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Research Article

Nitric oxide-mediated effects of L-ornithine-L-aspartate in acute toxic liver injury

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Abstract

This study was aimed to investigate nitric oxide-dependent mechanisms of L-ornithine-L-aspartate (LOLA) action in acute toxic liver injury in rats. Acute hepatitis was induced in Wistar rats using 50% oil solution of tetrachloromethane ($CCl_{4)}$ intragastrically (2 g/kg) twice in a 24 hour interval. Intraperitoneal treatment with LOLA (200 mg/kg) was started 6 hours after the second CCl_4 administration and maintained for 3 consecutive days. L-Nω-Nitroarginine Methyl Ester (L-NAME) was used intraperitoneally (10 mg/kg). In CCl_4-induced hepatitis, LOLA restores the structure of hepatocytes and prevents aminotransferases, alkaline phosphatase and gamma-glutamyl transferase elevation. It decreases total bilirubin concentration but does not affect increased cholesterol level. LOLA augments urea concentration, total protein level in blood and liver as well as serum and liver content of nitrite anions. LOLA enhances activity of catalase, glutathione S-transferase, manganese superoxide dismutase, increases reduced glutathione level and total antioxidant capacity and decreases thiobarbituric acid reactive substances level. The concomitant use of L-NAME inhibits the action of LOLA to enhance nitrite anions synthesis both in serum and liver, to delay the recovery of hepatocytes, to counteract LOLA effect against blood total protein reduction, to prevent the decline in aminotransferases, alkaline phosphatase,, gamma-glutamyl transferase and glutathione S-transferase activity and to reduce catalase activity and reduced glutathione level. Therefore, in CCl_4-induced hepatitis, LOLA effectively prevents cytolysis and cholestasis, improves liver metabolism and protects against oxidative stress. Partially, these changes occur in nitric oxide-mediated mechanism since the use of L-NAME declines most of LOLA effects.

Keywords

L-ornithine-L-aspartate, hepatoprotection, nitric oxide

Introduction

L-ornithine-L-aspartate (LOLA) is a well tolerated medicine effective in the treatment of patient with liver cirrhosis and chronic hepatic encephalopathy (HE) (Butterworth and Canbay 2018). LOLA induces hepatic urea synthesis, increases glutamine production in muscles and regulates the relationship between branched aromatic amino acids. The components of LOLA participate in the reactions of the ammonia molecule incorporation into urea and glutamine which underlies the application of LOLA as an effective ammonia-lowering strategy widely used for the management and treatment of HE (Kircheis and Lüth 2019).

LOLA exerts hepatoprotective effects by stabilizing peroxidant/antioxidant balance in the liver cells as it provides L-ornithine and L-aspartate as substrates for

glutamate production (Jalan et al. 2007). The product of LOLA-derived glutamate, namely, glutathione (GSH), is a potent antioxidant that has the requisite properties for the control of oxidative damage (Najmi et al. 2010).

Finally, since LOLA administration results in the accumulation of L-glutamate and L-arginine, it leads to the increase in nitric oxide synthase (NOS) production with the consequent enhancement in hepatic microperfusion (Ijaz et al. 2005). This mechanism was confirmed in patients with cirrhosis (Staedt et al. 1993) and in an experimental model of chronic liver failure (Rose et al. 1999). However, nitric oxide (NO)-dependent mechanism of LOLA action in acute toxic liver injury is not well understood.

Acute liver injury is associated with higher blood ammonia levels than in cirrhosis which correlates with increased risk of mortality, more severe encephalopathy, intracranial hypertension and cerebral herniation. This differences between ammonia levels are explained by the fact that in cirrhosis, there is some remaining liver cell mass that retains some capacity to detoxify ammonia, while in acute hepatitis large amounts of ammonia escape hepatic clearance (Acharya et al. 2009).

The experimental animal model, in which acute liver toxicity is induced by tetrachloromethane (CCl_4), is characterized by the specific biochemical and histopathological changes which represent the common features of liver injury in humans. CCl_4 causes direct liver injury due to cell necrosis caused by altering hepatocyte membrane permeability and induces up-regulation of pro-inflammatory mediators resulting in secondary hepatic injury (Díaz-Gómez et al. 2011). The toxicity of CCl_4 is also associated with free radical formation by the liver endoplasmic reticulum leading to a persistent oxidative stress (Martinelli et al. 2021).

