<u>ORIGINAL PAPER</u>

THE INDICES OF ENDOGENOUS INTOXICATION IN RATS WITH DIFFERENT MODELS OF HEPATOPULMONARY SYNDROME

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ABSTRACT

Introduction. The pathogenesis of hepatopulmonary syndrome (HPS) has not been clarified, but since the basis of HPS development is the dilation of inner lung capillaries, researchers suggest that HPS is caused by the prolonged action of biologically active compounds on the blood vessels of pulmonary circuit and a syndrome of systemic inflammatory response has also been implicated in its development.
The objective of the study was to evaluate the indices of endogenous intoxication and inflammation in blood serum and lung tissue of animals with different models of hepatopulmonary syndrome.
Material and methods. The first experimental model of HPS was made by imposing a double ligature on common bile duct and its further dissection with a scalpel. The animals of second experimental group were fed with a mixture of maize flour, lard, cholesterol, and alcohol plus subcutaneously injection with carbon tetrachloride oil solution for 8 weeks. Determination

RÉSUMÉ

Indices de l'intoxication endogène chez des rats aux différents types de syndrome hépato-pulmonaire

L'objectif de l'étude. La pathogenèse du syndrome hépato-pulmonaire (SHP) n'a pas été clarifiée, mais comme le développement du SHP est basé sur la dilatation interne des capillaires pulmonaires, les recherches suggèrent que le SHP est causé par l'action prolongée de composés biologiquement actifs sur les vaisseaux sanguins du circuit pulmonaire et, un syndrome de réponse inflammatoire systémique a également été impliqué dans la pathogenèse du SHP.
L'objectif de cette étude était d'évaluer les indices d'intoxication endogène et d'inflammation dans le sérum sanguin et le tissu pulmonaire d'animaux présentant différents types de syndrome hépato-pulmonaire.
Méthodes. Le premier modèle expérimental de SHP a été réalisé en imposant une double ligature sur le canal biliaire principal et en le disséquant à l'aide d'un scalpel. Les animaux du deuxième groupe

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of the middle mass molecules contents, TNF-α and CRP was carried out in blood serum and lung tissue.

Results. The modelling of HPS resulted in a statistically significant increase in endogenous intoxication, manifested by an increase in the content of MMM and inflammation indices in blood serum and supernatant of lung tissue homogenate. An increasing of middle mass molecules was more significant for a pool of MMM_2.

Conclusions. Comparing the indices of endogenous intoxication and inflammation in blood serum and lung tissue in both models of hepatopulmonary syndrome, we have found the synchronous development of destructive processes on systemic and local levels with predominance in lungs.

Keywords: endogenous intoxication, rats, hepatopulmonary syndrome.

Abbreviations: HPS – hepatopulmonary syndrome; MMM – middle mass molecules; TNF-α – tumor necrosis factor-α; CRP – C-reactive protein.

expérimental ont reçu un mélange de farine de maïs, de saindoux, de cholestérol et d'alcool, ainsi qu'une injection sous-cutanée d'une solution d'huile de tétrachlorure de carbone pendant 8 semaines. La détermination du contenu en molécules de masse moyenne, du FNT-α et de PCR a été effectuée dans le sérum sanguin et les tissus pulmonaires.

Résultats. La modélisation du SHP a entraîné une augmentation statistiquement significative de l'intoxication endogène, se traduisant par une augmentation de la teneur en MMM et des indices d'inflammation dans le sérum sanguin et le surnageant de l'homogénat de tissu pulmonaire. Une augmentation des molécules de masse moyenne était plus significative pour un pool de MMM2.

Conclusions. En comparant les indices d'intoxication endogène et d'inflammation dans le sérum sanguin et le tissu pulmonaire dans les deux types de syndrome hépato-pulmonaire, nous avons constaté le développement synchrone de processus destructeurs aux niveaux systémique et local, avec une prédominance dans les poumons.

Mots-clés: intoxication endogène, les rats, syndrome hépato-pulmonaire.

