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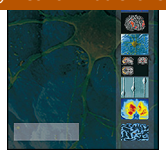

Effect of *N*-acetylcysteine on the spinal-cord glutathione system and nitric-oxide metabolites in rats with neuropathic pain

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HIGHLIGHTS

- NAC changes oxidative parameters in spinal cord of CCI rats.
- NAC has antinociceptive effect in rats with chronic constriction of sciatic nerve.
- NAC decrease nitric oxide metabolites in spinal cord of CCI rats.

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ABSTRACT

Since *N*-acetylcysteine (NAC) is a donor of cysteine, we studied the relationship between NAC and concentration of oxidized and reduced glutathione (GSH/GSSG ratio), and glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST) activities in the lumbosacral spinal cord of rats with chronic constriction injury (CCI) of the sciatic nerve that received NAC (150 mg/kg/day, i.p.) or 0.9% saline solution for 3 or 10 days. Hydrogen peroxide (H_2O_2) and nitric-oxide (NO) metabolites were also measured. Von Frey hair and hot-plate tests showed hyperalgesia at day 1 in CCI rats. Hyperalgesia persisted at all other times in saline-treated CCI rats, but returned to pre-injury values in NAC-treated CCI rats after 3 postoperative days. GST activity and the GSH/GSSG ratio increased in saline-treated CCI rats, while the NAC treatment increased GST and GPx activities at day 10, with no significant change in the GSH/GSSG ratio. NAC treatment did not affect H_2O_2 levels, but it reduced NO metabolites in CCI rats 3 days after the surgery. Thus, the anti-hyperalgesic effect of NAC appears not to involve its action as a cysteine precursor for GSH synthesis, but involves a decrease in NO.

1. Introduction

N-acetylcysteine (NAC) produces a broad range of effects, which include reduction in pain [1] and neuroprotection after spinal cord injury [2] and peripheral nerve lesion [3–5]. The beneficial effects of NAC appear to include modulation of the antioxidant redox system, reduction in several neuroinflammatory molecules, and decrease in nitric oxide [6,7].

NAC is a cysteine donor. Cysteine can cross the cell membrane and support the synthesis of glutathione (GSH) [8], which is a substrate of antioxidant enzymes such as glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST). GPx catalyzes the reduction of hydrogen peroxide (H_2O_2) and organic hydroperoxides at the expense of GSH [9]. GST plays a role in the detoxification of xenobiotics and products of lipoperoxidation [10]. The oxidation of GSH leads to the production of glutathione disulfide (GSSG). The GSH/GSSG pair forms the major redox couple in cells [11].

Since CCI can decrease GSH [1] and increase NO [12] in nerve tissue, we postulated that the analgesic effect of NAC would involve modulation of the GSH and NO in the spinal cord. Thus, our study assessed the relationship between mechanical and thermal hyperalgesia evoked by CCI and alterations in the GSH/GSSG ratio, GPx and GST activities and NO metabolites in the lumbosacral spinal cord of rats with CCI treated with NAC (150 mg/kg/d) for 3 or 10 days. Since peripheral nerve injury changes spinal-cord hydrogen peroxide (H_2O_2) levels and NAC reacts slowly with H_2O_2 [7], our study also determined

