THE ROLE OF NITRIC OXIDE AND FERRITIN IN THE PATHOGENESIS OF ALCOHOLIC LIVER DISEASE: A CONTROLLED CLINICAL STUDY

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Abstract

The role of ferritin in fibrogenesis of liver parenchyma in patients with alcoholic liver disease has been investigated in previous studies. Ferritin was shown to be an indirect marker of ferum deposition in liver parenchyma in alcohol liver disease. The aim of the present study was to examine the role of nitric oxide (NO) in the pathogenesis of alcoholic liver disease as well as the influence of NO on iron (ferritin) metabolism in patients with alcoholic liver disease.

Serum concentrations of NO and iron markers (iron, total iron binding capacity, ferritin) were measured in 30 male patients (aged 20–60 years) with alcoholic liver disease, as well as from a control group (30 male patients (aged 20–60 years) without liver disease). NO concentration was detected by measuring production of nitrates and nitrites using classical colorimetric Griess reactions.

There was a statistically significant increase in serum NO concentration in patients with alcoholic liver disease compared to the control group (mean ± SEM; 41,2 ± 25,3 vs. 28,9 ± 12,3 mmol/dm^3, respectively; p<0,03). Similarly, serum iron levels (18,7 ± 8,2 vs. 13,2 ± 10,2 g/100 cm^3, respectively; p<0,03) and serum total iron binding capacity (51,3 ± 13,9 vs. 41,4 ± 11,4 μmol/dm^3, respectively; p<0,005) were also significantly higher in patients with alcoholic liver disease compared to control patients. The serum concentration of ferritin was 27% higher in patients with alcoholic liver disease than in the control group; however this was not statistically significant (283,2 ± 291,0 vs. 222,9 ± 252,0 g, respectively; p<0,4). There was no correlation between NO and ferritin in the investigated groups.

These results suggest a possible role of NO and iron in the pathogenesis of alcoholic liver disease. NO and iron may be used as non-invasive predictors of liver damage. Also the role of iron in sera, and its deposition in liver parenchyma, could be used in clinical practice, especially in regards to assessing the fibrogenesis of liver parenchyma induced by ferritin.

KEY WORDS: alcoholic liver disease, nitric oxide, ferritin, fibrogenesis

Introduction

Acute consumption of alcohol (in high doses and with toxic effects) as well as chronic consumption of alcohol results in biochemical changes in liver metabolism that can be of a different intensity. Besides these alcohol caused changes of liver metabolism, depending on amounts and period of use of alcohol morphological changes are noticeable and morphological modification developed on cellular and sub cellular level (1). Clinical presentation of alcoholic hepatitis is a reflection of inflammatory changes that characterize this disease. According to laboratory findings, 70% patients with mild or moderately expressed alcoholic hepatitis will have pathohistological liver cirrhosis (2).

NO is main inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitter in gastrointestinal system. NO released as a response to nerve stimulation of myenteric plexus causes relaxation of smooth muscles (3). NO is synthesized by activation of neuronal NO synthase (nNOS) in myenteric plexus. Released NO plays important physiological role in different parts of gastrointestinal tract. NO regulates muscle tonus of lower esophageal sphincter, pylorus, Odie's sphincter and anus. NO also regulates accommodation reflex of fundus and peristaltic reflex of intestines. Previous studies proved that inhibitors of NOS delay gastric flushing and transfer of content in large intestine. Reduction of nNOS expression conjoined with lower local production of NO can be sufficient for motility disorder in GI tract.Decreased release of NO and nNOS expression are additional factors for functional dyspepsia. The role of NO in pathogenesis of liver diseases and its complications was extensively studied lately. However still there are many controversies about its role in mentioned disease. The highest attention was focused to studying of role of NO in systemic circulation and liver microcirculation. NO is involved in different processes linked to the liver transplantation, including ischemic-reperfusion damages, acute cell rejection and circulatory changes characteristic for advanced liver disease (4). Based on many investigations it is concluded that increased level of NO synthase is responsible for increased dynamics of circulation in liver cirrhosis while on the other hand decreased production of NO in hepatic microcirculation can have important role in development of liver parenchyma damage and appearance of portal hypertension (5).