Our previous research demonstrates that in 7 days after CCl4-induced toxic hepatitis, LOLA prevents cytolysis and cholestasis, improves liver metabolism and protects against oxidative damage (Oleshchuk et al. 2021). Multiple randomized trials in patients with cirrhosis using LOLA have demonstrated its efficacy in lowering systemic ammonia levels (Stauch et al. 1998; Ytrebø et al. 2009) and suggested the effectiveness of LOLA in HE (Vela and Ramírez 2011; Kircheis and Lüth 2019), while its benefits in acute liver toxicity still remain unclear. Therefore, this study is aimed to investigate NO-dependent mechanisms of LOLA action in acute toxic liver injury induced by CCl_4 administration in rats.

Materials and methods

Animals

Adult Wistar strain albino rats were used for the study. Animals were supplied by Central Animal House Facility of Ternopil National Medical University and kept under standard laboratory conditions in polypropylene cages in 12-h light/dark cycle at 25 ± 2 °C. Animals were provided with standard diet and water ad libitum.

All the animals received humane care according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985). Experiments performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee on Bioethics of I. Horbachevsky Ternopil National Medical University.

Experimental protocol

The study was performed on 24 white male rats weighing 170–210 g. Animals were randomly divided into 4 groups as follows:

1. Control (C) group (n = 6);
2. CCl_4-induced hepatitis (H) group (n = 6);
3. CCl_4-induced hepatitis + treatment with LOLA (LOLA) group (n = 6);
4. CCl_4-induced hepatitis + treatment with LOLA and L-NAME (LOLA + L-NAME) group (n = 6).

To induce acute hepatitis, 50% oil solution of CCl_4 was administered intragastrically at a dose of 2 g/kg twice in 24 hour interval. Control animals received the equal amount of saline. Intraperitoneal treatment with LOLA at a dose of 200 mg/kg was started in 6 hours after the second CCl_4 administration and maintained for 3 consecutive days. L-Nω-Nitroarginine Methyl Ester (L-NAME), a competitive inhibitor of NOSes, was used intraperitoneally at a dose 10 mg/kg.

Histological study

6 rats from every group were used for the histology. 2–3 slices were cut from the liver for morphological analysis and immediately fixed in 10% formalin solution. Tissue processing was performed in a fully enclosed tissue processor of vacuum type Logos ONE. The paraffin blocks were prepared from the histological materials. Histological sections were prepared on a rotary microtome Amos AMR-400 with a thickness of 4–5 μm. Histological studies were performed using an Eclipse Ci-E microscope (Japan) with a Sigeta M3CMOS 14000 digital camera at different magnifications: × 100, × 200, × 400. The changes in the parenchyma and the main structural elements of the liver were evaluated. Histological liver sections were stained with haematoxylin and eosin according to the standard methods.

Biochemical analysis

Blood samples were obtained from the right ventricle via left anterior thoracotomy at the time of the sacrifice of the animal. Blood was collected with a sterile syringe without anticoagulant and centrifuged at 2000× g to separate the serum. The serum samples were stored at −20 °C until use for biochemical assays. Detection of the biochemical

parameters was performed using a photoelectric colorimeter "Lambda 25" or spectrophotometer "Lambda 25" depending on the kit. Serum alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (γ-GT), total bilirubin, alkaline phosphatase (ALP), total protein in serum and urea were estimated according to the standard protocols using standard kits of reagents "Spaynlab". Liver concentration of protein was determined according to Lowry et al (1951).

Oxidant-antioxidant markers

The copper/zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) activity was measured according to previously described methods (Beauchamp 1971). Assessment of catalase activity was performed according to Aebi method (Aebi 1974) and glutathione S-transferase (GST) activity – according to Habig et al (1974). Total antioxidant capacity (TAC) was measured as described before (Re et al. 1999). Reduced GSH was evaluated following Griffith et al (1980). Thiobarbituric acid reactive substances (TBARS) were estimated according to the reported procedure (Ohkawa et al 1979).

Evaluation of nitrite anions content

The content of nitrite anions (NO_2^-) in the liver and serum was determined by a highly specific spectrophotometric method using color reaction with Griess reagent described by Green (1982).

Statistical analysis

Comparison of multiple groups was performed by One-way ANOVA. The data are presented as mean ± SEM. The results were considered statistically significant if the P-value was 0.05 or less.