Abréviations: SHP – syndrome hépato-pulmonaire; MMM – molécules de masse moyenne; FNT-α – facteur de nécrose tumorale α; PCR – protéine C-réactive.

INTRODUCTION

The hepatopulmonary syndrome (HPS) is an important vascular complication because of systemic hypoxemia in patients with cirrhosis and/or portal hypertension. It is formed by a clinical triad of arterial oxygenation abnormalities induced by intrapulmonary vascular dilatations with liver disease[1-3]. True HPS is estimated to occur in at least 10% of all patients with cirrhosis and portal hypertension[4]. The most frequent clinical symptoms of HPS are progressive dyspnea and platypnea[5], but neither is specific for HPS. Digital clubbing, with a positive predictive value of 75%, was the best clinical sign associated with the presence of HPS, while a correlation between the presence of spider angiomas and HPS was also demonstrated recently, suggesting that they could be used as skin markers of HPS[6-7].

Cirrhotic patients with HPS have a significantly increased mortality rate in comparison with cirrhotic patients without HPS[4]. In a multicenter cohort of cirrhotic patients, being evaluated for liver transplantation at seven USA liver transplant centers, patients with HPS had a 2 to 2.4-times increased risk of mortality compared with all other patients being evaluated for transplantation[8].

The pathogenesis of HPS has not been clarified, but since the basis of HPS pathogenesis is the dilation of inner lung capillaries, researchers suggest that HPS is caused by the prolonged action of biologically active compounds on the blood vessels of pulmonary circuit. Possible role in resistant vasodilation has been suggested for many substances synthesized in the body (nitrogen (II) oxide, endothelin B and endothelin–1, prostaglandins E1 and I2, tumor necrosis factor–α, vasoactive intestinal polypeptide, substance P, calcitonin, glucagon, platelets activating factor, and others)[9].

Endogenous intoxication syndrome is characterized by metabolic, morphological and functional disorders of various organs and systems and occurs in response to various factors of the external and internal environment, as a result of toxic substances accumulation in tissues and biological fluids[10]. The endogenous intoxication syndrome concept is widespread, as a process within the syndrome of a systemic inflammatory response[11]. Concentration of middle molecular products of proteolysis, which are called

middle mass molecules (MMM), in biological liquids is an important and objective indicator of toxicity within the body systems, independent of the causes and signs of the diseases, and is thought to primarily reflect the extent of abnormal protein metabolism and correlate with main clinical and laboratory prognostic criteria for metabolic disorders[12].

THE OBJECTIVE OF THE STUDY was to evaluate the indices of endogenous intoxication and inflammation in blood serum and lung tissue of animals with different models of hepatopulmonary syndrome.

MATERIAL AND METHODS

The experiments were performed on 56 white nonlinear mature male rats, 180–220 g in weight, housed at 25±3°C and humidity of 55±2%, under a constant 12 h light and dark cycle. Water was available ad libitum.

All experiments were conducted in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes[13].

The experimental animals were divided into 4 groups: I – control group N 1 (n=12); II – experimental group N 1 (n=18); III – control group N 2 (n=12); IV – experimental group N 2 (n=14).

The first experimental model of HPS was made by imposition of double ligature on common bile duct and its further dissection with a scalpel[14]. In the control group of animals N 1, common bile duct was separated from the tissue, but not dissected. For all surgical procedures, rats were anesthetized with 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine, via intramuscular injection. Postoperative wound was sewed up completely in layers. On the 28th day post – surgery, the animals were taken out of experiment under thiopental anesthesia.

The animals of second experimental group were fed with a mixture of maize flour, lard, cholesterol, and alcohol, plus subcutaneously injection with carbon tetrachloride (CCl4) oil solution for 8 weeks. The CCl4 oil solution (400 g/L) was injected at 0.5 mL/100 g body weight at the first day of experiment and at 0.3 mL/100 g body weight from the third day on at an interval of two days until the experimental end. Lard was used only in the first two weeks accounting for 20% of the feeding. Cholesterol was appended at 0.5% of feeding for the whole experiment. Alcohol was used in the drinking water exclusively (300 mL/L) during the whole experiment[15].

