Neamon-Hepa on Carbon Tetrachloride-Induced Hepatotoxicity: Antioxidant Properties

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Abstract

The present study was undertaken to investigate whether Neamon-Hepa treatment provides antioxidant protection from chronic liver injury induced in mice by a long-term CCl_4 administration. The Neamon-Hepa capsule, a combination indigene drug, contains L-arginine, Spironolactone and biopreparation from the *Spirulina platensis* - BioR. Carbon tetrachloride chronic treatment for three weeks significantly decreased the hepatic antioxidant enzyme activities - almost twofold superoxide dismutase enzyme activity, catalase activity, glutathione peroxidase activity in liver tissue and induced a marked elevation almost twofold of thiobarbituric acid reactive substances levels in the plasma and liver tissue. Treatment with the Neamon-Hepa following experimental liver damage, in a dose-dependent way, resulted in a marked augmentation of antioxidant enzyme activities and reduction of lipid peroxidation levels.

Key words: Neamon-Hepa, induced hepatotoxicity, antioxidant properties

Антиоксидантные возможности фармацевтического препарата Неамон-Хепа при токсическом гепатите, индуцированным тетрахлоридом углерода

Целью данной работы является исследование антиоксидантного воздействия лекарственного препарата Неамон-Хепа у мышей с токсическим гепатитом, индуцированным тетрахлоридом углевода. В состав комбинированного медикамента Неамон-Хепа входит аргинина аспартат, БиоР и спиронолактон. Хроническая интоксикация в течении 3 недель с CCl_4 снизила в два раза активность супероксиддисмутазы, каталазы, глутатионпероскидазы в печеночной ткани и повысила вдвое степень тиобарбитуровой кислоты в печеночной ткани и в сыворотке крови. Применение препарата Неамон-Хепа значительно уменьшило степень процессов липидного пероксидирования и увеличило активность антиоксидативных энзимов.

Ключевые слова: Неамон-Хепа, токсический гепатит, антиоксидантный эффект

Introduction

The liver has versatile functions and plays several important roles in metabolism, including biosynthesis of plasma proteins, gluconeogenesis and detoxification. Although the liver has strong regenerative ability, when cellular loss exceeds a certain threshold, the insufficient functions cause hepatic failure, leading to liver disease.

Oxidative and nitrosative stress are common pathogenetic mechanisms contributing to the initiation and progression of hepatic damage in a variety of liver disorders. Highly reactive transient chemical species, i.e., superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^1O_2$) and nitric oxide ($^{\cdot}NO$), have been implicated in the aetiology of degenerative diseases, including some hepatopathies [1]. These molecules, which are the byproducts of normal aerobic metabolism, are highly reactive and thus lead to the denaturation of biomolecules such as proteins, lipids and nucleic acids, resulting in injury to cells, tissues and organs. Cell damage occurs when there is an excess of reactive species or a defect of antioxidant molecules. Experimental research on the delicately regulated molecular strategies whereby cells control the balance between oxidant and antioxidant molecules has progressed in recent years [2, 3]. On the basis of this evidence, antioxidants represent a logical therapeutic strategy for the treatment of chronic liver disease.

Carbon tetrachloride (CCl_4) is a well established and widely used hepatotoxin and the principle cause of CCl_4-induced liver injury is proposed to be lipid peroxidation by free radical derivatives of CCl_4. In the liver, CCl_4 is rapidly metabolized by mixed function cytochrome P450 oxygenases of the endoplasmic reticulum resulting in the generation of the trichloromethyl radical ($CCl_3^{\cdot}$). This radical can also react with oxygen to form its highly reactive derivative trichloromethyl peroxy radical ($CCl_3OO^{\cdot}$). Both radicals initiate a chain reaction leading to lipid peroxidation, changes in membrane permeability, and loss of calcium homeostasis. In addition, tissue lipid levels increase. The initial cellular injury subsequently takes the form of inflammation, and the chronic effects of CCl_4 exposure include fatty degeneration and fibrosis that may ultimately culminate in extensive cell damage and death [4].