Results

LOLA improves hepatocyte morphology and reduces the markers of liver injury in CCl_4-induced hepatitis.

Fig. 1 demonstrates the liver sections obtained from different experimental groups of rats.

In control animals group (C), it is found normal hepatic architecture and liver lobular structure with well-preserved cytoplasm, prominent nucleus, and nucleolus.

In 3 days after toxic liver injury (H), induced by CCl_4, the lobe structure was considerably altered. It is found the significant expansion of the portal fields due to severe lympho-histiocytic infiltration and moderate blood supply to vessels with focal erythrodiapedesis. Hepatocytes of centrilobular zones underwent significant toxic impact with the formation of fatty droplet dystrophy and a specific balloon cell structure.

Treatment with LOLA (LOLA) caused the progressive restoration of the liver structure. In the area of the portal tracts, only some single residual features of the liver injury in the form of individual cells with vacuolar inclusions are observed. The vessels of the triads can be structurally equated to normal, perivascular edema is not observed. The lobular structure of the liver is maintained, the trabecular structure is well observed. Sinusoids are visualized along the entire lobe, contained single erythrocytes and macrophages. Only in some hepatocytes, the single sings of dystrophic changes are observed, nuclei are contained in all cells, intercellular contacts remained preserved.

In rats administered LOLA and L-NAME (LOLA + L-NAME), positive changes in the liver are observed as well. The lobular structure is significantly restored. It is revealed the restoration of the trabecular structure of hepatocytes on the major area of parenchyma, dystrophic sings of hepatocytes are reversed and intercellular contacts are restored, especially, in the centrilobular zone. Cell nuclei with the minimal changes are seen in the majority of hepatocytes. However, perivascularly, mainly periportally, in the liver cells, the features of hepatotoxic effects in the form of large-drop and small-drop fatty degeneration were observed. The vessels remained dilated and full-blooded. In some areas, the sings of toxic injury of the vascular endothelium in the form of focal hyperplasia is observed.

Aminotransferase elevation is mainly related to liver cellular damage when hepatocytes undergo necrosis as a result of direct cellular damage or inflammation (Giuffrè et al. 2020). The major changes in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity are demonstrated in Table 1.

Table 1. Aminotransferases activity.

	Control	Hepatitis	LOLA	LOLA+L-NAME
ALT (U/L)	81.88 ± 1.79	149.67 ± 4.09***	110.65 ± 5.76§§	130.67 ± 3.87†
AST (U/L)	105.83 ± 2.24	440.83 ± 9.40***	216.78 ± 12.64§§§	382.5 ± 20.00†††

Notes. Values are presented as mean ± SEM. P-value < 0.05 is considered significant. ***P < 0.001 vs control, §§P < 0.01, §§§P < 0.001 vs hepatitis, †P < 0.05, †††P < 0.001 vs LOLA treatment. ALT, alanine aminotransferase, AST, aspartate aminotransferase.

In rats with hepatitis, aminotransferases activity was markedly augmented in 3 days: ALT – by 82.78% and AST – in more than 4 times. LOLA treatment significantly abolished aminotransferase elevation: ALT – by 26.07% and AST – by 50.82%. Co-administration of L-NAME reduced LOLA efficacy against ALT and AST increase.

LOLA prevents cholestasis in CCL_4-induced hepatitis

Cholestasis results in increased concentration of all bile constituents, such as cholesterol, bile acids, and bilirubin (Rothuizen 2009), and also of enzymes such as alkaline phosphatase (ALP) and gamma-glutamyl transferase (γ-GT) (Malaguarnera et al. 2012).

In rats with CCl_4-induced hepatitis, ALP and γ-GT activity was significantly increased more than 3 and 5 folds,

