was followed by a decrease in activities of lysosomal cysteine (cathepsin B) and aspartyl proteases (cathepsin D) studied (Table 3). The observed changes were possibly associated with a decrease in the number and functional activity of Kupffer cells.

We studied the role of selective suppression of liver Kupffer cells (induced by gadolinium chloride, in a dose of 14 mg/kg, intravenously) in the development of intrahepatic cholestasis in CBA/C57BL/6 mice (intraperitoneal injection of ANIT in a single dose of 200 mg/kg).

Index	Control (intact mice)	Gadolinium chloride, 14 mg/kg, 24 h
ALT activity in serum, U/L	54.20 ± 1.55	44.6 ± 6.10
Cathepsin B activity (liver)	0.27 ± 0.02	0.20 ± 0.02*
Cathepsin L activity (liver)	0.46 ± 0.05	0.46 ± 0.03
Cathepsin S activity (liver)	0.030 ± 0.002	0.030 ± 0.001
Cathepsin D activity (liver)	0.10 ± 0.08	0.060 ± 0.007*
Total MMP-2 concentration in serum, ng/mL	201.5 ± 4.66	181.70 ± 4.64*
Serum TIMP-1 concentration, pg/mL	8000.0 ± 1443.4	8450.0 ± 1472.2

Table 3. Effect of gadolinium chloride on activity of lysosomal enzymes in liver and serum concentrations of MMP-2 and TIMP-1 in mice (M ± m)

Note: * $p < 0.05$ vs control. Activity of cathepsin B (against fluorogenic substrate Z-Arg-Arg-MCA), cathepsin L (substrate Z-Phe-Arg-MCA), cathepsin S (substrate Z-Val-Val-Arg-MCA, Sigma-Aldrich, USA) in liver homogenates expressed in nmol MCA/min per mg of protein. Cathepsin D activity is expressed in arbitrary units (A_{366} /min per g of protein). MMP-2 and TIMP-1 concentration was measured using commercial ELISA R&D kits. * $p < 0.05$ as compared to intact animals

In the separate experiment it was shown that preliminary administration of gadolinium chloride to ANIT-treated animals was followed by an increase in activities of ALT (133.5 ± 37.6 vs. 74.70 ± 9.18 U/L in the ANIT group, $p = 0.005$), alkaline phosphatase (269.1 ± 9.1 vs. 158.6 ± 11.1 U/L in the ANIT group, $p = 0.0001$), and γ -glutamyl transpeptidase in urine samples (243.0 ± 10.0 vs. 135.8 ± 30.7 U/L in the ANIT group, $p < 0.05$). Hence, the severity of cholestasis and hepatocyte injury increased after combined treatment with gadolinium chloride and ANIT. Aggravation of cholestasis under macrophage suppression in ANIT-treated animals, probably, was associated with the increased secretion of TNF- α . Gadolinium chloride exposure was accompanied by changes in protease secretion from Kupffer cells and stellate cells, remodeling of the extracellular matrix, and repopulation of liver macrophages. Cholestasis and hyperbilirubinemia in ANIT-treated rats had similar characteristic (Ferreira et al., 2003) as in mice. In general, this process was more significant than the signs observed in patients with cholestasis due to medical treatment by different drugs (phenothiazine derivatives, methyltestosterone, estrogens, oral contraceptives, etc.). So, in experiment in mice and rats pretreatment with gadolinium chloride increased the severity of cholestasis and aggravated liver damage. Gadolinium accumulation in the liver (with peak after 24 h) was accompanied by a decrease in activity of proteases (cathepsin D and cathepsin B) and concentration of matrix metalloprotease-2 (Table 3) possibly, as a result of decreased liver macrophage number. Our observations confirm the hypothesis that normal function of Kupffer cells and extracellular matrix plays an important role in cholestasis. Administration of gadolinium chloride also serves as a convenient model to study the side effects, toxicity, and safety of lanthanides as nanoparticles.

The model of selective liver macrophage depression was characterized by decreased activity of serum chitotriosidase, whereas macrophage stimulation (by zymosan or chito-carboxymethyl β -1,3-glucans), on the contrary, increased chitotriosidase activity (Korolenko et al., 2008). The uptake of gadolinium by liver cells during preliminary (before gadolinium chloride) administration of β -1,3-glucans was also increased. It was suggested that the model of selective liver macrophage depression is useful for study the protective effects of different biological response modifiers such as polysaccharides (β -1,3-glucans) *in vivo*.

