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A novel chronic cirrhosis TAA-induced model in rats

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Abstract

Experimental models for hepatic lesions are necessary so the effectiveness of new drugs in reversing cirrhosis or fibrosis can be tested. This is the case of liver cirrhosis, which requires animal models. Three drugs widely used to induce cirrhosis in animal models are carbon tetrachloride (CCI4), Dimethylnitrosamine (DMN), and Thioacetamide (TAA). TAA has been largely used, but a model that confirms the occurrence of chronic cirrhosis in rats has not yet been well defined. In this study we developed a model of chronic cirrhosis induced by TAA in rats. Our cirrhotic protocol used 200 mg of TAA in 4% aqueous solution per kg of body weight applied intraperitoneally three times per week for 14 weeks in Wistar rats. On the seventh week the dose was increased by 20%. Our protocol caused low mortality rate and represents an alternative and valid model for chronic cirrhosis.

Key Words: animal model, fibrosis, chronic cirrhosis, rat, thioacetamide.

Introduction

Experimental models for hepatic lesions are necessary so the detailed mechanism of cirrhosis progression and the effectiveness of new drugs in reversing cirrhosis or fibrosis can be tested. Thus, animal models are needed to produce cirrhosis, the rat being the most common model adopted (14). Experimental cirrhosis induced by carbon tetrachloride (CCI4) significantly increases the activity of the alaninetransaminase (ALT), aspartatetransaminase (AST) and alkalinephosphatase (ALP). A decrease in total protein, albumin and hematocrit is also observed (7).

CC14-induced cirrhosis can be obtained in animal models by intraperitoneal injections given three times a week for periods that vary between 11 and 14 weeks (7, 24, 30). However, CC14 does not induce cirrhosis in all the animals from a test group, which shows that CC14 has limited efficacy in cirrhosis development. Additionally, CC14-induced cirrhosis is not homogeneous, it shares little similarity with the condition occurring in humans, it produces high mortality rate and it is considered as a toxic chemical hazard for the researcher doing the manipulations (10, 14).

Dimethylnitrosamine (DMN), also known as N-Nitrosodimethylamine (NDMA) or N-metil-N-Nitrosomethylamine, is a mutagenic and carcinogenic substance and it can be absorbed by ingestion or inhalation. Even short exposures to this compound may cause skin and eye irritations and affect the respiratory tract. It can also affect the liver, and longer exposures may lead to cirrhosis and the development of tumors (13). It is used in models for hepatic lesions, causing high mortality rates when given in high doses. Lower doses given for longer periods may cause fibrosis but not cirrhosis. DMN causes ascites, high serum levels of billirubin, alkaline phosphatase (AP), -glutamiltransferase and ALT, and low serum levels of total protein, albumin, and plasma globulin, in animals. Cirrhosis with DMN is usually induced with intraperitoneal injections for 3 consecutive days during four weeks at a dosage of 10 µg/kg (26, 31). Although DMN can be used for cirrhosis induction in rats, its high mortality, necrosis and severe inflammation are not representative of most cases of cirrhosis observed in humans or in animals (21, 22, 28, 32).

Thioacetamide (TAA) is known to be a hepatotoxic and carcinogenic compound in animals and most likely in humans as well, although no studies have shown these effects clearly. The cirrhosis model induced by TAA in rats produces histopathological changes that are similar to those found in humans and animals and is considered as a valid model (1, 4, 6, 9, 14, 18). Although histological and hemodynamics analysis show that the similarities between the TAA-induced cirrhosis in experimental models and that in humans are greater than that observed in CC14-induced cirrhosis (8), the TAA-induced cirrhosis is still not completely understood.

TAA-induced cirrhosis became a model widely used for studying fibrosis in rats (5, 16, 29) and its characteristics are similar to those caused by viral hepatitis (3). The lesions are characterized by cellular necrosis, fibrosis, micronodular reorganization of the parenchyma, duct proliferation, transaminase increase (3, 27, 12), peroxidation of lipid membranes (25), increase of PA (3, 17), and macronodular cirrhosis (8).

TAA-induced cirrhosis requires easier induction, is less toxic than other chemicals, shows low mortality rates, is reproducible and is similar to that observed in humans and in non-experimental animals (1, 20), although it requires a longer induction period than other models (23). Some different protocols exist for TAA-induced liver fibrosis, the most common one being administration of TAA in different concentrations by intraperitoneal route, three times a week for periods that vary between four and seven weeks. However, protocols for a well-developed cirrhosis induction are still controversial (8). Most of these models employ oral administration of TAA in drinking water (12), although some authors believe they are less efficient and less reproducible (4). Administration of TAA by oral route for 30, 24 and 12 weeks has caused cirrhosis after 30 weeks (4, 17, 23), although other authors have reported it in seven weeks (6).