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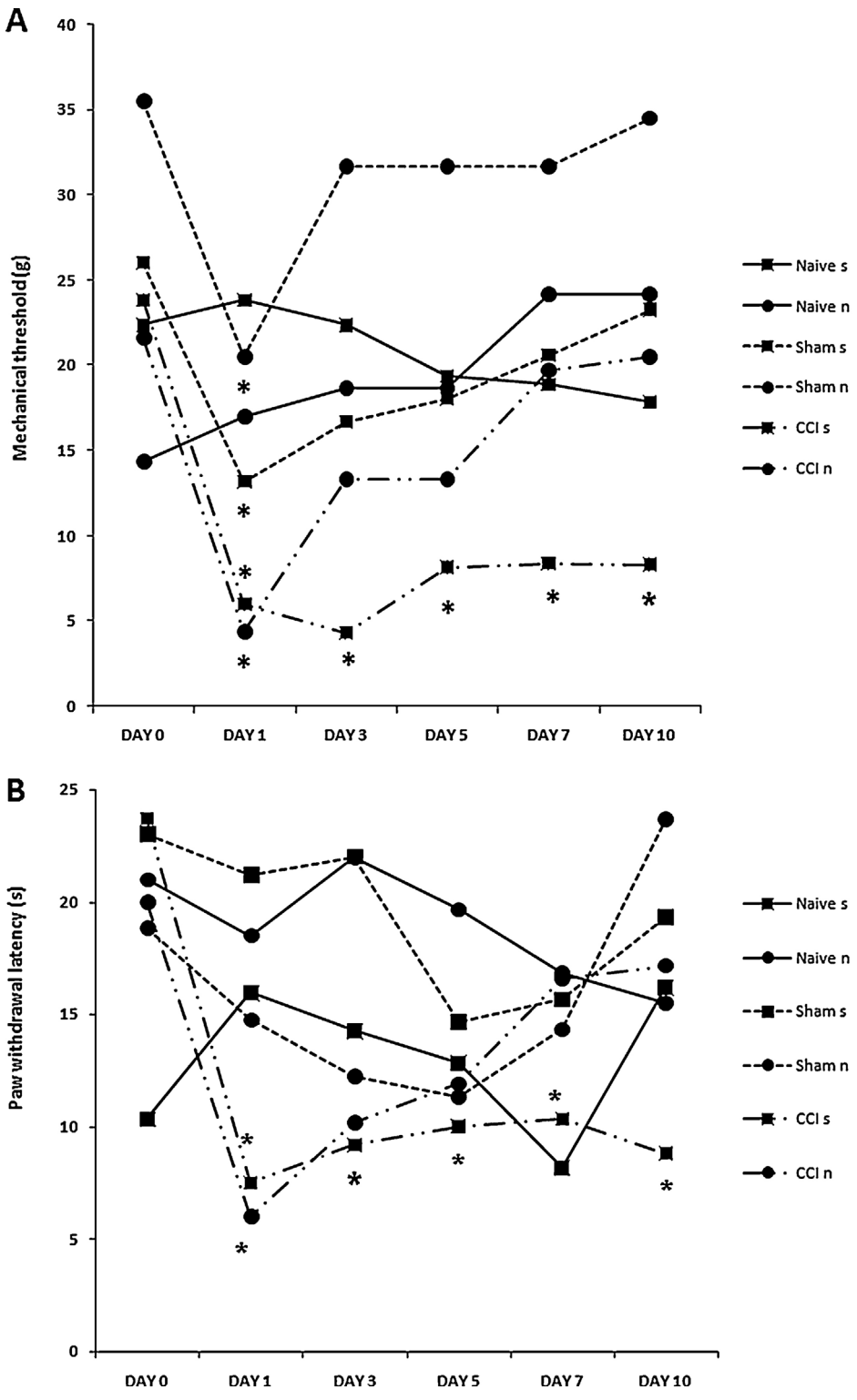

Fig. 1. Assessment of behavioral hypersensitivity after chronic constriction injury (CCI) of the sciatic nerve in rats treated with *N*-acetylcysteine (NAC) (150 mg/kg/day, i.p.) for 3 or 10 days. Graphs show the mechanical hypersensitivity measured by von Frey test (A) and the latency time against heat stimuli (B). Data represent the means ± SEM ($n = 6$ for each group). * Indicates a significant difference compared to pre-nerve lesion values (repeated-measures ANOVA, $p < 0.05$). Naive-s, saline-treated naive rats; Naive-n, NAC-treated naive rats; Sham-s, saline-treated sham rats; Sham-n, NAC-treated sham rats; CCI-s, saline-treated CCI rats; CCI-n, NAC-treated CCI rats.

the levels of H_2O_2 in the spinal cord of the animals. We also evaluated blood parameters including gamma-glutamyltransferase (gamma-GT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and creatinine, as well as hepatocyte morphology, because NAC treatment is associated with mild side effects [8].