Independently of NO role in pathogenesis of liver cirrhosis of non-alcoholic etiology there are only a few clinical studies that would clarify the role of NO in pathogenesis of alcohol induced liver disease. According to some studies alcohol induced damage to hepatocytes is joined with decreased level of NOS in sinusoidal (nonparenchimal liver cells), but also with increase in hepatocytes and centrilobular zones (5).Based on these findings it is believed that NO has a double role in liver damage: NO produced in sinusoidal spaces is protective and maintains tissue perfusion while the production of NO in hepatocytes is a compensatory mechanism that causes direct damage to the cells.

In patients with alcoholic liver disease compared to control group an increased production of NO was observed by the lymphomononuclear cells from peripheral blood. This production can be linked to the systemic vasodilatation in patients with liver cirrhosis (6). It is believed that NO released from monocytes can play important role in pathogenesis of alcoholic liver disease, especially alcoholic hepatitis (7). Contrary to still not well defined role of NO, the role of ferritin in pathogenesis of alcoholic liver disease is well examined. Serum iron, saturation of transferrin and serum ferritin are often increased in patients with alcoholic liver disease (8,9).

Three molecular mechanisms are proved that are important for toxic effects of free iron to cellular metabolism. First is increased creation of intermediary reactive oxygen radicals with release of lysosomal enzymes and indirect stimulation of fibrogenesis. Second one is direct stimulation of collagen synthesis with emphasize to fibrogenesis, and third one is changes in DNA with possible carcinogenesis induction (10). The role of ferritin in fibrogenesis of liver parenchyma is well characterized (11).

Serum ferritin is valuable as indirect marker of stored iron in liver parenchyma in patients with different types of liver damages, and as of that kind is valid in evaluation of different levels of liver damages (12). Recent studies of investigation of relation between ferritin and NO in serum are done in inflammatory conditions. It is established that reduced form of NO which in interaction primary with iron leads to increase of number of transferrin receptors and decreased synthesis of ferritin. Iron intracellular homeostasis is regulated with iron regulatory proteins (Iron Regulatory Proteins IRP1 i IRP2) (13). Iron regulatory proteins control synthesis of transferring

receptors and increased ferritin (14,15). Recent discoveries suppose that cellular response to increased NO production should be decreased synthesis of ferritin, due to the binding of IRP1 to ferritin mRNA, and increase of synthesis of transferring receptors due to binding of IRP1 to transferrin mRNA (16). NO has high affinity for iron. Degradation of proteins IRP1 and IRP2 caused by NO has a major role in iron metabolism during inflammation (17). There is no data in available literature on NO correlation and serum iron tests in alcoholic liver disease.

Materials and Methods

Patients

Control group of participants consisted of 30 male patients age 20 to 60 who had no pathological functional liver tests based on symptoms and objective findings of general state of health. Group of patients with alcoholic liver disease consisted of 30 male patients age 20 to 60 who were treated at the Institute for alcoholism of the University of Sarajevo Clinics Centre or hospitalized at the Gastroenterohepatology Clinic of the University of Sarajevo Clinics Centre.

Methods

Biochemical analysis and other findings

All participants underwent following laboratory findings by use of standard methods: laboratory demonstrator of stored iron, a level of serum iron, TIBC (Total Iron Binding Capacity), UIBC (Unsaturated Total Iron Binding Capacity) and saturation index as well as level of serum ferritin.

