Effects of nutritional therapy or n-Acetyl-Cysteine (NAC) treatment on biochemical markers and liver histology in dogs with CCL₄-induced hepatic necrosis

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SUMMARY

The purpose of this study is to evaluate the potential beneficial effects of nutritional therapy or N-acetyl Cysteine (NAC) treatment on hepatic necrosis experimentally induced by CCL₄ in dogs. Eighteen healthy mixed breed dogs of both sexes were randomly divided into 3 equal groups and received a single oral dose of CCL₄ (2.5 ml/kg body weight). Dogs of control group and of experimental group I were fed with maintenance diet (Hills) and with canine prescription k/d diet (Hills) respectively, once a day. Dogs of the experimental group II were fed with maintenance diet and were treated by NAC (140 mg/kg in the first oral dose followed by 70 mg/kg 6 hours later for 30 days). Serum enzyme activities (ALT and ALP) activities and bilirubin concentrations (total and direct) were monitored on the 1st, 5th, 10th, 20th and 30th days. Ultra-sonographies, BSP clearance and liver biopsies for histological evaluation were performed on the 10th, 20th and 30th days. Marked increases of serum enzyme activities were noticed since the 1st day and persisted until the 15th - 20th days in all CCL₄-intoxicated dogs. Total and unconjugated bilirubin concentrations also slightly increased in serum of control dogs and dogs of the experimental group I (nutritional therapy) from the 1st day to the 15th day, whereas in dogs treated by NAC, significant fluctuations have persisted until the 30th day. BSP retention rates were markedly extended from the 1st to the 20th days in all groups, but the BSP clearance was significantly more depressed in control dogs and in dogs treated by NAC than in those received nutritional therapy (p<0.05). Morphological changes (hyperechogenecity of the liver parenchyma) and tissue lesions (hydropic degeneration and hepatic necrosis) were evidenced in all dogs. Nevertheless, degenerative changes were less severe (only grades 1 and 2, particularly on the 20th and 30th days) and were associated with an increase of hepatocyte regeneration in experimental group II. These results showed that the intensity of liver injury could be assessed through BSP retention test and histological analysis and that nutritional therapy, not NAC treatment, partially attenuated CCL₄ - hepatotoxicity in dog.

Keywords : dog - hepatitis - CCL₄ - N-acetyl cysteine - nutritional therapy.

RÉSUMÉ

Effets biochimiques et histologiques d’une thérapie nutritionnelle ou d’un traitement par la N-acétyl-cystéine (NAC) chez des chiens présentant une nécrose hépatique induite par CCL₄. Par I. SEN, K. KURGUT, M. OK, M.M. KIRAN, H. GUZELBEKTES, M. ORTATATLI, F.M. BIRDANE et V. ALTUNOK.