Nevertheless, a standard protocol has not yet been established for a TAA-induced cirrhosis that is stable, reproducible, and chronic. An important aspect that will allow more reproducible models is the intraperitoneal injection, where the quantity of TAA that each rat receives can be more closely controlled than the oral administration.

Here we aimed to study a TAA-induced chronic cirrhosis model presenting the aforementioned characteristics using rats as the experimental model and to verify the progression of the lesions after the treatment with TAA stopped, by comparing the liver morphology after 24 days of the end of the experiment with a previous biopsy of the same rat.

Materials and Methods

Animals

A total of 40 female Wistar rats (*Rattus norvegicus*) with 200 g were used in this study. The rats were kept in groups of five in standard plastic cages containing sawdust and were divided into two groups: the control group (15 animals) and the TAA group (25 animals), the last one received thioacetamide and develops liver cirrhosis. They received 50 g of food daily and water *ad libitum*. The bed was changed every five days and animals were kept under natural photoperiod (19). The rats were provided by the Department of Pathology of the School of Veterinary Medicine and Animal Science of the São Paulo University, and the study was approved by the Bioethics Committee of the same University under the number 510/2004.

Procedures

The TAA group contained 25 animals. In this group, each animal received 200 mg/Kg of thioacetamide in a 4% aqueous solution applied intraperitoneally three times a week for 14 weeks, as described by Oe (18). On the seventh week the concentration of thioacetamide was increased by 20%. Ten days after cirrhosis induction all animals in the TAA group underwent hepatic biopsy for collection of liver samples. Twenty days after biopsies the animals were euthanized and liver samples were collected. A control group was maintained in parallel until the end of the experiment.

The rats in both groups were weighed weekly until euthanatized.

Anesthesia

Before euthanasia, the animals were anesthetized with isoflurane in oxygen 100% through a modified Magill circuit.

Blood collection and determination of serum concentration of albumin, total protein, Aspartateaminotranspherase (ALT), bilirubin, Total bilirubin, Creatinin, Triglycerides, Alkaline Phosphatase (AP) and Gama-glutamiltranspeptidase (GGT)

Blood was collected by cardiac puncture under isoflurane anesthesia using 5 ml syringes with 25x7 mm needles. Serum samples were analyzed using a VET/TEST® blood chemistry analyzer (IDEXX Laboratories Inc., USA).

Determining liver weight

The volume of the liver was calculated using a beaker full of water and weighting the volume of water displaced. The carcass was also weighted after all the small organs were removed in order to obtain the hepatocarcass index.

Fixation and embedding for light microscopy

The fixation procedure was carried out by immersing the liver samples in metacarn fixative for 12 hours, after which they were embedded in paraplast. Some liver samples were frozen in liquid nitrogen and kept at -80 °C.

Light microscopy staining

For histopathological studies, the liver sections were stained by hematoxylin-eosin (HE) and periodic acid-Schiff reactive methods (2). The parenchymal

collagen visualization and quantification was done using slides stained by the Picrossirius method (11).

Quantifying Parenchymal Collagen

Sections from ten animals from both the TAA and control group were randomly selected for collagen quantification. The volumetric ratio between collagen fiber and the parenchyma was calculated in picrossirius-stained sections. A total of 15 fields were measured by slide in 4 random slides/animal. The image of the collagen fibers from each field was separated from the background and binarized by a threshold filter and the total area of the collagen fibers in the field was calculated in order to obtain the percentage of area from each field occupied by collagen fibers. The mean percentage of collagen fibers in all fields from each animal liver section corresponds to the volumetric proportion of the liver that is occupied by the collagen in each animal. The images used in the quantification method were obtained with an Olympus BX 60 optical microscopy with a 40X objective and were analyzed by the morphometric program KS-400 (Zeiss).

Regenerating nodules quantification

A total of ten animals in the TAA groups were randomly selected for quantification of the regenerating nodules. The regenerating nodules were quantified per corresponding area in mm^2, in each slide, using a 4x objective.

Scanning Electron Microscope (SEM)

Liver of control animals (2 animals) and of cirrhotics (2 animals) were analyzed with SEM. Liver fragments were processed in two ways: a) Visualization of the parenchymal surface through freeze-fracture: the fragments were fixed in modified Karnovisk fixative (2.5% glutaraldehyde, 2% formaldehyde in 0.1 M phosphate buffer) for 2 days, followed by post-fixation in osmium 1% diluted in 0.2 M cacodylate buffer for 2 hours and dehydration. Between procedures, the materials were washed with distilled water (3x for 10 minutes). b) Visualization of the stromal surface of the liver: the fragments were soaked in 5% and 10% NaOH for two days. Next, it was fixed in modified Karnovisk for 30 min, post-fixed in osmium for 2 hours and dehydrated. Materials were washed in distilled water between passages. Both process included the critical point procedure, stubs assembly and gold metallization of the samples.