Functional liver tests were performed on all participants (albumins with total proteinogram, prothrombin time, alanine aminotranspherase, aspartate aminotranspherase, bilirubine)

Measurement of NO concentration

Blood sampling

Blood samples for determination of NO concentration were drawn by cubital venepunction. Right after sampling the samples were treated with 30% $ZnSO_4$ in order to deproteinized blood and release nitrate linked to haemoglobin (18). To one milliliter of heparinized blood dissolved with physiological solution 1:1 a 0,05 cm^3 30% solution of $ZnSO_4$ was added and after couple of minutes a sample was centrifuged for 10 minutes on 700 g. Extracted supernatants were stored in freezer at temperature of -20°C up to determination of nitrate concentration.

Procedure for NO determination

NO concentration in full blood was performed by measuring of nitrates and nitrites production using classical colorimetric Griess reaction (19). NO concentration was determined by conversion of nitrate into nitrite with help of elementary zinc and than with measurement of nitrite concentration colorimetric. In one milliliter of sample of deproteinized blood 8 mg of elementary zinc was added suspended in 0,4 ml of distilled water. Sample was also added more 0,032 cm^3 5% of vinegar acid and distilled water up to two milliliters. Sample was mixed on room temperature and centrifuged 2,5 minutes on 700 g. 1 milliliter of supernatant was taken and than 1 cm^3 of freely prepared Griess reagent (Griess reagent is a mixture of equal parts of 0,1% solution of naphtyl ethylene diamine-diklonda in distilled water and 1% sulfanilamide in 5% solution of H_3PO_4 which is mixed 12 hours before use and than left on cool place). After 10 minutes of mixture on vibrator at room temperature a light absorption was measured (optic density) by the spectrophotometer at 546 nm. NO concentration was read out of standard curve with known concentration of $NaNO_2$ (from 1,56 to 100 mmol/dm^3). Distilled water was used as a blind probe with addition of Griess reagent. Determination of NO concentration in serum of patients and control group was done at the Institute for Physiology and Biochemistry of Faculty of Medicine in Sarajevo.

Results

Results of measuring of amount of serum concentration of nitric oxide in patients with alcoholic liver disease are presented in Figure 1.

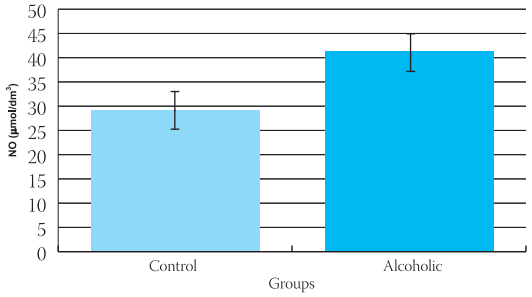

FIGURE 1. Nitric oxide concentration in serum of patients with alcoholic liver disease. Average values (X±SEM) of nitric oxide concentrations are presented in serum in control group of healthy participants and group of patients with alcoholic liver disease. Control – healthy male participants (N=30); Alcoholic liver disease – male patients with alcoholic liver disease (N=30); p – probability

Results presented in Figure 1. demonstrate that the values of NO in serum of patients with alcoholic liver disease (41,2 ± 25,3 mmol/dm^3), were higher for 42,4% compared to values determined control group of healthy participants (28,9 ± 12,3 mmol/dm^3). This difference was statistically significant (p<0,03).

Results of measuring of serum iron levels in patients with alcoholic liver disease are presented in Figure 2.

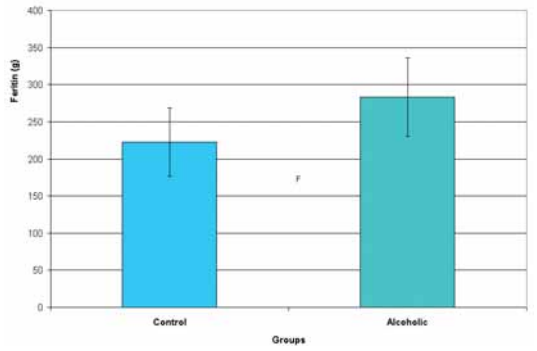

FIGURE 2. Concentration of iron in patients with alcoholic liver disease. The average values (x±SEM) of serum iron levels (µmol/dm^3) are presented for control group of healthy participants and group of patients with alcoholic liver disease. Control – healthy male participants (N=30); Alcoholic liver disease – male patients with alcoholic liver disease (N=30); p – probability