Neamon-Hepa is a multicomponent pharmaceutical product thought to have hepatoprotective properties and

can prevent the progression of chronic liver diseases. The Neamon Hepa capsule contains L-arginine, Spironolactone and biopreparation from the *Spirulina platensis* - BioR. In a previous study we have shown that treatment of mice with the Neamon Hepa concomitant with the CCl_4 produced an alleviation of the hepatic injury to a considerable extent which was reflected by the ability of the compound to lower the elevated liver/body weight index and the raised serum enzyme activities resulting from the administration of CCl_4 alone [5].

The present study was undertaken to investigate whether Neamon Hepa treatment provides antioxidant protection from chronic liver injury induced by a long-term CCl_4 administration.

Material and Methods

Male C57/Bl6 mice, aged 12 weeks and weighing 27-30 g, were used in the present study. The animals were housed according to our institution's animal-care guidelines. The cages were placed in ventilated closed rack at constant temperature and humidity with a 12-hour light/dark cycle. They were fed using commercially standard mouse diet and sterilized water ad libitum.

Mice were divideded into the following five study groups:
Control (n = 6);
CCl_4 (n = 6);
CCl_4 received Neamon-Hepa 10 mg/kg (n = 6);
CCl_4 received Neamon-Hepa 50 mg/kg (n = 6);
CCl_4 received Neamon-Hepa 100 mg/kg (n = 6);

Animals received an subcutaneos 1 ml/kg injection of CCl_4 (Sigma) in sesame oil (1:1 ratio) twice weekly (Monday and Thursday) for three weeks. Control animals were injected with an equivalent amount of sesame oil. Neamon-Hepa (Eurofarmaco SA) capsules (834 mg) were dissolved initialy in 4.2 ml etanol and then removed from it (ex. 168 µl for group V) was added in to 100 ml of drinking water.

Subsequently, on the fifth day after last CCl_4 injection, the mice of each group were sacrificed by exsanguination, the blood was collected and plasma was separated from it and frozen for laboratory tests. Livers were quickly excised under ice-cold conditions. A portion of the liver was thoroughly washed three times with saline to remove maximal possible residual blood. The samples were frozen and stored at -70°C until analysis.

Lipid peroxidation was estimated by measurement of thiobarbituric acid reactive substances (TBARS) in the liver tissue by the method of Uchiyama and Mihara [6]. Plasma TBARS level were measured according to the method of Buege and Aust [7]. It is expressed as micromoles per liter for plasma and as micromoles per gram weight liver tissue.

Superoxide dismutase (SOD) enzyme activity was analyzed according to the method of Beauchamp and Fridovich [8]. Catalase (CAT) activity was estimated by the procedure of Thomson et al. [9]. Glutathione peroxidase (GPx) activity was measured by the method of Paglia and Valentine [10]. SOD, CAT and GPx activities were expressed as international units per milligram protein.

Results are presented as means ± standard error of the mean (SEM). Comparisons between groups were carried out using t-test. Differences with $P < 0.05$ were regarded as statistically significant.

Results

Carbon tetrachloride chronic treatment significantly decreased the hepatic antioxidant enzyme activities. The SOD, CAT, and GPx activities in CCl_4-treated mice were 52.1%, 57.4% and 66.6% respectively, compared to the control mice. At the same time, CCl_4 chronic treatment induces a marked elevation of TBARS levels, which were assessed as an indicator of lipid peroxidation. The level of TBARS were more than twofold greater in the plasma and liver tissue from CCl_4-treated group.

