ABSTRACT – Background - Hepatic cirrhosis is the final stage of liver dysfunction, characterized by diffuse fibrosis which is the main response to the liver injury. The inhalatory carbon tetrachloride is an effective experimental model that triggers cirrhosis and allows to obtain histological and physiological modifications similar to the one seen in humans. Aim - To investigate the effects of N-acetylcysteine (NAC) on the fibrosis and oxidative stress in the liver of cirrhotic rats, analyzing liver function tests, lipoperoxidation, activity of glutathione peroxidase enzyme, collagen quantification, histopathology, as well as the nitric oxide role. Methods - The animals were randomly in three experimental groups: control (CO); cirrhotic (CCl4) and CCl4 + NAC. Evaluate the lipid peroxidation, the glutathione peroxidase enzyme, the collagen and the expression of inducible nitric oxide synthase (iNOS). Results - The cirrhotic group treated with N-acetylcysteine showed trough the histological analysis and collagen quantification lower degrees of fibrosis. This group has also shown less damage to the cellular membranes, less decrease on the glutathione peroxidase levels and less expression of inducible nitric oxide synthase when matched with the cirrhotic group without treatment. Conclusion - N-acetylcysteine seems to offer protection against hepatic fibrosis and oxidative stress in cirrhotic rat livers.


INTRODUCTION

Cirrhosis is a progressive chronic liver disease which constitutes an irreversible stage of liver dysfunction, characterized by a process of diffuse fibrosis and formation of micro and macro nodules, with distortion of the normal architecture of the hepatic parenchyma. Both the fibrotic cicatization and the hepatocellular regeneration constitute the main responses of the liver tissue to the numerous aggressions of inflammatory, toxic, metabolic or congestive nature that it suffers. Besides the functional and morphologic alterations, studies indicate alterations in the antioxidant defense mechanisms of cirrhotic livers. These alterations, by increasing the generation of reactive oxygen species and free radicals, enhance the lipoperoxidation process, which may make the liver tissue more susceptible to damage. Alterations in the hepatic oxidant/antioxidant mechanisms have been proposed. The imbalance, favoring the oxidants, largely contributes to hepatic necrosis. The oxidative stress resulting from the imbalance of pro-oxidant/antioxidant mechanisms leads to oxidative alterations in the lipids of cell membranes, composed of polyunsaturated fatty acids, which can lead to the loss of functionality through alterations in cell homeostasis. The chain propagation of lipid peroxidation forms reactive compounds with oxygen or nitrogen, enhancing its damage. This aggression interferes with the cell mechanisms, enzyme mechanisms, genic expression and second messenger mechanisms. The oxidative stress generated by various conditions activates the hepatic stellate cells and it has a possible link between the hepatic chronic damage and liver fibrosis, leading to cirrhosis as a final endpoint.

A classic example of oxidative damage to the liver tissue is a model of inhalatory carbon tetrachloride (CCl4). This model is effective in triggering fibrosis and in the long run cirrhosis, and allows to obtain histologic and hemodynamic modifications that are characteristic of the liver cirrhosis and portal hypertension seen in humans. Its action is based on the potent hepatotoxicity of CCl4, which leads to hepatic damage by means of free radicals formed during its metabolism: trichloromethyl (-CCl3) and trichloromethyl peroxy (-OCCl). These free radicals, when metabolized by the p-450 cytochrome, generate oxidative stress, which leads to hepatic damage and a subsequently irregular hepatic regeneration (fibrosis). The use of antioxidants can minimize the oxidative stress and contribute to the therapeutic in cirrhotic livers. Among the various antioxidants, N-acetylcysteine (NAC) is a small molecule which, by being freely filterable, has prompt access to the intracellular compartments. This drug has a diversity of applications, largely because of the chemical properties of the thiol moiety present in its structure. The ability of the reduced thiol moiety to sweep reactive...
oxygen species is well-established with NAC. In addition to this marked antioxidant capacity, NAC exerts an indirect protection to the liver by being hydrolyzed into cysteine, thus serving as a substrate for reduced glutathione increasing its levels. With this, it is applicable in intoxication by acetaminophen, as well as in adult respiratory distress syndrome, chronic bronchitis and cystic fibrosis.

Using a modified experimental model of liver cirrhosis induced by CCl4 inhalation, the present study was designed to evaluate the fibrosis and the oxidative stress in the liver of cirrhotic rats, through the collagen quantification, the histopathologic aspect of the hepatic tissue, the liver function tests, the damage to cell membranes through lipoperoxidation, the activity of enzyme glutathione peroxidase as well as the nitric oxide role in this process.

**METHODS**

**Animals**

The procedures with the animals complied with the guidelines of the Health Research and Ethics Committee of the Research and Postgraduate Group of the “Hospital de Clínicas de Porto Alegre, RS, Brazil”.