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Ethics Committee of the Federal University of Rio Grande do Sul (#19037). Adult male

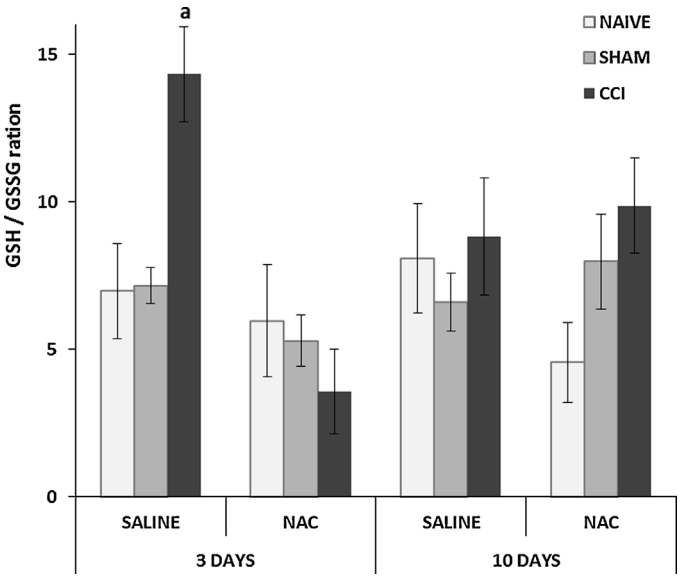

Fig. 2. GSH/GSSG ratio in the spinal cord of rats with chronic constriction injury (CCI) of the sciatic nerve treated with *N*-acetylcysteine (NAC) (150 mg/kg/day, i.p.) for 3 or 10 days. Data represent the means ± SEM ($n = 6$ for each group). (a) Indicates a significant difference compared to naive and Sham groups (three-way ANOVA followed by Tukey post-hoc test, $p < 0.05$).

Wistar rats, weighing 200–300 g, were divided into three experimental groups (Naive, Sham and CCI, $n = 24$ in each group), and each was further divided into four subgroups ($n = 6$ in each subgroup), which received NAC ((Fluimucil®, Zambon Laboratórios Farmacêuticos) at a dose of 150 mg/kg/d or 0.9% saline solution, intraperitoneally, for 3 or 10 d. The administration started 4 h after the surgical procedure and continued once daily until termination, using a technique described elsewhere [3,5]. Rats were not anesthetized during the injections.

2.2. Induction of chronic constriction injury (CCI)

After anesthesia (90 mg/kg ketamine and 10 mg/kg xylazine), the right common sciatic nerve was exposed proximal to its trifurcation, and four ligatures (4.0 Shalon Chromic Catgut) were tied loosely around it as described by Bennett and Xie [13] with slight modifications according to Goecks et al. [14]. To expose the sciatic nerve in Sham rats, all surgical procedures involved in CCI were used except the ligature.

2.3. Behavioral assessment

Rats were subjected to sensitivity assessments before the surgical procedure (day 0) and at 1, 3, 5, 7 and 10 days after surgery. To measure mechanical sensitivity, responses of the injured hind paw to a range of applied innocuous von Frey filaments (North Coast Medical, Inc., USA) were evaluated. The minimum and maximum stimulus intensity was 1 g and 64 g, respectively. The first stimulus was always initiated with the lowest filament. If there was no positive response, the next higher filament was applied. This testing pattern was continued until a withdrawal response was recorded [14]. Thermal hyperalgesia was measured by placing the rats on a hot plate maintained at 50 °C (±2 °C). Withdrawal latency was considered as when the animal jumped or licked a hind paw, independently of side.