3.2 Liver injury and cholestasis

Bile duct obstruction, cholestasis development is associated with hepatic accumulation of leukocytes and liver injury (Gehring et al., 2006). According to our data obtained, the development of acute CCl₄-hepatitis was accompanied by hepatocyte cytolysis and increase in serum ALT activity (72 h after hepatotoxin administration to mice 9.50 ± 0.42 , $n=10$ vs control 3.50 ± 0.29 U/L, $n=10$, $p < 0.01$), however cholestasis was not found in this model (no changes of alkaline phosphatase and GGTP activity in serum of mice comparatively to intact animals). During combined treatment by Triton WR 1339 and CCl₄ the cholestasis was more significant as compare to the group of mice received only Triton WR 1339. The aggravation of liver injury induced by Triton WR 1339, possibly, was related to increased damage of lysosomes by detergent and cholestasis development. It was shown in combined administration of Triton WR 1339+ CCl₄, that activity of β -galactosidase (116.8 ± 0.49 $\mu\text{mol MUF/L per min}$) and chitotriosidase (8.4 ± 0.55 $\mu\text{mol MUF/L per min}$) in bile was higher compared to intact mice (Table 2). The role of lysosomal enzymes in bile secretion in mice is still not clear. Previous studies showed that in intact mice "synchronous" exocytosis of lysosomal glucosidases (β -galactosidase, β -glucuronidase and N-acetyl- β -D-glucosaminidase) into bile occurred in intact rats (Korolenko et al., 2008). The function of lysosomal enzymes in the bile remains unclear. Glucosidases are probably involved into degradation of glucuronides. Dramatic increased in β -galactosidase activity and some changes in β -hexosaminidase and chitotriosidase in bile of mice with the both models of experimental cholestasis (induced by ANIT and Triton WR 1339) probably reflected the increased exocytosis of enzymes into bile. Transport disorders and concentration of lysosomal enzymes in bile can potentiate the effect of different compounds (lipids and fatty acids) excreted into bile independently of lysosomal enzymes. Hepatocytes and bile duct cells probably are connected with bile secretion in mice, but in general cellular source of lysosomal enzymes in bile and serum is not clear. The main part of plasma proteins in mammals (mice and rats) are synthesized in liver cells. In humans, serum chitotriosidase was shown to originate partially from macrophages and PMN, however in mice, on the contrary, chitotriosidase localized mainly in cells of the gastrointestinal mucosa (Korolenko et al., 2007; Aerts et al., 2008) and cellular source of this enzyme in serum and bile is still not clear.

3.3 Chitotriosidase activity in liver pathology

The special attention was devoted to chitotriosidase and intrahepatic cholestasis. Chitotriosidase is a new enzyme which was found in human macrophages (Aerts et al., 2008). There is limited information on the biological role of chitotriosidase in laboratory animals (mice, rats, rabbits); as in humans, the main function of this enzyme is related to innate immunity. It was shown that serum chitotriosidase activity in patients with type I Gaucher disease increased up to 100-1000 times and now serves as a diagnostic surrogate marker of this disorder. In humans, enzyme activity markedly varies (9-195 nmol MUF/mL h) in healthy persons and increases with age (Aerts et al., 2008). Chitotriosidase activity is very low in 6.2% young individuals and high in 3.1% individuals. Enzyme activity moderately increases (by 2-3 times) in patients with inborn lysosomal storage diseases (Krabbe disease, GMI gangliosidose, and Niemann-Pick, type A and B disease). As was noted before, human chitotriosidase is mainly synthesized in macrophages; stimulated

macrophages produce considerable amounts of chitotriosidase mRNA (Aerts et al., 2008). In neutrophil precursors chitotriosidase is synthesized and accumulated in specific granules. Chitin, a component of cell walls and membranes in various microorganisms, is the natural substrate for chitotriosidase; enzyme cleaves also the synthetic substrate 4-methylumbelliferyl-(MUF)- β -D-N,N',N''-triacetylchitotrioside. Chitinases of plants protect them from various pathogenic fungi, this function is similar to mammalian chitotriosidase. Probably, mammalian chitotriosidase also has some protective properties. Functions of chitotriosidase need further studies. The role of this enzyme is connected with innate immunity and removing of foreign bacteria and fungi, whose membranes include chitin; this function of chitotriosidase is of particular interest. In mammals, the biological function of *serum* chitotriosidase, as well as its cell origin in experimental lysosomal storage syndrome, is poor understood (possibly, enzyme is originated from the stimulated macrophages). Earlier we have shown the increase of chitotriosidase activity in serum of experimental animals (mice and rats) in macrophage stimulation (by zymosan, carboxymethyl β -glucan), which were taken up by Kupffer cells and stimulate liver macrophages and also lung and spleen macrophage pools. Triton WR-1339 administration in mice was shown to increase the number of liver macrophages and reproduce lysosomal storage syndrome, elevating serum chitotriosidase activity 5-7 days after the single drug administration. Thus, macrophage stimulation by β -glucans, Triton WR 1339 moderately (1.5-2-times) increased serum chitotriosidase activity in experimental animals (mice and rats). However, in these experimental models stimulation of macrophages in rats and mice did not dramatically increase chitotriosidase activity as in type I Gaucher disease in human serum (i.e. more than by 100-1000-times).