Results presented in Figure 2. demonstrate that the values of iron levels in serum of patients with alcoholic liver disease (18,7 ± 8,2 g/100 cm^3), were higher for 41,7% compared to values determined for control group of participants (13,2 ± 10,2 g/100 cm^3). This difference was statistically significant (p<0,03). Results of TIBC measuring (TIBC-Total Iron Binding Capacity) in serum of patients with alcoholic liver disease are presented in Figure 3.

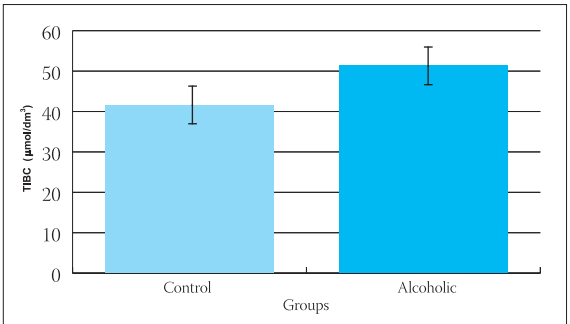

FIGURE 3. TIBC concentration (Total Iron Binding Capacity) in patients with alcoholic liver disease. The average values of TIBC concentration (X+SEM) in serum (µmol/dm^3) are presented for control group of healthy participants and group of patients with alcoholic liver disease. Control – healthy male participants (N=30); Alcoholic liver disease – male patients with alcoholic liver disease (N=30); p – probability

Results presented in Figure 3. demonstrate that the values of TIBC in serum of patients with alcoholic liver disease (51,3 ± 13,9 µmol/dm^3) were higher for 24% compared to values determined for control group of participants (41,4 ± 11,4 µmol/dm^3). This difference was statistically significant. (p<0,005).

Results of measuring of serum ferritin concentration in patients with alcoholic liver disease are presented in Figure 4.

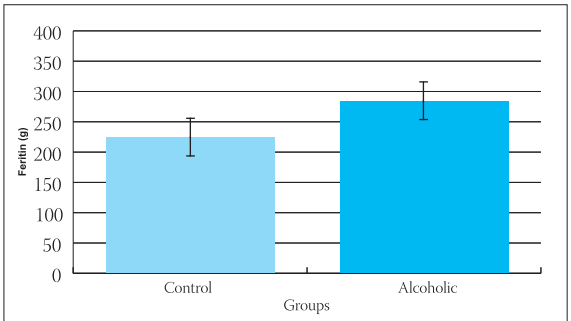

FIGURE 4. Concentration of ferritin in serum of patients with alcoholic liver disease. The average values (X+SEM) of ferritin concentration (g) are presented for control group of healthy participants and group of patients with alcoholic liver disease. Control – healthy male participants (N=30); Alcoholic liver disease – male patients with alcoholic liver disease (N=30); NS – non significant

Results presented in Figure 4. demonstrate that the values of serum concentration of ferritin in patients with alcoholic liver disease (283,2 ± 291,0 g) were for 27% higher than those determined for control group of participants (222,9 ± 252,0 g). However, this difference was not statistically significant.

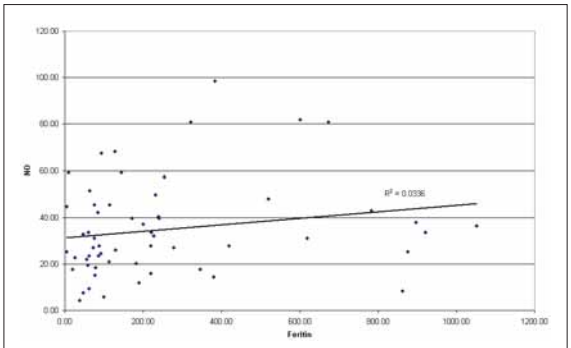

FIGURE 5. Correlation between concentration of NO and ferritin in serum of patients with alcoholic liver disease. Single results of NO concentration and ferritin are shown in serum of patients with alcoholic liver disease.