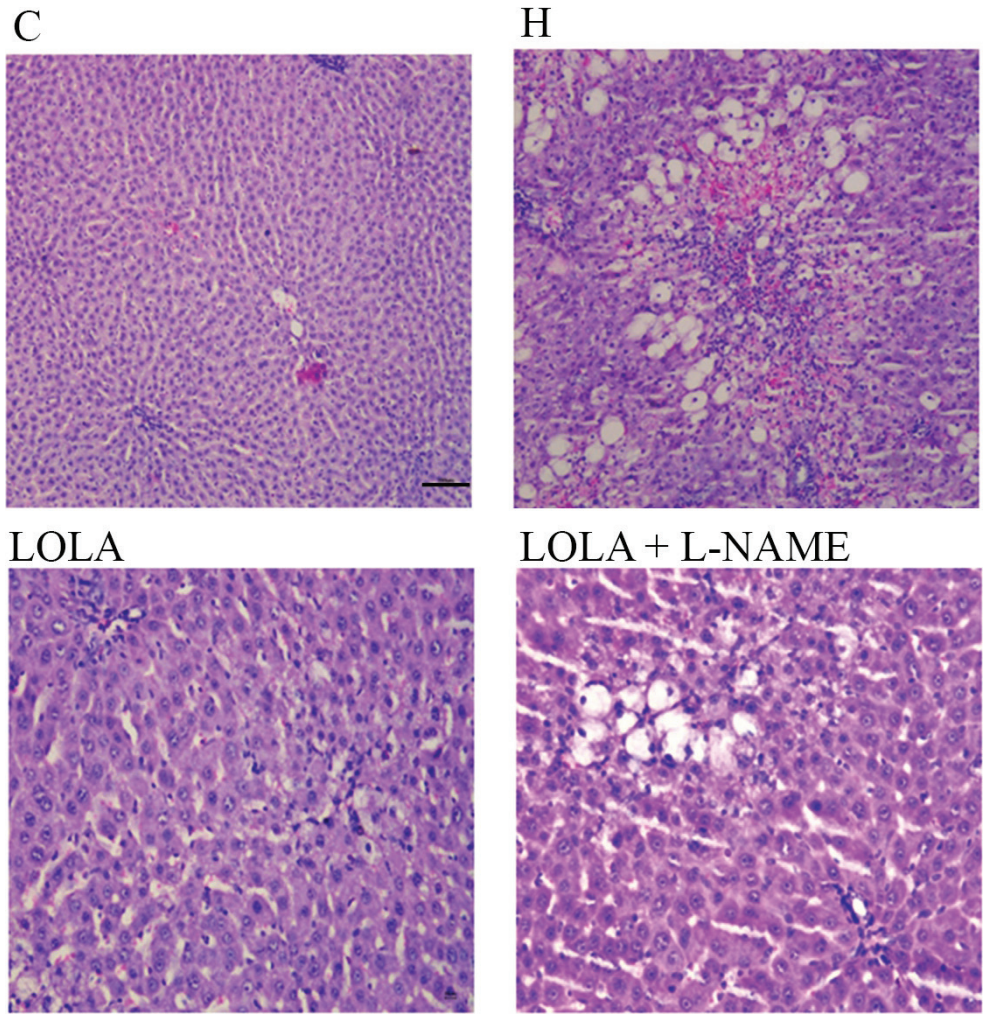

Figure 1. Representative images of rat liver tissues stained with haematoxylin and eosin. **C** (control) – normal hepatic architecture and liver lobular structure; **H** (CCl_4-induced hepatitis) – expansion of the portal fields due to severe lympho-histiocytic infiltration, moderate blood supply to the vessels with focal erythrodiapedesis, fatty droplet dystrophy and a specific balloon cell structure; **LOLA** (CCl_4-induced hepatitis + LOLA treatment) – progressive restoration of the liver structure, **LOLA+NAME** (CCl_4-induced hepatitis + LOLA treatment + L-NAME) – partial restoration of the liver.

respectively. In LOLA treated animals, ALP activity was reduced by 64.18%, and γ-GT activity was decreased 2.67 times. L-NAME diminished LOLA effectiveness in ALP and γ-GT activity reduction (Table 2).

Total bilirubin concentration increased fourfold in rats with hepatitis. Administration of LOLA caused the reduction of total bilirubin concentration by 54.30%. Concomitant administration of L-NAME did not affect the ability of LOLA to decrease concentration of total bilirubin (Table 2).

A significant increase in total cholesterol concentration by 82.65% was observed in animals treated with CCl_4, and neither LOLA administration alone nor in combination with L-NAME reversed this effect (Table 2).

Table 2. Parameters of cholestasis.