β -Hexosaminidase is one of glucosidase, which had been studied earlier in serum of humans and mice. However, there is limited number of works devoted to secretion of this enzyme into bile (possibly, by exocytosis). The most of investigations were devoted to study this enzyme in humans. Human plasma contains a high-molecular weight enzyme precursor (63kDa) of β -hexosaminidase A and B. The presence of 38 kDa mature enzymes in liver lysosomes of mammals is related to processing inside of these particles. The bile of mice probably contains mature β -hexosaminidase, which is secreted by exocytosis after enzyme processing in lysosomes.

The development of cholestasis in humans and experimental animals may be also associated with metabolic disorders of lipids and lipoproteins and accompanies liver injury of different etiology. Releasing of lysosomal enzymes can increase the severity of hepatocyte damage under the influence of toxic components from the bile (fatty acids salts), which can exert modulatory effect on apoptosis of liver cells. Administration of ANIT is rapidly (8-24 h after) followed by reversible damage of the epithelium of bile ducts and serves as a widely used model of intrahepatic cholestasis in rats and mice. Macrophage depression by gadolinium chloride aggravated the cholestasis development in mice induced by ANIT.

Preliminary administration of Triton WR 1339 in mice and rats, aggravated the development of acute toxic hepatitis induced by CC_4 . The development of CCl_4 -induced hepatitis (after 72 h) was accompanied by hepatocyte cytolysis and increase in plasma ALT activity (9.50 ± 0.42 U/L per h, $n=10$; vs. 3.50 ± 0.29 U/L per h in the control, $n=10$; $p < 0.01$). Cholestasis in acute toxic hepatitis was not found under isolated CCl_4 administration in mice. However, aggravation of cholestasis and especially, liver injury was observed after preliminary

administration of Triton WR 1339 to mice with acute toxic hepatitis. The aggravation of cholestasis after combined treatment by Triton WR 1339 and CCl_4 was probably associated with effect of Triton WR 1339 on liver lysosomes. Activities of β -galactosidase (116.80 ± 0.49 $\mu\text{mol MUF/L per min}$, $n=5$) and chitotriosidase (8.40 ± 0.55 $\mu\text{mol MUF/L per min}$ in bile samples from treated animals were significantly higher compared to intact mice and animals with isolated administration of Triton WR 1339.

4. Conclusion

Intrahepatic cholestasis was shown to develop during treatment by different medical drugs (antibiotics, phenothiazine derivatives, anti-inflammatory drugs), some of them possessed by lysosomotropic action (these drugs are taken up and selectively concentrated *in vivo* inside of lysosomes). The lysosomotropic action of drugs was shown in medical clinics using some antibiotics, iron compounds. In experiment we compared the cholestasis development in mice with help of known ANIT model and lysosomotropic agent Triton WR 1339 administration. We compared these models to reveal the common features of intrahepatic cholestasis, during study of bile and serum enzymes in animals with intrahepatic cholestasis. Intrahepatic cholestasis development was confirmed by elevation of serum alkaline phosphatase and γ -glutamyl transferase activity in the both cases. According to morphological (electron microscopic) study of liver cells enlargement of bile canaliculi and bile ducts was noted as well as other morphological signs of intrahepatic cholestasis. Both models of intrahepatic cholestasis can be used for reproduction in mice (and rats). It was concluded that drug-induced intrahepatic cholestasis is manifested in increased secretion of lysosomal enzymes into the bile. Bile components can aggravate liver cells damage by affecting the process of hepatocyte apoptosis and necrosis. Selective depression of Kupffer cells, enriched by lysosomes, reproduced *in vivo* (with help of gadolinium chloride) was followed by the aggravation of cholestasis and liver damage. Our results confirm the hypothesis that normal function of liver macrophages and their lysosomes play the important role in cholestasis development. These data can be useful in further investigation of the role of lysosomes of hepatocytes and liver macrophages in bile secretion and, possibly, in prevention of cholestasis development in medical clinics used some drugs with lysosomotropic action (phenothiazines, some antibiotics, polymers, iron compound, chloroquine etc.).

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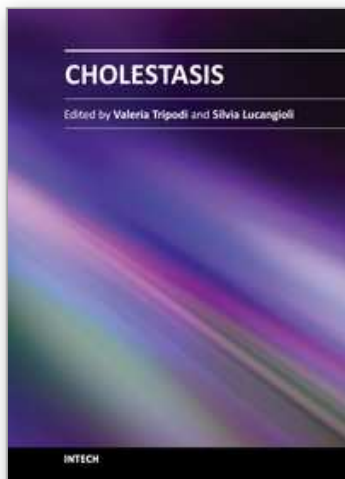
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This book covers different aspects on the understanding of mechanisms, effects, and management of cholestasis. This unique compendium contains important citations, an invaluable amount of research work, and many applications, which are outstanding resources for clinicians, pharmacists, biochemists, upper-level undergraduate, graduate, and continuing-education students who are dedicated to discovering new knowledge on cholestasis.

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