The control group of animals N 2 was on a standard diet of the vivarium and was administered intragastric the equivalent amount of olive oil. The main advantage of this model is its non-invasiveness and multifactorial aspect, which makes it closer to the real causes in patients.

During the simulation of the HPS 8 animals died.

Determination of the middle mass molecules contents was carried out according to the methodology of Andreychyn[16]. An acid-soluble fraction was isolated from the blood serum (supernatant of lung tissue homogenate), which was obtained by adding 1.8 mL of the trichloroacetic acid 10% solution to 0.2 mL of biological liquid. The next centrifugation was carried out at 3000 rpm for 30 min. 0.5 mL of the isolated fraction was diluted with distilled water at a ratio of 1:10 and determined the optical density at a wavelength of 254 nm (the chain amino acids are determined, MMM_1) and 280 nm (the aromatic amino acids are determined, MMM_2) vs distilled water on a spectrophotometer SF-46. The results were expressed in conventional units, which are numerically equal to the extinction indices.

TNF-α and CRP concentrations were measured using commercially available ELISA kits.

All of the data were processed using the software package Statistica 6.1 for Windows. Intergroup comparisons were performed using Mann–Whitney–Wilcoxon U test. The median (Me) and interquartile range (IQR [Q25-Q75]) were deduced. Differences with p-value < 0.05 were considered as significant.

RESULTS

In rats of the first experimental group (on the 28th day after the ligation of the common bile duct), we recorded an increase in the blood serum content of MMM_1 by 2.7 times (p<0.001) vs control group of rats N 1 (Table 1). In rats of the second experimental group (carbon tetrachloride-induced cirrhosis), the blood serum MMM_1 content was increased by 2.2 times (p<0.001) vs control group of rats N 2. When comparing this index in blood serum of animals of both experimental groups, we determined that it predominates by 23.3% (p<0.001) in the rats on the 28th day after ligation of the common bile duct.

An increasing of middle mass molecules was more significant for a pool of MMM_2, indicating a pronounced increase in aromatic amino acids in the middle molecule composition. Thus, the content of MMM_2 in the blood serum increased by 3.1 times (p<0.001) in the rats of the first experimental group. In the rats of the second experimental group, the content of MMM_2 in the blood serum increased by 2.8 times (p<0.001). When comparing this index in blood serum of animals of both experimental groups, we determined that it predominates by 18%

Table 1. The indices of endogenous intoxication syndrome in blood serum and lung tissue of rats with hepatopulmonary syndrome, Me [Q25-Q75].

Group of animals	Control group N 1 (n=12)	Experimental group N 1 (n=12)	Control group N 2 (n=12)	Experimental group N 2 (n=12)
Blood serum				
MMM_1, conventional units	0.336 [0.311;0.370]	0.908 [0.840;0.970] $p_1<0,001$	0.330 [0.293;0.365]	0.736 [0.675;0.810] $p_1<0,001$ $p_2<0,001$
MMM_2, conventional units	0.122 [0.100;0.149]	0.378 [0.317;0.438] $p_1<0,001$	0.114 [0.876;0.143]	0.320 [0.280;0.371] $p_1<0,001$ $p_2<0,002$
Supernatant of lung tissue homogenate				
MMM_1, conventional units	0.218 [0.197;0.245]	0.655 [0.606;0.732] $p_1<0,001$	0.223 [0.198;0.265]	0.580 [0.517;0.657] $p_1<0,001$ $p_2<0,001$
MMM_2, conventional units	0.630 [0.475;0.815]	0.221 [0.180.;0.270] $p_1<0,001$	0.590 [0.432;0.748]	0.184 [0.136;0.232] $p_1<0,001$ $p_2<0,01$

Note:
p_1 – significant difference if compared to the control animals;
p_2 – significant difference between experimental animals.