Fifteen male Wistar rats (mean weight = 250 g) were used. They were obtained from the laboratory animals facility of the Federal University of Rio Grande do Sul and were housed in polypolyethylene cages, in groups of five, and kept under a 12h light/dark cycle and controlled room temperature. The animals were fed with rat chow in a quantity of 16 g per animal/day (Purina-Nutripal, Porto Alegre, RS, Brazil). The water was given ad libitum.

**Groups and treatment**

The animals were divided in 3 groups: I- control; II- cirrhotic; III – cirrhotic + NAC.

NAC (Sigma, St. Louis, MO, USA) was administered i.p. at a dose of 10 mg/kg/day. It was initiated at week 10th, when histological analyses and liver function tests indicated that the animals were already cirrhotic, and was carried out until the date of sacrifice.

**Cirrhosis induction**

For enzymatic induction and to shorten the time needed for the development of cirrhosis, phenobarbital (0.3 g/L) was added to the animal’s drinking water 7 days before the first inhalation and, later, throughout the experiment. The CCl4 group was exposed to the aggressive agent twice a week (mondays and fridays), according to JIMENEZ and CLARIÁ. Fifteen male Wistar rats (mean weight = 250 g) were used at a concentration of 1 mg/mL, according to LOWERY et al. The samples were measured in the spectrophotometer at 625 nm, being expressed in mg/mL, for expression of the values of antioxidant enzymes and TBARS.

**Biochemical analyses**

The animals were sacrificed at week 16th. First they were anesthetized with ketamine (100 mg/kg) and xylazinë (50 mg/kg) i.p.. Then blood was sampled from their retro-ocular sinus for determination of total (TB) and direct bilirubin (DB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP) and albumin (ALB). Serum levels of TB, DB, AST, ALT, AP and ALB were determined by commercial kits. The liver was removed and stored at -70°C for posterior quantification of thiobarbituric acid reactive substances (TBARS), enzymes, collagen, nitrites and nitrates, as well as histology.

**Homogenate**

After complete excision of the liver and withdrawal of the material for fixation in formalin, the region corresponding to the medial portion of the right lobe (1.5 x 0.5 x 0.5 cm) was resected for homogenization. The liver fragment, before homogenization, was kept at a temperature of 2°C in physiological solution. Nine milliliters of phosphate buffer (140 mM KCl, 20 mM phosphate, pH 7.4) per gram of tissue was placed into the homogenization tube. The liver was homogenized with Ultra-Turrax (IKA- WERK) at a temperature of 2°C for 30s. This homogenate was centrifuged (SORVALL RC-5B Refrigerated Superspeed Centrifuge) at 3.000 rpm for 10 min. The supernatant was transferred by pipette to Eppendorf tubes and the precipitate was discarded. The samples were conditioned at -70°C for later use in the several techniques used.

**Proteins**

In this method a bovine albumin solution (SIGMA) was used at a concentration of 1 mg/mL., according to LOWRY et al. The samples were measured in the spectrophotometer at 625 nm, being expressed in mg/mL, for expression of the values of antioxidant enzymes and TBARS.

**TBARS (evaluation of thiobarbituric acid reactive substances)**

The amount of aldehydic products generated by lipid peroxidation was quantified by the thiobarbituric acid reaction using 3 mg of protein per sample. Results were referred as TBARS. The samples were incubated at 90°C for 30 min after adding 500 µL of 0.37% thiobarbituric acid in 15% trichloroacetic acid, then centrifuged at 4°C at 2000 x g for 15 min. Spectrophotometric absorbance was determined in the supernatant a 535 nm.

**Glutathione peroxidase**

The activity of antioxidant enzyme glutathione peroxidase was evaluated by the NADPH oxidation rate in the presence of reduced glutathione (GSH) and glutathione reductase (GR). The sodium azide (NaN₃) was added to inhibit the activity of catalase. Finally, the decrease in NADPH absorbance at 340 nm was
measured. The activity of enzyme glutathione peroxidase is expressed as $\mu$mol/min/mg of protein$^{33}$.

Collagen

Collagen concentration was determined by measuring the hydroxyproline content in fresh liver sections after acid digestion. It is read at 560 nm in the spectrophotometer and is expressed as $\mu$g/mg of protein$^{33}$.

Western Blot – iNOS

Determination of protein iNOS was performed through Western Blot using system Laemmli, 1970. The technique demands homogenization of the liver in lysis buffer (140mM NaCl, 15mM EDTA, 10% glycerol, 20mM Tris; pH 8.0) adding a mixture of protease inhibitors (protease inhibitor cocktail tablets; Roche; ref. 1836170). After homogenization it is incubated for 30 min at 4ºC, subsequently centrifuged for 30 min at 17,000 x g and 4ºC, and the supernatant is withdrawn at aliquots.