	Control	Hepatitis	LOLA	LOLA+L-NAME
ALP (U/L)	197.2 ± 10.93	662.45 ± 18.99***	237.32 ± 9.17§§§	320.42 ± 23.39†
γ-GT (U/L)	1.00± 0.09	5.15 ± 0.38***	1.93 ± 0.31§§§	3.03 ± 0.24†
Total bilirubin (mmol/L)	2.98 ± 0.22	12.77 ± 1.01***	5.84 ± 0.47§§§	5.73 ± 0.18^{NS}
Total cholesterol (μmol/L)	1.14 ± 0.03	2.09 ± 0.10***	1.85 ± 0.19^{NS§}	1.79 ± 0.14^{NS}

Notes. Values are presented as mean ± SEM. P-value < 0.05 is considered significant. ***P < 0.001 vs control, §§§P < 0.001 vs hepatitis, †P < 0.05 vs LOLA treatment, ^{NS§}P > 0.05 vs hepatitis, ^{NS}P > 0.05 vs LOLA treatment. ALP, alkaline phosphatase, γ-GT, gamma-glutamyl transferase.

LOLA regulates liver metabolism in rats with acute hepatitis

The significance of hepatic urea synthesis resides in the removal of potentially toxic ammonium ions (Kircheis and Lüth 2019). In rats with CCl_4-induced hepatitis, urea level in the blood was significantly decreased by 49.55%. LOLA caused two-fold augmentation of urea concentration as compared to untreated rats which was not reversed by L-NAME administration (Table 3).

Almost all of serum proteins are synthesized by hepatocytes under physiological conditions. In liver injury, this process might be affected (Rothuizen 2009). In the present study, total protein concentration was significantly decreased by 24.01% in the blood and by 25.14% in the liver,

Table 3. Parameters of liver metabolism.

	Control	Hepatitis	LOLA	LOLA+L-NAME
Urea (mmol/L)	6.45 ± 0.10	3.25 ± 0.20***	7.15 ± 0.11$^{\$\$\$}$	6.70 ± 0.15^{NS}
Total protein in blood (g/L)	64.55 ± 1.37	49.05 ± 1.66***	61.9 ± 0.69$^{\$\$\$}$	56.12 ± 1.14††
Total protein in liver (mg/g)	135.30 ± 2.54	101.29 ± 4.92***	118.98 ± 2.85$^{\$}$	127.00 ± 2.22^{NS}
Serum NO_2^- (μg/l)	1.23 ± 0.06	3.29 ± 0.24***	4.05 ± 0.23$^{\$}$	2.07 ± 0.20†††
Liver NO_2^- (mg/l)	2.14 ± 0.07	1.85 ± 0.09*	2.35 ± 0.17$^{\$}$	1.24 ± 0.08††

Notes. Values are presented as mean ± SEM. P-value < 0.05 is considered significant. *P < 0.05, ***P < 0.001 vs control, $^{\$}$P < 0.05, $^{\$\$\$}$P < 0.001 vs hepatitis, ††P < 0.01, †††P < 0.001 vs LOLA treatment, ^{NS}P > 0.05 vs LOLA treatment. NO_2^-, nitrite anion.

respectively. Treatment with LOLA caused the significant augmentation in total protein concentration both in the blood (by 26.20%) and in the liver (by 17.47%) as compared to untreated rats. L-NAME administration prevented LOLA-induced increase in total protein concentration in the blood while LOLA influence on the liver concentration of total protein was not affected by L-NAME (Table 3).

Since NO_2^- exists as a stable metabolite of nitric oxide, its content could represent NO-synthesizing potency of the body tissues (Oleshchuk et al. 2019). The content of NO_2^- was elevated after CCl_4 administration in the serum by 168%. On the contrary, the content of NO_2^- in the liver was significantly decreased by 13.5%. LOLA treatment resulted in the increased production of NO_2^- in the serum by 23%, and in the liver by 26.51%. The concomitant use of L-NAME in animals with CCl_4-induced hepatitis inhibited the action of LOLA to enhance NO_2^- synthesis both in serum and liver (Table 3).

LOLA preserves antioxidant system in CCL_4-induced hepatitis

Catalase is one of the crucial antioxidant enzymes which plays an important role by breaking down hydrogen peroxide and maintaining the cellular redox homeostasis (Nandi et al. 2019). The activity of catalase in the liver was significantly decreased in group of rats with hepatitis by 21.2%. Administration of LOLA markedly increased catalase activity by 18.78% while concomitant use of L-NAME reversed the ability of LOLA (Table 4).