Treatment with the Neamon-Hepa following experimental liver damage resulted in a marked augmentation of antioxidant enzyme activities and reduction of lipid peroxidation levels. As is shown in the table, the most consistent evidence for beneficial effects came from a maximal daily dosage of Neamon-Hepa. All doses of Neamon-Hepa increased the decreased antioxidant enzyme activities in CCl_4-treated mouse liver. This effect was dose dependent, with the highest level of antioxidant enzymes activities occurring in the group that received Neamon-Hepa 100 mg/kg. For example, in this group SOD, CAT and GPx activities increased more than 65%, 55% and 30% respectively, compared to the CCl_4-treated group. On the other hand, dose-dependent treatment with Neamon-Hepa significantly decreased the increased level of TBARS in both the plasma and liver tissue. As a result, treatment

Table 1

Effects of Neamon-Hepa on antioxidant enzymes (SOD, CAT, GPx) activities in the liver and TBARS in the liver and plasma of CCl_4-treated and control mice

Group	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	TBARS	
				Liver (µM/g tissue)	Plasma (µM/L)
I	25.7 ± 0.96	18.8 ± 0.80	11.1 ± 0.52	0.48 ± 0.05	0.24 ± 0.02
II	13.3 ± 1.55#	10.8 ± 0.72#	7.4 ± 0.52#	1.27 ± 0.08#	0.62 ± 0.09#
III	16.8 ± 0.90*	14.2 ± 0.87*	7.9 ± 0.25	1.03 ± 0.06*	0.40 ± 0.08*
IV	19.4 ± 0.85**	15.8 ± 0.83**	8.9 ± 0.35*	0.84 ± 0.05**	0.36 ± 0.08*
V	22.3 ± 1.18**	17.0 ± 0.55**	9.7 ± .37**	0.67 ± 0.06**	0.32 ± 0.07**

The values are mean ± S.D.
# $p < 0.01$, as compared to the control group;
* $p < 0.05$, ** $p < 0.01$, as compared to the second group.

with 100 mg/kg of Neamon-Hepa decreased TBARS levels from 90% to 70% in the liver tissue and plasma respectively compared to CCl_4-treated mice. Although, CCl_4-induced lipid peroxidation effcts were significantly attenuated by Neamon-Hepa treatment, value were still higher than those found in the control group.

Discussion

The antioxidant activity of Neamon-Hepa was measured by its protection against CCl_4-induced liver damage in mice. Liver damage induced by carbon tetrachloride is the best characterized system of xenobiotic-induced hepatotoxicity and is a commonly used model for the screening of in vivo antioxidant and hepatoprotective activity of drugs. Several mechanisms underlying this toxicity have been suggested. The hepatotoxic effects of CCl_4 are largely due to its active metabolite, trichloromethyl radical. These activated radicals bind covalently to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides. This lipid peroxidative degradation of biomembranes is one of the principle causes of hepatotoxicity of CCl_4 [4, 11].

Previously, we have shown that Neamon-Hepa treatment decreased CCl_4-induced liver damage in mice. The activity of serum alanine aminotransferase, aspartate aminotransferase and level of total bilirubin decreased more than twofold in the group of animals that received 100 mg/kg of Neamon-Hepa daily. At the same time, we found a marked lowering of cholesterol level, lactate dehydrogenase and alkaline phosphatase activities [5].

In the present investigation, exposure to chronic doses of CCl_4 caused decreases in the hepatic SOD, CAT and GPx activities. The glutathione-dependent enzymes SOD, CAT, and GPx represent co-ordinately regulated major cellular defense systems against oxidative stress. Glutathione content in the liver contributed to the maintenance of cell homeostasis by scavenging free radicals resulting from physical or chemical injuries. It has been suggested that the lipid peroxides generated after CCl_4 intoxication are eliminated by GPx in the presence of glutathione, thus curbing the propagation of lipid peroxidation [12]. In our experiments, the significant decrease in hepatic GPx activity following CCl_4 exposure was partially restored by Neamon-Hepa therapy.

SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. Decrease in enzyme activity of SOD is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in live injury [13]. SOD acts as a cellular deference element against potentially harmful effects of superoxide ions by catalyzing the dismutation of these ions. Superoxide radical is one of the main causes of oxygen cytotoxicity, for it is the first oxygen radical produced *in vivo*, and lasts for longer time than other radicals. Simultaneously, Neamon-Hepa treatment causes a significant increase in hepatic SOD activity; more than 65% in the group that received a higher dose.