Results

Gross anatomy

All animals submitted to TAA treatment developed severe cirrhosis in 14 weeks. The mortality rate was 4%; hair fall was observed in five animals and spleen hypertrophy in two. The liver of cirrhotic rats was less lobated (compacted) when compared to the liver of normal animals. Cirrhotic animals showed a nodular surface with increased nodular presence in the visceral face than the diaphragmatic face of the liver.

Differences in the nodule aspect caused by cirrhosis were observed but most animals showed macronodular cirrhosis.

Animal weight

The initial average weight of the animals submitted to TAA (TAA group) in the beginning of the study was 200.2 ± 6.09 g. Animals with cirrhosis in the TAA group lost weight until the fourth week, when they started to recover weight. The weight gain between the fourth and seventh weeks was 5%. After the seventh week, the TAA dose was increased by 20%, and the animals started to lose weight again until the 11th week, when they began to regain weight between the 12th and 14th weeks. After the end of the period of cirrhosis induction by TAA, the total weight gain from the beginning to the end of the experiment was 4.1%. Animals in the control group showed a total weight gain of 14.5% at the end of the study (Figure 1).
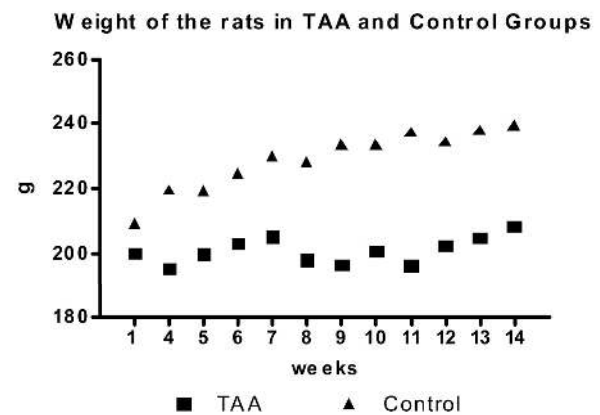

Figure 1. Follow up of the weight changes of the animals during induction of cirrhosis (TAA group) as compared to controls (control group).

Liver weight, volume and liver/carcass ratio

Liver weight of cirrhotic animals (TAA) (8.9 ± 1.1 g) was 29.27% higher than the weight of livers in animals in the control group (7.4 ± 0.9 g) (p < 0.05). The weight of the carcass in animals in the TAA group was (169.3 ± 6.5 g), which was 9% lower than that observed in the control group (186 ± 8.7 g; p < 0.05). The volume of the liver in animals in the TAA group (8.2 ± 1.0 cm^3) was 20.60% higher than that in control group (6.8 ± 0.8 cm^3) (p < 0.05). The density of the liver was similar in both groups (1.1 g/cm³). The liver/carcass ratio was significantly higher in TAA group (5.4) than in the control group (3.9) (p > 0.05).

Hepatic functions

Table 1 shows the results of the serological analysis.

Table 1 – Analysis of hepatic enzymes in animals in both groups, control and TAA.

	GroupC (n=15)	GroupTAA (n=20)
Triglicerydes (mg/dl)	72.5 (16.7)	81.01 (39.2)
Albumin (g/dl)	3.3a (0.2)	3.1b (0.1)
ALT (U/L)	44.7a (14.9)	62.5b (15.6)
Alkaline Phosphatase (U/L)	88.5a (35)	203.7b (47)
GGT (U/L)	0.17a (0.25)	6.7b (5.7)
AST (U/L)	111.3a (27.2)	142.5b (26.4)
Total Bilirubin (mg/dl)	0.16a (0.03)	0.27b (0.12)
Bilirubin (mg/dl)	0.02a (0.01)	0.17b (0.080)
Creatinine (mg/dl)	1.07 (0.12)	1.06 (0.09)

Averages in the same column that are followed by different letters indicate statistical difference, considering = 0. 05 with Student T statistical test. Standard deviation is indicated in parenthesis. ALT=Aminotransferase Alanine; GGT = Gama-glutamiltranspeptidase; AST = Aspartate Aminotransferase.

Microscopy

Histology. Animals in the control group showed characteristic hepatic normal gross anatomy. Lobules had central centrolobular veins to which hepatic sinusoids and cords converged (Figure 2A). In the hepatic triad, it was easy to see the bile ducts, hepatic veins and arteries (Figure 2B).