Polyacrylamine gel electrophoresis (10%) was performed. The sample was placed into each groove, comparing all the groups at the same gel. After this procedure, proteins were transferred to a nitrocellulose membrane and, after blocking unpecific bindings, it was incubated with anti-iNOS polyclonal antibody obtained from rabbit (NOS2, H-174; Santa Cruz Biotechnology). Immunoquantification was done by comparing densitometrically the values of control samples with their corresponding ones in each of the other groups considered. The results were expressed relative to the percentage of the control (100% or 189735 pixels).

Histology

For the histological examination the material was included in paraffin blocks and subsequently cut into 6 $\mu$m-thick sections on a rotating microtome. The technique of picrosirius staining in paraffin blocks and subsequently cut into 6 $\mu$m-thick sections (

The degree of fibrosis was assessed according to the score/ scale below:

- 0: Normal liver with no fibrosis
- I: Thick perivenular collagen and few collagen septa
- II: Thin septa with incomplete bridges across the portal regions
- III: Thin septa and extensive bridges
- IV: Thick septa with complete bridges across portal regions and nodular appearance
  - Mild degree: compromising of 25% of portal spaces
  - Moderate degree: compromising of 25%-75% of portal spaces
  - Severe degree: compromising of over 75% of portal spaces

Statistical analysis

The results were expressed as mean ± standard error (SEM) of (n) values. ANOVA, followed by Student Newman Keuls, was used for group comparison, and P<0.05 was considered as significant. A non-parametric test (Mann-Whitney) was used for the anatomic-pathological analysis.

RESULTS

Biochemical analysis

Concerning the markers of liver damage AST and ALT, a significant difference was observed (P<0.05) between the control (179/109 U/l) and cirrhotic groups had higher plasma levels (465.6/653.2 U/l). The NAC no reduced significantly the increase in transaminase activities in this experiment (Table 1). In the other tests performed, direct bilirubin, total bilirubin, albumin and alkaline phosphatase, this difference was not observed as all the other groups were paired.

Lipid peroxidation

The analysis of the values obtained by the method of TBARS shows a 54% increase in lipoperoxidation in the CCl4 group as compared to CO group. As the CCl4 group is compared to the CCl4 + NAC group, a decrease of 48.5% is observed, returning to the levels of the control groups (Table 1 and Figure 1).
Glutathione peroxidase

Comparing the levels of enzyme glutathione peroxidase, a decrease of 58.5% in the CCl$_4$ group was observed as compared to the control group. The CCl$_4$ + NAC group presented a 93% increase in the levels of this enzyme as compared to the cirrhotic group, levels similar to those of the control animals (Table 1 and Figure 2).

Collagen

Collagen concentration increased 5.6-fold in the cirrhotic group as compared to the control group and only 1.6-fold in the cirrhotic + NAC group, values that were statistically significant (Table 1 and Figure 3).

Histology

The histology of the animals at the 10th week shows installed cirrhosis in the animals of the CCl$_4$ groups, due to the presence of nodular fibrosis. In week 16 animals, cirrhosis becomes more severe in the CCl$_4$ group, while in the CCl$_4$ + NAC group it regresses to mild or moderate fibrosis, with alterations only in periportal spaces. No histological alterations were seen in control animals (Figure 4 A, B, C, D, E and F).

Western Blot - iNOS

The expression of iNOS was elevated in the animals of the cirrhotic group, returning to control levels in NAC- treated animals (Figure 5 A and B).
**DISCUSSION**