Results shown on Figure 5. demonstrate that there is no established correlation between concentration of NO and ferritin in serum of patients with alcoholic liver disease (R2=0,03).

Discussion

The results of our investigation showed that iron concentration in patients with alcoholic liver disease is significantly larger compared to the control group. This increase

was statistically significant. However values of serum ferritin concentration in patients with alcoholic liver disease were higher compared to values of control group, but this increase what was not statistically significant. Even though the values of serum iron and TIBC were significantly increased in patients with alcoholic liver disease in comparison to the control group, the level of serum ferritin was not significantly increased. These results are not in accordance to investigation done by other authors (20,21). These authors demonstrated that increase of serum iron and saturation of transferrin are usually linked to the severness of alcoholic liver disease. Contrary to this, serum ferritin can be increased in absence of significant liver damage, considering that alcohol stimulates ferritin synthesis. Experimental studies demonstrated that alcohol and iron are weakly fibrinogens when administered one by one, but if applied together contribute development of fibrosis or liver cirrhosis (22,23). These authors consider that histochemically positive iron in liver and serum ferritin are significant as indirect markers of stored iron in liver of patients with moderately expressed hepatocellular damage. Results of our investigation partly confirm results of quoted authors. We also detected increased levels of serum iron, ferritin and TIBC in patients with alcoholic liver disease as well as positive correlation between Fe and TIBC, but contrary to mentioned investigation we established negative correlation between iron and ferritin. By analyzing results of our investigation as well as former investigation of other authors we believe that the damage of liver parenchyma can be attributed to fibrogenesis of liver parenchyma caused by the serum iron and ferritin. While investigating other potential causes to liver damage caused by the alcohol, many investigations, especially in recent years, have been undertaken about the controversial role of NO in pathogenesis of liver disease with alcoholic etiology. Results of our study demonstrated that patients with alcoholic liver disease had higher values of NO in serum compared to the values of the control group of patients. This increase was statistically significant. Precise mechanism by which ethanol causes release of NO and place on which NO acts under the ethanol influence is still unknown. Results of our investigation are in accordance to the results of authors who in cases of increased concentration of NO in serum proved lower concentration of ferritin than expected. According to the results of our investigation between the concentration of NO and ferritin there is no established correlation. Since the influence of NO to ferritin concentration as indirect predictor of liver damage is not investigated it is hard to give an explanation of mechanism based on our investigation by which NO reduces concentration of ferritin in serum and decreases iron storage in liver parenchyma and therefore fibrogenesis of liver parenchyma. We believe that increased concentration of NO in hypoxia present in alcohol metabolism with interaction with inflammatory cytokines in liver parenchyma during alcoholic hepatitis leads to increased number of transferrin receptors and therefore increased binding of iron to transferrin. In conditions of complete saturation of transferrin with iron its serum concentration is decreased and its storage to pools. According to mentioned, besides many negative effects expressed by NO and which lead to damage to the liver parenchyma in alcoholic liver disease its role in iron metabolism in this disease can be characterized as positive taken into consideration that iron participates in fibrosing of liver parenchyma.

Conclusion

These results suggest a possible role of NO and iron in the pathogenesis of alcoholic liver disease. NO and iron may be used as non-invasive predictors of liver damage.

Also the role of iron in sera, and its deposition in liver parenchyma, could be used in clinical practice, especially in regards to assessing the fibrogenesis of liver parenchyma induced by ferritin.

References

(1) Kuntz E., Kuntz H.D. Hepatology, Principles and Practice 2002;28: 470-485.

(2) Levitsky J., Maillard M.E. Diagnosis and Therapy of Alcoholic Liver Disease. Semin. Liver Dis. 2004; 24 (3): 233-247.