2.4. Sample preparation

Rats were killed by decapitation and their lumbosacral spinal cord was promptly dissected out and divided transversely into

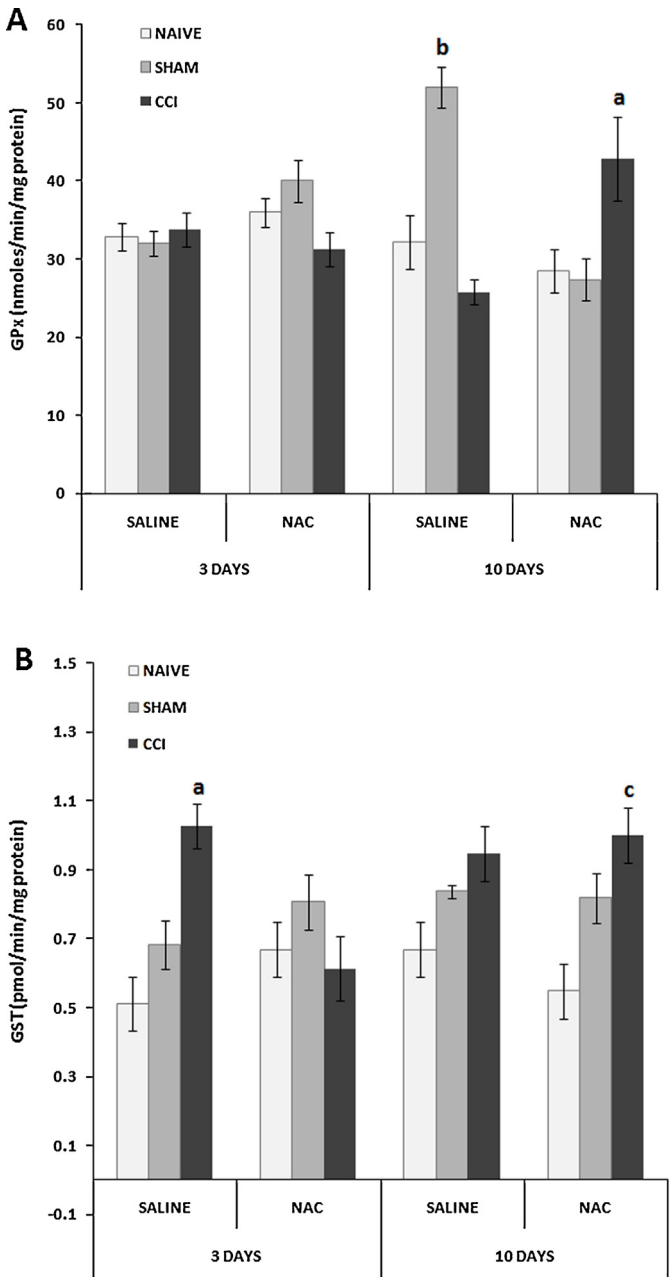

Fig. 3. GPx (A) and GST (B) activities in the spinal cord of rats with chronic constriction injury (CCI) of the sciatic nerve treated with *N*-acetylcysteine (NAC) (150 mg/kg/day, i.p.) for 3 or 10 days. Data represent the means ± SEM ($n = 6$ for each group). (a) Indicates a significant difference compared to naive and Sham groups. (b) Indicates a significant difference compared to naive and CCI groups. (c) Indicates a significant difference compared to naive group (three-way ANOVA followed by Tukey post-hoc test, $p < 0.05$).

three parts. The same portion always received the same preparation. One part was cooled in liquid nitrogen and processed to determine H_2O_2. Another part was homogenized in 1.15% KCl diluted 1:5 (w/v) containing 1 mmol/L phenylmethylsulfonyl fluoride, centrifuged at 800 × *g* for 20 min at 4 °C, and the supernatant was used for assays of GPx and GST activities and NO metabolites. A third part was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 min at 1000 × *g*, and the supernatant was neutralized with 2 mol/L KOH and used to determine the GSH/GSSG ratio.

The blood was centrifuged for 20 min at 1000 × *g* and the plasma used to determine gamma-GT, AST, ALT, bilirubin and creatinine. Commercially available kits (LABTEST) were used for these assays.