L’objectif de cette étude est d’évaluer les effets éventuellement protecteurs d’un traitement nutritionnel ou d’un traitement par la N-acétyl-cystéine (NAC) chez le chien, sur des lésions de nécrose hépatique induites expérimentalement par du CCL₄. Dix-huit chiens tout-venants, en bonne santé, des 2 sexes, ont été répartis au hasard en 3 groupes égaux et ont reçu par voie orale pendant 30 jours (groupe I), une ration de maintenance (thérapie nutritionnelle) ou de NAC (1ère dose : 140 mg/kg puis 70 mg/kg 6 heures après pendant 30 jours (groupe II). Les activités sériques de l’ALAT et des PALs, les concentrations sériques de bilirubine (totale et directe) et la clairance de la BSP ont été mesurées les 1er, 5ème, 10ème, 15ème, 20ème et 30ème jours. Des échographies et des biopsies hépatiques en vue d’une analyse histologique ont été réalisées les 10ème, 20ème et 30ème jours. De fortes augmentations des activités enzymatiques sériques ont été notées dès le 1er jour et ont persisté 15-20 jours chez tous les chiens intoxiqués par CCL₄. Les concentrations sériques de la bilirubine totale et directe ont augmenté chez les chiens du groupe contrôlé et du groupe I (thérapie nutritionnelle) tandis que, dans le groupe II (traitement par la NAC), des variations faibles et significatives ont été observées jusqu’au 30ème jour. Les taux de rétention de la BSP ont été intensément et précoce-ment modifiés du 1er au 20ème jour dans tous les groupes. Cependant, le ralentissement de la clairance de la BSP a été significativement plus important chez les chiens contrôles et traités par la NAC que chez les chiens ayant eu une thérapie nutritionnelle (p<0.005). Des modifications morpho-physiques (hypéréchogénicité du parenchyme hépatique) et des lésions tissulaires (dégénérescence hydropique et nécrose hépatique) ont été détectées chez tous les chiens. Néanmoins, dans le groupe I, les lésions dégénératives ont été moins sévères (seulement des grades 1 et 2) que dans les 2 autres groupes, particulièrement les 20ème et 30ème jours, et ont été associées à une augmentation de la régénération des hépatocytes. Ces résultats ont montré que l’intensité des lésions hépatiques pouvait être évaluée par le test de rétention de la BSP et par analyse histologique, et qu’une thérapie nutritionnelle, et non un traitement par la NAC, pouvait atténuer l’hépatotoxicité de CCL₄ chez le chien.


Introduction

Carbon tetra chloride CCL₄ induced liver injury, histologically characterized by hepatic steatosis, hydropic degeneration, and centrilobular necrosis in liver, and has been used as a model to evaluate the effects of various medications [14, 18, 24]. Although mechanisms of liver injury and fibrosis are multifactorial, oxidative/peroxidative damage of cell and organite membranes, proteins, and enzymes are mainly involved [6]. The published literature reveals different
approaches for preventing the harmful effects of CCL\textsubscript{4} to hepatocytes; vitamin E therapy has been used in treatment of acute CCL\textsubscript{4}-induced hepatic injury in rats, whereas hepatic stimulator substance from wealing rats was used in mice, and adenosine in rats [6,14].

Many nutraceutical, conditionally essential nutrients and botanical extracts have been proposed as potentially efficient in management of liver disease. The provision of adequate calorie, protein, and micronutrients is critical to support hepatocyte regeneration [20]. Consequently, nutritional therapy plays a supportive role in the management of most hepatic diseases [16, 17]. The primary goal for dietary management of hepatobiliary disorders includes maintaining metabolic balance while providing nutrients for healing and regeneration of damaged tissue. Other important objectives include: 1) correcting and preventing malnutrition, 2) reducing the need for hepatic ‘work’, and 3) avoiding production of hepatotoxics and neurotoxic compounds [17, 20]. General recommendations have been made about the levels of key nutritional factors in foods for patients with hepatobiliary disease [9, 16]. Prescription diet canine k/d have been produced for preventing diseases such as renal failure, hepatic disease and early heart failure by Hills manufacture.

Cytoprotective agents would be able in part, to inhibit inflammation and fibrosis, initiation of apoptosis, and also to preemptively protect tissues against oxidative injury, and ensure ability for maintaining an appropriate redox balance. The benefit of N-acetylcysteine (NAC) therapy also has been suggested in the treatment of acute hepatic failure. N-AC, a substance necessary for the glutathione synthesis, has potent antioxidant effects [3, 11, 27]. It enhances oxygen delivery and consumption, exerts relaxing effect on vascular smooth muscle, inhibition of leukocyte chemotaxis, and inhibits platelet aggregation and adhesion [28]. KIGAWA et al [15] suggested that N-AC effectively improved hepatic circulation and hepatic function in dogs with obstructive jaundice. However, the role of N-AC in treatment of sepsis and septic complications still remains controversial and frequency of administration of N-AC also has not been evaluated in dogs [6].