Liver cirrhosis induced by CCl₄ inhalation is an effective experimental method in which the hepatic lesion originates from free radical formation. The model used in this work was a modified version of the one proposed by JIMÉNEZ and CLÁRIA⁵¹; the main difference being the gradual increase in the gassed time – by 30 seconds every three sessions – thus producing progressive disarray in the hepatic parenchyma without leading to the pronounced mortality as initially observed, when 1-minute increases in the inhalation time were employed⁶⁰. Lipoperoxidation is a marker for the damage to cell membranes and, in the TBA-RS method, it is measured through the formation of an intermediate compound of lipoperoxidation, malondialdehyde. The levels of this compound were quite increased in the cirrhotic group, showing a great increase in the oxidative stress as compared to the control group. This increase may be ascribed to the generation of trichloromethyl radicals (·CCl₃), which combine with oxygen and form the trichloromethyl peroxy radical (·O₂CCl₃), after carbon tetrachloride is metabolized in the p-450 cytochrome enzymatic complex. Once formed, these compounds trigger a cascade of reactions that culminate in lipoperoxidation⁵⁰⁶. On the other hand, the cirrhotic group with NAC presented significantly decreased values, similar to those of the control group. This finding was also verified by SIMILE et al.⁶¹, using antioxidant 5´Methylthioadenosine. NAC administration seems to protect the hepatic parenchyma against the action of trichloromethyl and trichloromethyl peroxy radicals, due to its antioxidant potential. NAC acts as an antioxidant which effectively reduces oxygen reactive species. It presents potent ability to interact directly with oxidant agents, acting as a scavenger of free radicals, and it exerts an indirect effect on the antioxidant mechanism, since it contributes to restore glutathione. This drug is defined as a precursor for the synthesis of this antioxidant enzyme⁶². Glutathione peroxidase is a key enzyme in the antioxidant defense system, and it acts by catalyzing the transformation of hydrogen peroxide into water, being dependent on selenium and reduced glutathione⁶³. This enzyme also plays a major protective role in the hepatic necrosis produced by acetaminophen⁶⁴. It is depleted in the cirrhotic group, in the attempt to stabilize the oxygen reactive species formed by the high lipoperoxidation index observed in this group. In contrast, in the group treated with NAC, the activity levels of this enzyme were found to be similar to those of control animals. This may be accounted for by the fact that NAC serves as a substrate for glutathione, thus restoring the hepatic levels of this enzyme⁶⁴. On the collagen quantification, our results show a 5.6-fold elevation in the cirrhotic group as compared to the control group, while in the treated group there was a pronounced decrease (1.6-fold as compared to controls) in the levels of this build-up. This may be explained by NAC being suppressing the expression of collagen-promoting gene α, thus decreasing its synthesis. Another mechanism would be NAC’s proven ability to partially inactivate NF-κB, which is implied in the activation of collagen-producing stellate cells⁶⁵. Another factor that may be contributing in the decreased production of collagen is the decrease in lipoperoxidation triggering the cicatricial response.

Although the relationship between increased lipoperoxidation and increased collagen synthesis has been studied, some mechanisms have not yet been completely clarified. Several chronic liver diseases are characterized by a progressive accumulation of conjunctive tissue suffering fibrosis degeneration⁶⁶. It is known that there is an important relationship between chronic liver damage and liver fibrosis, and that the latter is possibly represented by oxidative stress associated with activation of stellate cells⁶⁷. When activated, stellate cells leave the quiescent state and become similar to myofibroblastic cells, which are dominant producers of the extracellular matrix⁶⁸. Several studies have demonstrated that lipoperoxidation stimulates collagen synthesis in fibroblasts and stellate cells⁶⁹. Specifically referring to the collagen increased levels observed in CCL₄–treated animals, studies have shown the decreased activity of collagenases, something which may be implied as well in the mechanism of liver fibrosis⁷₀, ⁷¹. The results observed in the measurement of collagen levels are corroborated by the histologic analysis, where a quite pronounced nodular delimitation is observed in cirrhotic animals and a decrease of it in the treated group.

The tests of liver integrity show greater tissue injury in the cirrhotic group and indicate that injury is reduced in the treated cirrhotic animals, which presented values close to those of the controls. The histological examination shows that cirrhosis induction was effective among the evaluated animals. With the technique of picrosirius staining, less fibrosis was observed in the CCl₄ + NAC group than in the CCl₄ group, suggesting protection by NAC. Nitric oxide is a vasodilator detected in the vascular system, but it is nevertheless found in all tissues. It is synthesized from L-arginine by an enzyme, nitric oxide synthase, which possesses three isoforms: NOS-1 (ne-NOS), a constitutive isoform isolated from neuronal sources; NOS-2 (iNOS), an induced isoform that is stimulated through inflammatory cytokines such as the tumor necrosis factor and interleukins 1 and 6; and NOS-3 (eNOS), a constitutive isoform isolated from endothelial cells⁷². In cirrhotic animals, iNOS shows increased expression due to liver damage and the inflammatory mediators released as result of it. In our study we observed that generic expression of iNOS is increased in the animals of the cirrhotic group, probably as a result of the liver injury stemming from the toxic metabolites of CCl₄, as already demonstrated by PAVANATO et al.⁷³. In the group treated with NAC, a lesser expression of iNOS was observed, possibly due to a reduction of the inflammatory process triggered by carbon tetrachloride. This decrease is due, as remarked by PASTOR et al.⁷⁴, to the direct and indirect antioxidant actions of NAC, inhibiting the expression/liberation of cytokines and the expression of adhesion molecules and of NFκB⁷⁵.
CONCLUSION

In conclusion, NAC seems to offer protection against hepatic fibrosis in the liver of cirrhotic rats, decreasing lipoperoxidation and iNOS expression and regenerating glutathione levels.

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