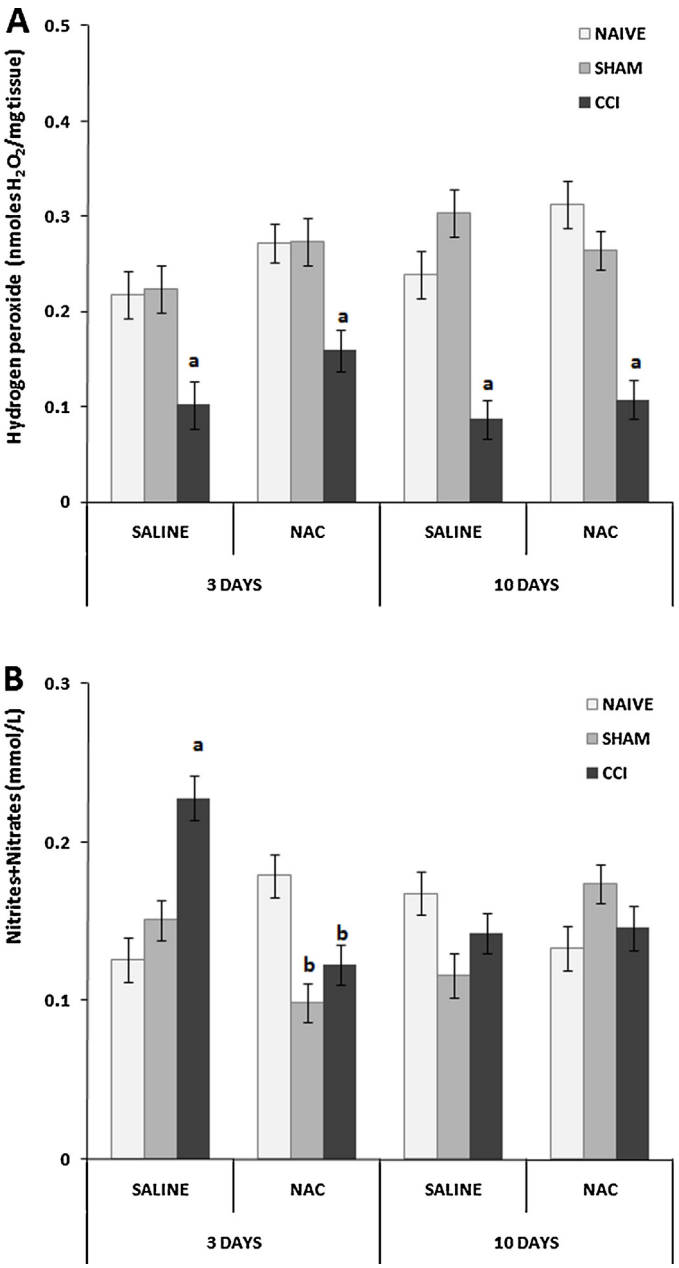

Fig. 4. Hydrogen peroxide (A) and nitric oxide metabolites (nitrites + nitrates) (B) in the spinal cord of rats with chronic constriction injury (CCI) of the sciatic nerve treated with *N*-acetylcysteine (NAC) (150 mg/kg/day, i.p.) for 3 or 10 days. Data represent the means ± SEM ($n = 6$ for each group). (a) Indicates a significant difference compared to naive and Sham groups. (b) Indicates a significant difference compared to naive group (three-way ANOVA followed by Tukey post-hoc test, $p < 0.05$).

Liver samples were immersed in Bouin solution, embedded in paraffin, and cut in 20 μm sections. Staining was done using the hematoxylin and eosin method.

2.5. Determination of antioxidant activities, H_2O_2 and NO metabolites

GPx activity was measured by following the nicotinamide dinucleotide phosphate acid (NADPH) oxidation at 340 nm [15]. GST activity was measured by the rate of formation of dinitrophenyl-*S*-glutathione at 340 nm [16]. The GSH/GSSG ratio was determined based on the procedure described by Akerboom and Sies [17].