The purpose of this study was to evaluate the effects of nutritional therapy (Prescription diet K/D, dry) and N-acetylcysteine in the dogs with experimentally induced hepatic necrosis by CCL\textsubscript{4}.

**Materials and Methods**

**A) STUDY DESIGN**

Eighteen healthy mixed breed dogs of both sexes (1-4 year old, weight between 8 to 23 kg) were used in this study. They were randomly divided into three groups of 6 dogs each. All dogs were fed before the experiment once a day for ten days with a maintenance diet (Hills). Hepatocellular damage were induced by oral single dose of CCL\textsubscript{4} administration (2.5 mL/kg of body weight) [26]. Antiemetics (Metoclopramid HCl, 5 mg/kg of body weight, Metpamid) and analgesics (Xylasin Hydroclorur, 0.5 mL/10 kg of body weight, Rompun 2%) were given to minimize the discomfort of the dogs during CCL\textsubscript{4} intoxication.

Eighteen dogs were randomly divided into three equal groups (n = 6). Control group was fed with maintenance diet (Hill’s, Table I) according to body weight (Table II). The dogs of experimental group I were fed with prescription diet K/D (Hill’s, Table I) according to body weight (Table II). Finally, the dogs of experimental group II received N-AC (140 mg/kg of body weight, loading dose at sixth hour, followed by 70 mg/kg of body weight, maintenance dose, six hours intervals, for 30 days PO) and were fed with maintenance diet (Hills) according to body weight (Table II).

Blood samples were taken from the vena cephalica antebrachii. Separations of serum from coagulum were taken place immediately within an hour by centrifugation for 15 minutes at 1500g at room temperature. Collected serum were stored at -20°C before analysis.

**B) CLINICAL AND LABORATORY EXAMINATIONS**

The daily clinical status was monitored and serum alkaline phosphatase (ALP), alanine aminotransferase (ALT) enzyme activities, total (TB) and direct bilirubin (DB) concentrations were determined by autoanalyser (Medikom tecinom) before experiment and on days 1, 5, 10, 15, 20, and 30 of the experiment. Blood samples were collected before administration of Bromosulfophthalein solution (BSP). BSP retention testing was performed with a 5% solution (50 mg/mL) of bromosulfophthalein sodium salt in sterile water at a dosage of 5 mg/kg BSP (Sigma chemical Co) /kg body weight. The calculated dose of BSP solution was administered intravenously and heparinized venous blood sample was collected 30 minutes later. Blood samples were centrifuged at 1500g for 10 minutes at room temperature, and the plasma removed. The plasma BSP concentrations were determined within six hours with method described by FLATLAND et al. [10].

**C) MORPHOLOGICAL AND HISTOLOGICAL EXAMINATIONS**

Ultrasonographic examinations were performed with 5.0 MHz real-time linear transducer (Pie Medical, Scanner 480) on all dogs before feeding once in a week. The animals were scanned in dorsal recumbence and longitudinal and transversal planes were used for the liver examinations as described by NEYLAN and BERNARD [20]. Ultrason sound recordings were evaluated for hepatic contour and internal architecture including alterations in echogenicity (focal or diffuse) and intensity (compared to that of the kidney) and the appearance of hepatic vessels. Dimensions, shape, wall and content of the gall bladder were also evaluated on ultrasonographic images.

Liver biopsy specimens using a biopsy needle with ultrasound-guide were obtained according to the method described by SELCER and CORNELIUS [22] on the 10\textsuperscript{th}, 20\textsuperscript{th} and 30\textsuperscript{th} days of the experiment. Liver specimens were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 5 \um, and stained with hematoxylin and eosin (HE). Liver
biopsy specimens were evaluated on the basis of hepatocellular degeneration, necrosis (single or focal), regeneration and increase of connective tissue.