Cathalase is a haemoprotein and it protects cells from the accumulation of hydrogen peroxide by dismutating it to from N_2O and O_2 or by using it as an oxidant in which it works as a peroxidase [14]. A large reduction in the CAT activity in our CCl_4-treated mice may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. In contrast, administration of Neamon-Hepa show a significant dose-dependent increasing in CAT activity. Liver TBARS level is considered to be a valuable indicator of toxicant induced hepatic damage from production of free radicals [14]. Elevated level of TBARS observed in our CCl_4-treated mice indicates excessive formation of free radicals and activation of lipid peroxidation system resulting in hepatic damage. The significant decline in the concentrations of these constituents in the liver and plasma of Neamon-Hepa-treated mice unveils its antioxidant efficacy. We suppose that all three essential components L-arginine, Spironolactone and BioR (biopreparation from the Spirulina platensis) inserted in the Neamon-Hepa together are able to increase antioxidant activity and to lower TBARS levels.

It has been reported that the use of Spironolactone in the combined therapy effectively attenuates oxidative stress in patients with chronic kidney disease [15] and prevents oxidative stress in uremic rats [16]. Also, it was shown that intervention with antioxidants and l-arginine reduced the activation of redox-transcription factors and increased eNOS expression in cells and in vivo [17, 18].

The antioxidant property of various *Spirulina platensis* extracts under CCl_4-induced hepatic damage had been reported [19, 20]. Recently, new experimental data were presented on the influence of the complexes of Fe(III), Mn(II), Zn(II), Co(II) on the synthesis of antioxidants in the biomass of spirulina and dunaliella. Simultaneously, they define a new procedure for obtaining antioxidant preparations on the basis of spirulina's and dunaliella's biomass [18]. Further studies find a significant decline in malondialdehyde, lipid hydroperoxides and conjugated dienes with rise in SOD, catalase, GPx, glutathione, vitamin-E and vitamin-C levels in the hepatic and renal tissues when rats were given therapy with purified cyanobacterial phycoerythrin together with CCl_4 intoxication for 4 weeks [19]. A more detailed scientific report confirms the fact that Phycocyanin, a biliprotein from Spirulina platensis is a potential therapeutic agent in oxidative stress-induced diseases. Furthermore, it was able to scavenge alkoxyl, hydroxyl and peroxyl radicals and to react with peroxinitrite and hypochlorous acid. It also inhibits microsomal lipid peroxidation induced by Fe^{+2}-ascorbic acid or the free radical initiator 2,2' azobis (2-amidinopropane) hydrochloride in vitro and reduces CCl_4-induced lipid peroxidation *in vivo* [20].

In conclusion, the results in this study indicate that Neamon-Hepa treatment can ameliorate CCl_4 stress-induced oxidative liver injury. Nevertheless, further research must be carried out to elucidate the mechanisms of the hepatoprotective antioxidant effect by Neamon-Hepa at the molecular level.