(p<0.002) in the rats on the 28th day after ligation of the common bile duct.

While investigating the MMM_1 content in a lung tissue of the first experimental group rats, we recorded a 3-fold increase in this index (p<0.001) if compared to the control group of rats N 1. In the rats of the second experimental group, the content of MMM_1 in the lung tissue increased by 2.6 times (p<0.001) vs control group of rats N 2. When comparing this index in the lung tissue of animals of both experimental groups, we determined that it predominates by 13% (p <0.001) in the rats on the 28th day after the ligation of the common bile duct.

An increasing of middle mass molecule content in the pulmonary tissue was also more significant for the MMM_2 pool. Thus, the content of MMM_2 in the lung tissue was increased by 3.5 times (p<0.001) in the rats of the first experimental group. In the rats of the second experimental group, the content of MMM_2 in the lung tissue increased by 3.1 times (p<0.001). When comparing this index in the blood serum of both experimental groups animals, we determined that its prevalence was 19.9% (p<0.01) in the rats on the 28th day after ligation of the common bile duct.

In rats after the ligation of the common bile duct, we determined an increase in the blood serum content of TNF-α by 7.3 times (p<0.001) vs control group of rats N 1 (table 2). In rats with carbon tetrachloride-induced cirrhosis, the blood serum TNF-α content was increased by 4.5 times (p<0.001) vs

control group of rats N 2. When comparing this index in blood serum of animals of both experimental groups, we determined that it predominates by 42.5% (p<0.01) in the rats after the ligation of the common bile duct. In supernatant of lung tissue homogenate we also observed a pronounced increase of TNF-α content (by 9.6 (p<0.001) and 6.9 times (p<0.001) respectively). In the similar manner, the concentration of CRP was changed. When comparing this index in blood serum of animals of both experimental groups, we determined that it predominates by 36.6% (p<0.002) in the rats after the ligation of the common bile duct, but in lung tissue the changes were not significant (p>0,05).

DISCUSSION

The modelling of HPS resulted in a statistically significant increase in endogenous intoxication, manifested by an increase in the content of MSM in blood serum and supernatant of lung tissue homogenate. This clearly points to an increase in destructive processes, as well as inhibition of the detoxifying function of the body, causing disruption in neutralization of endogenous toxins and, consequently, accumulation of intermediate metabolic products. In the case of liver cirrhosis, excess of bacterial endotoxins enters the systemic circulation as a result of increased permeability of the intestinal wall.

Comparing the severity of endogenous intoxication in the blood serum and lung tissue of the rats with

Table 2. The indices of inflammation severity in blood serum and lung tissue of rats with hepatopulmonary syndrome, Me [Q25-Q75].

Group of animals	Control group N 1 (n=12)	Experimental group N 1 (n=12)	Control group N 2 (n=12)	Experimental group N 2 (n=12)
Blood serum				
TNF-α, pg/mL	5.35 [2.11;9.55]	39.11 [28.19;51.09] $p_1 < 0,001$	6.15 [3.71;10.09]	27.45 [18.68;36.28] $p_1 < 0,001$ $p_2 < 0,01$
CRP, mg/L	0.30 [0.24;0.35]	1.53 [1.40;1.64] $p_1 < 0,001$	0.28 [0.21;0.32]	1.12 [1.01;1.32] $p_1 < 0,001$ $p_2 < 0,002$
Supernatant of lung tissue homogenate				
TNF-α, pg/mL	2.42 [1.90;4.50]	23.29 [14.96;32.28] $p_1 < 0,001$	2.03 [1.12;2.80]	14.07 [8.76;19.32] $p_1 < 0,001$ $p_2 < 0,01$
CRP, mg/L	0.18 [0.13;0.23]	1.26 [1.11;1.44] $p_1 < 0,001$	0.21 [0.14;0.25]	1.22 [1.11;1.44] $p_1 < 0,001$ $p_2 > 0,05$

Note:
p_1 – significant difference if compared to the control animals;
p_2 – significant difference between experimental animals.

modeled hepatopulmonary syndrome, we observed a pronounced increase of MMM in the lung tissue.