Hepatocellular degeneration was graded as follows:

0 : Normal
1 : Slight hepatocellular swelling and granular cytoplasm in centrilobular areas is evident
2 : Hepatocytes in centrilobular and midzonal areas show moderate swelling and pale staining
3 : Diffuse and severe hepatocellular swelling and cytoplasmic paleness. Cytoplasmic rupture and pyknosis are evident in some areas.

D) STATISTICAL ANALYSIS

All data were analyzed with a one-way ANOVA. Results were considered as significant when p values were less than 0.05. In addition, variance analyses (ANOVA) was used to group comparisons. Results are expressed as mean ± standard deviations (SD).

Results

From the first day, all dogs became ill and showed clinical symptoms of CCL4 intoxication (anorexia, apathy, lethargy). These symptoms culminated on the 1st to the 3rd days. Thereafter, gradual improvement of the clinical signs was observed in all dogs until the first week of the experiment.

1. BIOCHEMICAL RESULTS:

Since the 1st day, serum ALT activities dramatically increased in all 3 groups (p<0.001), were maximal on the 1st day then gradually decreased until the 30th day for reaching comparable values to initial enzyme activities. In the 2 experimental groups, significant declines of serum ALP, ALT activities noticed on the 15th day were more rapid than in control group (Table III).

Serum ALP activities were also markedly increased in all CCL4-intoxicated dogs on the 1st day (p<0.001, Table III). The enzyme activities were maximal on the 1st day in serum of dogs of the control group and of the experimental group I, while the highest values were observed on the 5th day for dogs of the experimental group II. Thereafter, ALP activities progressively declined (p<0.05) and no more significant difference with pretreatment values was obtained on the 10th day for control group, and on the 15th day for the 2 experimental groups (Table III).
FIGURE 1. — Liver histological modifications observed in dogs intoxicated by CCL₄ (control group) and received nutritional therapy (experimental group I: prescription k/d diet) or NAC treatment (experimental group II). A: moderate hydropic degeneration in hepatocytes (grade 2), control 30th day, H&E X 460. B: slight hydropic degeneration (cloud swelling) in hepatocytes (grade 1), experimental group I, 30th day, H&E X 460. C: Severe hepatocellular swelling (hydropic degeneration, grade 3), experimental group II, 10th day, H&E X 460. Note quite narrowed sinusoids. D: coagulation necrosis in the liver, control, 10th day, H&E X 460.
Serum total and direct bilirubin concentrations varied according to time in the same way in untreated dogs and in dogs with nutritional therapy. They rapidly and markedly increased from day 1 to day 15, then reached basal values on the 20th day and significantly decreased on the 30th day from pretreatment values (p<0.05). In CCL4-intoxicated dogs treated with NAC (experimental group II), a first significant elevation was evidenced on the 5th day, followed by a second increment on the 20th day (p<0.05) (Table III). Whereas no significant variation of direct bilirubin concentrations was evidenced for control and experimental group II, this marker slightly increased from the 1st to the 15th days in experimental group I (Table III).

The plasma BSP retention rates markedly increased (p<0.001) on the 10th day of the experimental in all groups (Table IV), then gradually decreased in all groups and returned back to normal range of values on the 30th day. Nevertheless, maximal values obtained on the 10th day were significantly lower (p<0.001) in dogs with prescription K/D diet (experimental group I) than in the 2 other groups, and BSP retention rates declined more quickly in this group (Table IV). No adverse reactions to BSP were observed in this study.

2. MORPHOLOGICAL AND HISTOLOGICAL RESULTS:

Characteristic ultrasonographic changes were observed in all dogs at the 5th day of the experiment. These included enlarged liver, tightly packed echoes and loss of echogenicity of portal vein walls. The liver had an increased echogenicity in comparison with spleen and the renal cortex. It showed a bright hyperechoic, blurred echogenic structure and less visible vasculature in comparison to pre-experimental findings. Subsequently the liver gradually almost completely regained its normal ultrasound appearance in all groups.