Table 1
Effect of the treatment with N-acetylcysteine (NAC) (150 mg/kg/day, i.p.) by 10 days on rat blood parameters.

	Saline	NAC
TGO (mmol/L)	103.66 ± 4.50	120.61 ± 9.89
TGP (mmol/L)	55.81 ± 2.78	57 ± 5.50
GAMA-GT (mmol/L)	54.21 ± 9.18	29.86 ± 4.22
Total bilirrubin (mmol/L)	1.08 ± 0.10	1.16 ± 0.15
Direct bilirrubin (mmol/L)	0.75 ± 0.17	1.11 ± 0.14
Creatinine (mmol/L)	0.12 ± 0.04	0.11 ± 0.04

TGO: aspartate amino transferase.
TGP: alanine amino transferase.
GAMA-GT: gamma-glutamyltransferase.

The assay for H_2O_2 was based on the procedure described by Guedes et al. [18]. To measure NO metabolites, the Griess reagent was used [19].

2.6. Protein measurement

Protein was measured by the method of Lowry et al. [20], using bovine serum albumin as the standard.

2.7. Statistical analysis

The biochemical results were analyzed using three-way ANOVA (factors: lesion, treatment and time) followed by Tukey post-hoc test. The results of the von Frey and hot-plate tests were analyzed by repeated measures ANOVA. Differences were considered statistically significant when p was <0.05.

3. Results

3.1. Behavioral assessment

One day after CCI, all CCI and Sham rats showed mechanical (Fig. 1A) and thermal hyperalgesia (Fig. 1B). This response was not found at the other times in the Sham groups. At day 3 postoperatively, the administration of NAC attenuated mechanical and thermal hyperalgesia. This effect was also found at days 5, 7 and 10. The sensitivities remained higher in saline-treated CCI rats.

3.2. Effects on antioxidant, oxidative and nitrosative parameters

At day 3, the spinal-cord GSH/GSSG ratio increased (13%) in the saline-treated CCI rats. This increase was not found at day 10. No increase occurred when NAC was administered to CCI rats (Fig. 2). No change was found in Sham animals.

While no change was found in GPx activity in the CCI groups at day 3, the activity of this enzyme was increased (40%) in the NAC-treated CCI group at day 10 (Fig. 3A). GPx activity did not change in the Sham groups at day 3. However, an increase (56%) was found in saline-treated Sham rats at day 10.

GST activity increased (100%) in saline-treated CCI animals at day 3, but not in animals that received NAC treatment (Fig. 3B). At 10 days, a significant increase (50%) was found only in NAC-treated CCI rats. No significant change occurred in the Sham groups.

At days 3 and 10 post-CCI, H_2O_2 levels were significantly reduced in the spinal cord (Fig. 4A). NAC did not change this parameter. H_2O_2 levels did not change in Sham rats.

Three days after CCI, NO metabolites were higher (192%) in the spinal cord of the saline-treated rats. This increase was not present in the group that received NAC, which showed a reduction of around 20% (Fig. 4B). Whereas no change was found in saline-treated Sham rats at day 3, NAC administration caused a reduction (33%) in NO

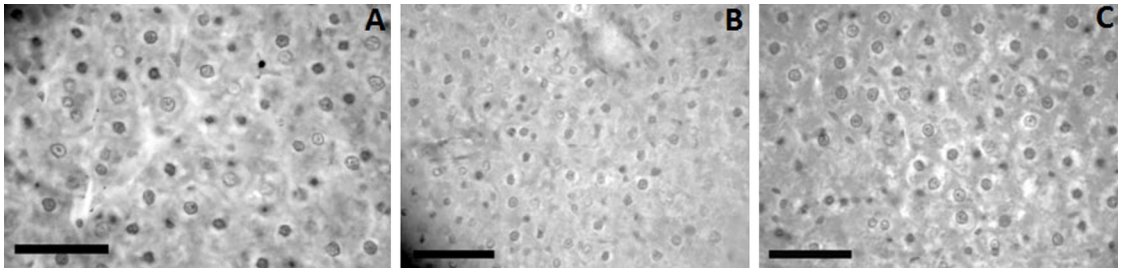

Fig. 5. Cross sections of the liver from rats without (A) and with chronic constriction injury (CCI) of the sciatic nerve treated with *N*-acetylcysteine (150 mg/kg/day, i.p.) for 3 (B) or 10 (C) days. Liver sections were stained with hematoxylin and eosin. Calibration bar: 5 μm (A–C).

metabolites in these rats. At day 10, no significant change was found in the CCI and Sham groups.