Several authors examined a significant role of intestinal endotoxemia and bacterial translocation in the pathogenesis of HPS[17-18]. Liver damage makes it more difficult for the organ to filter blood from the portal vein, which leads to the appearance of portosystemic shunts and a decrease in the hepatic phagocytic capacity. As a result, the lung filtrates systemic blood to compensate for the decrease in hepatic phagocytosis, and the increase in the lung phagocytic activity results in macrophage accumulation in the pulmonary endothelium and increases cytokine and NO levels in the extracellular environment[19]. Phagocytes also produce the superoxide anion radical, which oxides the plasmatic membrane and produces a substantial amount of reactive oxygen species (ROS)[20-21]. These molecules control many physiological functions including vascular tonus regulation. Tieppo et al assessed the lipoperoxidation of pulmonary tissue following the experimental model of common bile duct ligation; using the techniques of thiobarbituric acid reactive substances and chemiluminescence, the authors identified a significant increase in the lipid peroxidation, which may be explained by the action of phagocytic cells when fighting against the process of bacterial translocation[22]. Vallance and Moncada suggested that endotoxemia in liver cirrhosis is caused either directly by the movement of bacteria through the intestinal mucosa or indirectly through the cytokine cascade stimulates vascular endothelial iNOS, which increases the production of NO[23].

There is evidence that in patients with liver cirrhosis the level of tumor necrosis factor-α increases, playing an important role in the accumulation of macrophages in the lumen of the pulmonary vessels. In turn, these macrophages stimulate another NO-producing enzyme, inducible NO-synthase, thus likely causing pulmonary vasodilation[24]. However, other studies have found that while the level of circulating TNF-α was significantly increased in thioacetamide-induced liver cirrhosis, the accumulation of macrophages in the lumen of pulmonary vessels was minimal and HPS did not develop[25]. Studies suggest that neutralization of tumor necrosis factor-α improves the course of experimental HPS[26]. Zhang et al showed a significant increase of endotoxin and TNF-α levels in plasma and increased number of Gram-negative microorganism colonies in rats with HPS, which suggests that intestinal endotoxemia is indeed implicated in the pathogenesis of experimental HPS[27]. TNF-α plays a role in the damage of the intestinal barrier that results in intestinal endotoxemia and bacteremia. Subsequently, intestinal endotoxemia can cause intrapulmonary vasodilation and hypoxia. Hypoxemia can exacerbate intestinal barrier damage due to the release of a number of cytokines (including TNF-α). Thus, a „vicious circle" develops in which hypoxemia and impaired barrier function of the intestinal mucosa intensify each other[27,28].

Plasma CRP is an acute-phase protein and increases markedly with acute invasive infections. Several studies were performed on the association of CRP with the severity of inflammation in liver disease, such as fatty liver, chronic hepatitis C and liver cirrhosis[29-31]. El-Awady et al suggested that CRP is a reliable test of bacterial translocation, and it is a predictor of vascular breach and severity of bacterial translocation[32].

THE LIMITS OF THE STUDY were the small rat population included in the study. It is therefore essential to validate our findings with greater sample sizes to determine the features of endogenous intoxication syndrome. Second, the present study investigated only 2 experimental hepatopulmonary syndrome models, which don't reflect all real causes in patients.

CONCLUSIONS

We showed increased endogenous intoxication, manifested as an upsurge in the content of MSM and inflammatory indices in blood serum and lung tissue, in rats with different models of hepatopulmonary syndrome. Herewith more pronounced intensification of endogenous intoxication was observed in rats on the 28th day after the common bile duct ligation. Comparing the indices of endogenous intoxication and inflammation in blood serum and lung tissue in both models of hepatopulmonary syndrome, we have found the synchronous development of destructive processes on systemic and local levels with predominance in lungs.

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article"

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study"

„All institutional and national guidelines for the care and use of laboratory animals were followed"

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