TBARS level, which serves as a marker of lipid peroxidation (Tóthová et al. 2013), was increased after CCl_4 administration by 21.26%. LOLA treatment resulted in decreased formation of TBARS by 9.26%, but this change was not significant. L-NAME did not markedly change LOLA action (Table 4).

Reduced GSH is the most important intracellular scavengers of free radicals, thereby decreased GSH levels may reflect depletion of the antioxidant reserve (Annuk et al. 2001). GSH conjugation of the products of membrane lipid peroxidation by GST, the enzyme involved in cellular detoxification, is generally regarded as one of the major cellular defense mechanisms against toxicity (Raza et al. 2002).

In rats with hepatitis, the level of reduced GSH was slightly but not significantly decreased by 13.40% and GST activity was inhibited by 17.42%. LOLA treatment augmented reduced GSH level by 76.99%, and GST activity by 8% as compared to untreated rats. L-NAME administration prevented these effects of LOLA: reduced GSH level was reduced by 31.46% and GST activity by 15.70% as compared to LOLA treatment only (Table 4).

Table 4. Parameters of oxidant-antioxidant system.

	Control	Hepatitis	LOLA	LOLA+L-NAME
Catalase (μmol/min/mg)	203.01 ± 10.02	159.96 ± 9.75*	190.01 ± 5.62$^{\$}$	147.44 ± 7.40††
TBARS (μmol/kg)	704.32 ± 6.36	854.06 ± 30.48**	774.99 ± 14.50$^{\$\$}$	744.25 ± 8.45^{NS}
Reduced GSH (μmol/g)	2.71 ± 0.21	2.35 ± 0.22^{NS*}	4.15 ± 0.24$^{\$\$}$	2.85 ± 0.28†
GST (μmol/min/mg)	1.81 ± 0.07	1.49 ± 0.03**	1.61 ± 0.03$^{\$}$	1.36 ± 0.02†††
Mn-SOD (U/mg)	5.24 ± 0.20	4.99 ± 0.10^{NS*}	5.97 ± 0.30$^{\$}$	5.55 ± 0.18^{NS}
Cu, Zn-SOD (U/mg)	5.21 ± 0.20	2.54 ± 0.21***	3.17 ± 0.28$^{NS\$}$	2.62 ± 0.30^{NS}
TAC (μmol ABTS•+× g^{-1}	55.20 ± 0.80	46.03 ± 2.08**	57.70 ± 2.53$^{\$\$}$	61.63 ± 0.15^{NS}

Notes. Values are presented as mean ± SEM. P-value < 0.05 is considered significant. *P < 0.05, **P < 0.01, ***P < 0.001 vs control, $^{\$}$P < 0.05, $^{\$\$}$P < 0.01 vs hepatitis, †P < 0.05, ††P < 0.01, †††P < 0.001 vs LOLA treatment, ^{NS*}P > 0.05 vs control, $^{NS\$}$P > 0.05 vs hepatitis, ^{NS}P > 0.05 vs LOLA treatment. TBARS, thiobarbituric acid reactive substances, GSH, glutathione, GST, glutathione S-transferase, Mn-SOD, manganese superoxide dismutase, Cu,Zn-SOD, copper-zinc superoxide dismutase, TAC, total antioxidant capacity.

The copper/zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) could effectively eliminate reactive oxygen species (ROS) and maintain the redox balance (Prochazkova et al. 2001).

In rats with CCl_4-induced liver injury, activity of Mn-SOD was decreased (not significantly) by 4.74% and Cu, Zn-SOD activity was reduced twice. LOLA administration caused the significant increase in Mn-SOD activity by 19.75% which was not reversed by L-NAME. Activity of Cu, Zn-SOD was 25.12% higher (not significantly) in LOLA treatment as compared to untreated rats. L-NAME administration has not reversed LOLA effect against Mn-SOD and Cu, Zn-SOD (Table 4).

Low TAC could be indicative of oxidative stress or increased susceptibility to oxidative damage (Young 2001). In rats with hepatitis, total antioxidant capacity was reduced by 16.6%. LOLA treatment resulted in significant elevation of TAC by 25.34%. L-NAME did not counteract the ability of LOLA to increase TAC (Table 4).