Biopsy specimens of liver taken on 10th, 20th and 30th days of the experiment generally showed various degrees of hydropic degeneration (Fig. 1A,B,C), focal or single cell necrosis (Fig. 1D), regeneration characterized by hepatocytes which have eosinophilic cytoplasm and 2-4 nuclei (Fig. 2A), and increase of connective tissue (Fig. 2B). Histopathological lesions are summarized according to the biopsy specimens and given in Table V. Hydropic degeneration was obviously more severe in control group than in experimental groups, particularly on the 10th and the 20th days. Moreover, no grade 3 lesions was observed in the experimental group I (nutritional therapy) and regeneration pictures were more often visualized in this group, especially at the 30th day.

FIGURE 2. — Liver histological modifications observed in dogs intoxicated by CCL4 (control group) and received nutritional therapy (experimental group I: prescription k/d diet) or NAC treatment (experimental group II). A: Regeneration of hepatocytes, experimental group I, 30th day, H&E X 460. Note hepatocytes with eosinophilic cytoplasm and 2-4 nuclei (arrows). B: Increase of connective tissue from the portal area into lobules, control, 30th day, H&E X 290.

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Discussion

In this study, our results clearly indicated that CCL4 has induced hepatitis in dogs characterized by marked increases of serum ALT and ALP activities, by elevation in serum of total bilirubin and direct bilirubin concentrations and by delayed BSP clearance. Moreover, ultrasonographic changes such increased echogenicity of liver parenchyma and loss of echogenicity of vein walls, and hydropic hepatocyte degeneration associated to necrosis were also evidenced. The toxic effects of CCL4 on the liver have been known for years and studied extensively [4, 14, 18, 24] and induced severe hepatobiliary disorders (Table V). The observed changes were in agreement with earlier reports [2, 24]. Because of its morphology and diverse metabolic functions, the liver is subject to a variety of metabolic changes. Consequently, more than one hepatic function test may be required for complete evaluation of liver integrity [5, 10, 23]. Serum ALT activities are elevated during hepatic necrosis in dog and ALT is considered as a marker for hepatic cytolysis in this species [7]. Increased of serum ALP activity are essentially due to bile obstruction in dogs and cats modest elevations occur within one day during hepatic necrosis associated with secondary biliary obstruction [7]. As CCL4 mainly induced cytolysis in
liver, serum ALT and ALP activities have been monitored in this study. The enzyme GGT was excluded because its increases in serum are closely related to cholestasis and not to liver necrosis. Pretreatment values were closely related to the previously recommended references [1]. ALT activities were between 28 and 64 (usual values 0-69 U/L) and ALP activities 160 and 188 U/L (usual values: 39-222 U/L). Comparable increases of serum ALT and ALP activities rapidly occurred (since the 1st day) in all CCL4-intoxicated dogs, evidencing hepatic necrosis. The gravity of the induced lesions could not be directly assessed by serum enzyme activities because they are not proportional to the intensity of liver injury [7]. Moreover, increases of serum enzyme activities, particularly ALP, could also result from hepatic induction.

Hyperbilirubinemia is observed in dogs with hemolytic and hepatobiliary disorders and measurement of direct (i.e. conjugated) and indirect (i.e. unconjugated) bilirubin concentrations can partially allow distinction between hemolysis and hepatic diseases. Total bilirubin concentrations in serum of dogs are normally below 8.50 µmol/L [7]. In our study, slight elevations of total and indirect bilirubin concentrations were noticed in the serum of all CCL4-intoxicated dogs since the 1st-5th day, suggesting a partial and limited defect of hepatic uptake of pigments. Whereas changes of these biochemical markers were transient in untreated dogs and in dogs received nutritional therapy, significant variations were still observed in dogs treated by NAC until the end of experiment.