3.3. *Effects on plasmatic and histopathological parameters*

Gamma-GT, AST, ALT, bilirubin and creatinine levels did not change with NAC administration (Table 1). No change was found in liver morphology (Fig. 5).

4. Discussion

The analgesic effect of NAC is in line with previous studies [1,21]. However, it differs from a recent study where injections of NAC did not induce analgesia in CCI mice [22]. Although further studies are needed to explain this difference, the species used should be considered. Neuropathic pain behavior varies widely, not only between inbred and outbred strains, but also even between substrains [23].

While CCI elevated the GSH/GSSG ratio, this increase was not observed in animals that received NAC. NAC appears to have antioxidant activity [8], which may have contributed to the lack of increase in the ratio. However, injections of NAC reduced the expression of the catalytic subunit of the cystine/glutamate antiporter in the spinal cord [22]. Extracellular glutamate levels released as a consequence of increased cystine/glutamate antiporter activity are balanced by glutamate transporter activity, and the glutamate-induced GSH depletion is markedly reduced [24]. It has been demonstrated that CCI induces upregulation in the mRNA expression of the spinal glutamate transporters [25], and the expression and activity of the cysteine/glutamate antiporter are increased against a rise in NO and reactive oxygen species [24], which are increased in pain conditions [26]. Therefore, the cooperative action of the cystine/glutamate antiporter and excitatory amino-acid transporters may have contributed to GSH biosynthesis in the spinal cord of the saline-treated CCI rats.

NAC, in turn, decreased NO metabolites in spinal cord of the NAC-treated CCI and sham rats. NAC exerts a strong inhibitory effect on the expression of the inducible nitric oxide synthase (iNOS) gene, one of the enzymes that catalyze NO production [7]. This effect might have contributed to the reduction in NO. Since iNOS increases in the spinal cord after CCI [12] and this enzyme plays a role in the nociceptive process [27], its reduction might have contributed to the anti-hyperalgesic effect of the NAC. However, NAC appears to suppress the c-Jun N-terminal kinase and p38 proteins, which are involved in the induction of peripheral and central sensitization that occurs in pain [28,29]. Thus, at the moment it is impossible to identify the factors involved in the analgesic effect of NAC. Research on this question is under way in our laboratory.

NAC induced a late increase in GPx activity in CCI rats. The lack of change at day 3 may be due to an increase in the catalase activity induced by CCI [14]. It may have contributed to the availability of GSH to maintain optimum intracellular conditions. The increase in GPx activity in the saline-treated sham rats at day 10 may be related to the increase (398%) in lipid hydroperoxides [14]. The recruitment of GST activity at day 3 would contribute to prevent the establishment of an oxidative stress condition in saline-treated CCI rats. In these rats, the lipid hydroperoxides increased in this period [14]. The GST increase in NAC-treated CCI rats may be related to cell detoxification, due to the longer time period of the treatment.

The reduction in the H_2O_2 levels was related to neuropathic pain and not to the NAC treatment. NAC reacts slowly with H_2O_2 and this route appears to be insignificant under physiological conditions [8], which explains the lack of a relationship between NAC and H_2O_2.

The lack of changes in the plasma indicators and hepatocyte morphology suggests that NAC treatment appears not to have a toxic effect at the dose used in our study.

Thus, our study suggests that the anti-hyperalgesic effect of NAC does not involve its action as a cysteine precursor for GSH synthesis, but does involve a decrease in NO.

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