Discussion

LOLA is a stable salt of ornithine and aspartate which activates urea cycle in the liver (Kircheis and Lüth 2019), decreases blood ammonia levels (Ytrebø et al. 2009) and delays the onset of HE (Rose et al. 1999). However, to date, there is no study regarding the mechanism of LOLA action in acute toxic liver injury. In this study we evaluated hepatoprotective effects of LOLA in acute hepatitis induced by CCl_4 administration which has caused the major changes common for acute toxic hepatitis: hepatocellular and cholestatic damage, specific histomorphological features as well

as activation of lipid peroxidation process. We have found that LOLA prevents hepatic cytolysis and cholestasis, improves metabolic functions of the liver and exerts significant antioxidant action. Furthermore, we provide the first evidence that LOLA protects liver, which undergoes acute toxic injury, mainly in NO-mediated mechanism.

During liver injury, hepatocellular permeability is increased, and consequently AST and ALT are released from the intracellular space into plasma (Lescot et al. 2012). Thus ALT and AST enzymes are of major importance in assessing and monitoring liver cytolysis (Yakubu et al. 2005; Datsko et al. 2020). Our findings suggest that LOLA's hepatoprotective activity might be due to its effect against cellular leakage and loss of functional integrity of the cell membrane in hepatocytes as it reduces CCL_4-induced rise in ALT and AST activity and restores the structure of the liver.

Intrahepatic cholestasis develops due to swelling of hepatocytes, which may occur in different liver diseases, causing occlusion of the canaliculi and bile ductules. The hepatocytes become overloaded with substances that cannot be adequately excreted, thus, they enter the blood circulation (Rothuizen 2009). Cholestasis results in increased concentration of all bile constituents, such as cholesterol, bile acids, and bilirubin (Rothuizen 2009), and also of enzymes such as alkaline phosphatase and γ-GT (Malaguarnera et al. 2012). Our data show that in rats with CCL_4-induced hepatitis, the major features of cholestasis developed while LOLA prevented the increase in ALP and γ-GT activity and reduced total bilirubin concentration, although total cholesterol concentration was not affected.

We have found significant antioxidant properties of LOLA as well. The oxidative damage is thought to be a basic mechanism underlying liver injury (Malaguarnera et al. 2012). The free radicals attack on cell membrane-bound polyunsaturated fatty acids resulting in formation of TBARS which are the markers of lipid peroxidation (Tóthová et al. 2013). Our study demonstrates the protective action of LOLA against LP since content of TBARS is decreased under its influence.

The body has a defense mechanism against oxidative stress which includes the antioxidant enzymes which can catalytically remove the reactive species. Mn-SOD is one of the mitochondrial targets of ROS, and thus its activity might become reduced with ROS exposure (Nandi et al. 2019). SOD converts superoxide into hydrogen peroxide which is in turn degraded by catalase, which maintains an optimum level of the H_2O_2 molecule in the cell and regulates cellular signaling processes. Catalase deficiency or malfunctioning is associated with the variety of diseases (Nandi et al. 2019). We have demonstrated that exposure to CCl_4 induces the failure of potent antioxidant enzymes such as catalase and Mn-SOD while LOLA-treatment enhances their activity in rats with hepatitis as compared to untreated rats.

Hepatoprotective effects of LOLA could be explained, in part, by antioxidant properties of its metabolic products glutamine and GSH (Butterworth and Canbay 2018). The mitochondrial GSH pool presents an important defense system against oxidative stress as it reacts covalently with ROS, and serves as a substrate for GST which provides protection against membrane LP by scavenging peroxides and their end products (Malaguarnera et al. 2012). Our data show that in rats with hepatitis, LOLA treatment increases the level of reduced GSH and activity of GST which were inhibited by CCl_4. This confirms previously published findings suggesting that LOLA corrects the loss of GSH in the serum of animals with liver failure (Najmi et al. 2018).

The measure of TAC considers the cumulative action of all the antioxidants present in plasma and body fluids (Ghiselli et al. 2000). TAC is decreased in conditions associated with oxidative stress (Young 2001). Our results show that in rats with hepatitis, TAC is significantly inhibited, whereas LOLA improves this parameter confirming its potent antioxidant effects.

The liver performs numerous biochemical functions. It is the site of metabolism for carbohydrates, fats and proteins where they are all broken down and synthesized. Metabolism in the liver usually leads to detoxification of environmental compounds (Allouche et al. 2011). We established that in CCl_4-induced hepatitis liver metabolism is significantly altered, which results in the reduction of blood and liver total protein content and urea concentration. LOLA administration prevented inhibition of liver metabolism.