BSP retention test is a non-invasive, sensitive diagnostic test for liver diseases (hepatic necrosis, cholestasis, fibrosis with depressed hepatic blood flow) in dogs, although moderate to severe hyperbilirubinemia, hypoalbuminemia and ascites would interfere with BSP metabolism [10]. This test explores the liver capacity of large organic anion uptake, conjugation and excretion into biliary ducts [10]. The plasma BSP retention rate increased (p<0.001) on the 10th day of the experiment in all groups (Table IV), and gradually decreased for reaching comparable values to treatment values on the 30th day. However, the plasma BSP clearance was less depressed in the group of dogs treated by nutritional therapy (group I) than in the 2 other groups, suggesting that CCL4-induced liver damage were reduced in this group.

Histopathological evaluation of liver tissue is often essential for accurate diagnosis, rational therapy, and appropriate prognosis in dogs with hepatobiliary disease, and also for determining the degree of hepatocellular regeneration [8, 13, 21, 22]. In our study, the effects of prescription diet K/D (Hills) or NAC treatment in dogs with experimentally induced hepatic necrosis were evaluated by determination of ALT and ALP serum activities but also by BSP retention rates and histopathologic findings. Fine needle aspiration and core biopsy techniques are used routinely for evaluation of canine and feline liver disease [21, 25]. Moderate and severe degenerative changes (hydropic hepatocyte degeneration: grades 2 and 3) associated with a low cellular regeneration were mainly observed in controls and in-group treated by NAC. By contrast, in dogs received nutritional therapy, hepatocyte degenerative lesions were less severe and less frequent and hepatocyte regeneration was more evident.

Consequently, the BSP retention test results and the histopathological findings show that NAC does not significantly reduce CCL4-hepatotoxicity in dogs, whereas nutritional therapy partially limits it. However, NAC (N-Acetyl-Cystein) is considered as an antioxidant compound because it enhances GSH and enzyme synthesis (glutathione S-transferase and glutathione peroxidase), promotes directly the detoxification of some hepatotoxins, and acts by quenching oxidant radicals [3, 11, 27]. NAC also increase the volume of portal blood flow and hepatic microcirculatory tissue flow [15]. In this way, BAKKER et al. [3] found that NAC administration in dogs with endotoxnic shock was well tolerated.
might increase oxygen availability to the tissues, and was associated with an attenuation of TNF release. In another study conducted in dogs with endotoxemia, NAC significantly increased glutathione peroxidase activity [28]. KIGAWA et al. [15] suggested that NAC given intravenously effectively improved hepatic circulation and hepatic function in dogs with obstructive jaundice. Increments in blood flow in mesenteric, renal and femoral artery together with higher cardiac index and a lower pulmonary vascular resistance in dogs treated with NAC were also determined [28]. Moreover, CENTER [6] affirmed that, despite significant alterations of the NAC pharmacokinetic in humans with hepatic dysfunction, conventional recommended doses of NAC did not induce toxic effects in dogs and cats suffering from severe liver disease. Nevertheless, prolonged NAC administration as a constant rate infusion must be avoided in hepatic insufficiency in order to avoid excessive cystein catabolism leading to proton accumulation. Indeed, large excess of H+ block urea formation and favor glutamine synthesis rather than ammonia detoxification through urea cycle [6].

The primary goal for dietary management of hepatobiliary disorders includes maintaining metabolic balance while providing nutrients for healing and regeneration of damaged tissue [20]. Other important objectives include correcting and preventing malnutrition, reducing the need for hepatic work and avoiding production of hepatotoxic and neurotoxic compounds [20]. The canine prescription k/d diet may decrease CCL4 hepatotoxicity by providing adequate daily energy and nutrient intake and consequently by keeping the liver function of detoxification and supporting tissue repair and hepatocyte regeneration.

In conclusion, principally delayed BSP clearance and liver histopathological findings have clearly evidenced that NAC, considered as a potent antioxidant, fails to protect liver from CCL4 toxicity, even partially, whereas nutritional therapy would reduce hepatic necrosis and promote tissue regeneration.

References