References

1. Rudic V. Bio R: studii biomedicale şi clinice. Chisinau, 2007;375.
2. Ahmed N, Thornalley PJ, Lüthen R, et al. Processing of protein glycation, oxidation and nitrosation adducts in the liver and the effect of cirrhosis. *J Hepatol*. 2004;41(6):913-9.
3. Urtasun R, Conde de la Rosa L, Nieto N. Oxidative and nitrosative stress and fibrogenic response. *Clin Liver Dis*. 2008;12(4):769-90.
4. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol*. 2003;33(2):105-36.
5. Parii, B. Todiraş, M, Parii S, et al. Efectele protectoare ale remediului autohton Neamon-Hepa în hepatita indusă cu tetraclorură de carbon. *Curierul medical.* 2010;2:31-35.
6. Uchiyama M, Mihara M. Determination of Malonaldehyde Precursor in Tissues by Thiobarbituric Acid Test. *Anal. Biochem*. 1978;86:271–8.
7. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol*. 1978;52:302-10.
8. Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem*. 1971;44(1):276-87.
9. Thomson JF, Nance SL, Tollaksen SL. Spectrophotometric assay of catalase with perborate as substrate. *Proc Soc Exp Biol Med.* 1978;157(1):33-5.
10. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med.* 1967;70(1):158-69.
11. Recknagel RO, Glende EA Jr, Dolak JA, et al. Mechanisms of carbon tetrachloride toxicity. *Pharmacol Ther*. 1989;43(1):139-54.
12. Nishida K, Ohta Y, Ishiguro I. Gamma-glutamaylcysteinylethyl ester attenuates progression of carbon tetrachloride induced acute liver injury in mice. *Toxicology*. 1998;126:55–63.
13. Curtis SJ, Moritz M, Snodgrass PJ. Serum enzymes derived from liver cell fractions. I. The response to carbon tetrachloride intoxication in rats. *Gastroenterology*. 1972;62(1):84-92.
14. Cabré M, Camps J, Paternáin JL, et al. Time-course of changes in hepatic lipid peroxidation and glutathione metabolism in rats with carbon tetrachloride-induced cirrhosis. *Clin Exp Pharmacol Physiol.* 2000;27(9):694-9.
15. Renke M, Tylicki L, Knap N, et al. Spironolactone attenuates oxidative stress in patients with chronic kidney disease. *Hypertension*. 2008;52:e132–e133.
16. Michea L, Villagrán A, Urzúa A, et al. Mineralocorticoid receptor antagonism attenuates cardiac hypertrophy and prevents oxidative stress in uremic rats. *Hypertension*. 2008;52(2):295-300.
17. de Nigris F, Lerman LO, Ignarro SW, et al. Beneficial effects of antioxidants and L-arginine on oxidation-sensitive gene expression and endothelial NO synthase activity at sites of disturbed shear stress. *Proc Natl Acad Sci USA*. 2003;100(3):1420-5.
18. Wallner S, Hermetter A, Mayer B, et al. The alpha-amino group of L-arginine mediates its antioxidant effect. *Eur J Clin Invest*. 2001;31(2):98-102.
19. Soni B, Visavadiya NP, Madamwar D. Ameliorative action of cyanobacterial phycoerythrin on CCl_4-induced toxicity in rats. *Toxicology*. 2008;248(1):59-65.
20. Romay Ch, González R, Ledón N, et al. C-phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. *Curr Protein Pept Sci*. 2003;4(3):207-16.

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The Spectrum of Mesenchymal Tumors of the Skin

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The Spectrum of Mesenchymal Tumors of the Skin

Cutaneous tumours of mesenchymal origin are relatively uncommon tumors, originating from non-epithelial skin structures and characterized by clinico-pathological heterogeneity. Being classified histologically according to the mature tissue they resemble, these heterogeneous mesenchymal neoplasms show a broad range of differentiation and form the largest group of skin tumors. The aim of the study was to analyze the incidence patterns and clinical peculiarities of cutaneous mesenchymal tumours according to the histologic type. Research trials were conducted in the Institute of Oncology of the Republic of Moldova and included 1121 patients with cutaneous tumors of mesenchymal origin, surgically treated in the period 2004-2008, including 1036 (92.4%) benign tumors and 85 (7.6%) malignant tumors. The most frequent benign tumors were hemangioma (52.4%) and dermatofibroma (33.7%). Kaposi sarcoma was the most common form of cutaneous malignant mesenchymal tumors, accounting for 43.53% of cases. 41.18% of skin tumors of mesenchymal origin were reprezented by dermatofibrosarcoma protuberans, which is a locally aggressive tumor with a high recurrence rate and little metastatic potential. This study demonstrated variation by age, sex and anatomic location in patients with cutaneous sarcomas according to the histologic type.

Key words: cutaneous mesenchymal tumors, Kaposi sarcoma, dermatofibrosarcoma protuberans.