(3) Takahashi T. Patophysiological significance of neuronal nitric oxide synthase in the gastrointestinal tract. J. Gastroenterology 2003; 38 (5):421-430.

(4) Shah V., Kamath P.S. Nitric oxide in liver transplantation:pathobiology and clinical implications. Liver Transpl. 2003; 9(1):1-11.

(5) Farzaneh-Far R., Moore K. Nitric oxide and the liver, Liver 2001;21:161-174.

(6) Congress Short Report Falk Symposium, Steatohepatitis (NASH and ASH) October, 2000.

(7) Hunt N.C., Goldin R.D. Nitric oxide production by monocites in alcoholic liver disease. J. Hepatol.1992; 14 (2-3):146-150.

(8) Vucelić B. Gastroenterologija i hepatologija 2002; II dio Hepatologija. Medicinska naklada, Zagreb. 2002; 1201-1208.

(9) Vrhovac B., Bakran I., Granić M., Jakšić B., Labar B., Vucelić B. Interna medicina. Medicinska biblioteka, Zagreb. 1997; 1040-1042.

(10) Kim S., Ponka P. Role of nitric oxide in cellular iron metabolism. Biometals 2003; 16 (1) 125-135.

(11) Graudal N., Milman N., Galloe A.M., Christoffersen P. Distribution of liver haemosiderin iron in 187 patients with various types of hepatic diseases. British Medical Journal 1996;104 (3):220-226.

(12) Wikstad I., Peeterson B.A., Elinder G. A comparative study of size and function of the remnant kidney in patients nephrectomized in childhood for Wilm's tumor and hydronephrosis. Acta Pediat. Scand. 1986;75: 408-414.

(13) Sangwon K., Prem P. Nitrogen monoxide-mediated control of ferritin synthesis: Implications for macrophage iron homeostasis. PNAS (Proceedings of the National Academy of Scienes of USA. 2002; 99: 12214-12219.

(14) Kim S., Ponka P. Nitric oxide-mediated modulation of iron regulatory proteins: implication for cellular iron homeostasis. Blood Cells Mol. Dis. 2002; 29 (3):400-410.

(15) Watts R.N., Ponka P. Effects of nitrogen monoxide and carbon monoxide on molecular and cellular iron metabolism: mirror-image effectors molecules that target iron. Biochem. J. 2003; 369 (3): 429-440.

(16) Bouton C., Drapier J.C. Iron regulatory proteins as NO signal transducers. SciSTKE 2003; (182):pe17.

(17) Mc Naughton L., Puttagunta L., Martinez-Cesta M.A., Kneteman N., Mayers I., Moqbel R. Distribution of nitric oxide syntethase in normal and cirrhotic human liver. Proc. Natl. Acad. Sci. USA 2002; 24:99(26):17161-17166.

(18) Lowry O.H., Rosenbrough N.J., Farr A.L., Randall R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1991; 193-265.

(19) Grees L.C., Wagner D.A., Glogowski J. Analysis of nitrate, nitrite and 15 N nitrate in biological fluids. Anal. Bichem. 1982; 126:131-138.

(20) Moirand R., Kerdavid F., Loreal O. Regulation of ferritin expresion by alcohol in a human hepatoblastoma cell line (HEP G2) Alcohol Clin. Exp. Res. 1990;14:847-852.

(21) Moirand R., Kerdavid F., Loreal O. Regulation of ferritin expresion by alcohol in a human hepatoblastoma cell line and in rat hepatocyte cultures. J. Hepatology 1995;23:431-439.

(22) Stal P., Hultcrantz R. Iron increases ethanol toxicity in rat liver. J. Hepatol. 1993; 17:108-115.

(23) Tsukamoto H., Horne W., Kamimura S. Experimental liver cirrhosis induced by alcohol and iron. J. Clin. Invest. 1995; 96:620-630.