Since urea synthesis is stimulated by LOLA, it confirms the other authors' conclusions (Kircheis et al. 1997; Ytrebø et al. 2009) suggesting this drug might serve as a potent tool in HE prevention. In acute liver injury, liver detoxification function is abruptly reduced due to the massive necrosis of hepatocytes. This leads to the hyperammonemia and development of HE (Ciećko-Michalska et al. 2012). Mode of ammonia-lowering actions of LOLA is defined by its ability to provide critical substrates for both urea genesis and glutamine synthesis. Activation of the urea cycle leads to the detoxification of ammonia (Kircheis and Lüth 2019).

Urea is a final metabolite of NO. The latter displays the liver synthesizing function and serves as an indicator of protein metabolism and correlates with the level of residual nitrogen in the body (Oleshchuk et al. 2019). NO is formed in the liver under the action of two isoforms – endothelial (eNOS) and inducible (iNOS) and the pattern of their expression and activity differs among physiological and pathological processes. In liver diseases, there is a significant increase in the activity of iNOS isoform (Oleshchuk 2014).

In the biological systems, NO exists as an unstable compound which could be quickly transformed into nitrite anion (NO_2^-). Products of nitrites, in turn, could be further involved into the process of free NO synthesis and release and take part in NO-related mechanisms. In liver injury, changes in the content of NO_2^- are frequently observed (Prochazkova 2001). We have found that the liver production of NO_2^- in acute hepatitis is reduced while serum NO_2^- is augmented.

Decline in liver NO_2^- production could be explained by a profound change in the cellular distribution of eNOS, which leads to its translocation into hepatocyte nuclei. At the same time the high serum nitrite level is likely to be due to the increased concentration of NO, which syn-

thesis is mediated by iNOS. Increased activity of iNOS is the result of proinflammatory cytokines release which is a prominent feature of in liver injury (Datsko et al. 2020). The increased concentration of NO in the flowing blood by the feedback mechanism dramatically inhibits the expression of eNOS. eNOS-induced deficiency of vasodilation develops, contributing to a decreased sinusoid diameter and increased overall portal vascular resistance. Thus, despite the overproduction of nitric oxide, there is a relative lack of intrahepatic microcirculation mediator, as evidenced by reduction of nitrite anion in the liver.

Notably, LOLA treatment resulted in the increased production of NO_2^- in the serum and liver. Previously it was established that in patients with cirrhosis (Staedt et al. 1993) and in an experimental model of chronic liver failure (Rose et al. 1999), LOLA treatment enhanced NOS synthesis. NO, produced due to the function of endothelial NOS (eNOS), exerts protective properties in liver injury through the regulation of sinusoidal diameter, prevention of neutrophil adhesion, inhibition of platelet aggregation and adhesion, and scavenging of reactive oxygen species (Peralta et al. 2001). The relevance of NO in hepatic microcirculation is supported by the fact that administration of eNOS inhibitors reduces microvascular perfusion and aggravates I/R injury, while the supplementation with L-arginine (NO precursor) or NO donors improves microcirculatory status and minimizes liver damage after I/R (Shah and Kamath 2003). However, NO-dependent mechanisms of LOLA action in acute toxic liver injury are not well understood.

To determine if the mechanisms of LOLA action in acute toxic liver injury depends on NO synthesis, we used L-NAME which is a competitive inhibitor of all NOSes with high selectivity to eNOS (Víteček et al. 2012).

The concomitant use of L-NAME in animals with CCl_4-induced hepatitis inhibited the action of LOLA to enhance NO_2^- synthesis both in serum and liver. Besides, we have found that the use of L-NAME delayed the recovery of hepatocytes and affected the reduction of AST and ALT, ALP and γ-GT caused by LOLA, counteracted LOLA effect against blood total protein reduction, prevented the decline in catalase and GST activity and reduced GSH level caused by LOLA.

Conclusion

Altogether these results indicate that in CCl_4-induced acute hepatitis, LOLA effectively prevents major syndromes which accompany acute liver injury, such as cytolysis and cholestasis, improves liver metabolism and protects against oxidative stress. Partially, these changes develop in NO-mediated mechanism since the use of L-NAME declines most of LOLA effects.

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