Histopathological evaluation. Loss of parenchymal architecture was observed in the liver of rats with TAA-induced cirrhosis, both at biopsy and after 17 days (Figure 2C), when compared with controls (Figure 2D). Fibrous scars forming bridges between portal areas (Figure 2C) were also observed. Centrolobular veins were of difficult identification in the parenchyma (Figure 2E). All animals showed bile duct hyperplasia and this proliferation could be found in 62.5% of the histological slides analyzed (Figure 2F). In the areas of proliferation of bile ducts we also found proliferation of oval cells without evidence of inflammation.

The liver samples of the TAA-induced group were strongly stained with PAS (Figure 2G). On the other hand, normal livers showed less positivity to PAS (Figure 2H), with greater intensity in the acinar zone III. We also observed hepatomegalocytosis and deposition of hemosiderin in the fiber septum, mainly in the region of the hepatic triad (Figure 2I). Another characteristic observed in cirrhotic livers was the presence of hepatocytes with nuclear lesion (Figure 2J), characterized by an intranuclear vacuole that stained weakly with HE method. Although the number of these nuclear lesions is not expressive, it may indicates degeneration of the nuclear material. These findings, together with the occurrence of citoplasmatic vacuoles in hepatocytes, indicate the occurrence of severe hepatic cirrhosis.
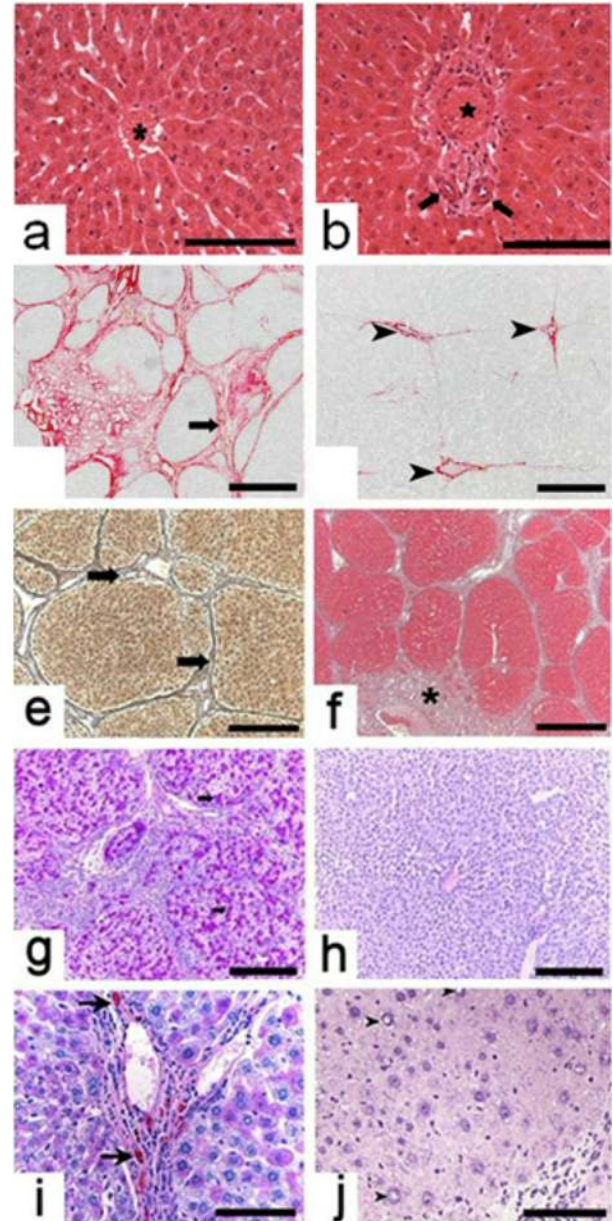

Figure 2. Photomicrography of livers of rats in group C (control) and group TAA (cirrhosis TAA-induced). a) Control liver showing the histology of a normal liver. Centrolobular vein (asterisk), to which the sinusoids discharge and where hepatic cords converge. b) Hepatic triads, formed by the portal vein (star), hepatic vein (arrow) and biliary duct. Staining with hematoxylin-eosin. Bars: 100 µm. c) Changed parenchymal structure with presence of regenerating nodules (star) and thick fibrous septum (arrows) in a liver from group TAA. d) Parenchymal structure in a control liver with presence of centrolobular veins and hepatic triads (arrowhead). Picrosirius staining. Bars: 200µm. e) Liver from an

animal in the TAA group showing the reticular fibers (arrow) circumscribing the regenerating nodules. Gomori stain for reticular fibers. Bars: 100 µm. f) Liver from an animal in the TAA group showing area of bile ducts proliferation (asterisk). Masson-trichrome staining. Bar: 100 µm. g and h) PAS-hematoxilin stain showing increased positivity in cirrhotic liver (g) when compared with the control liver (h). Bars: 100 µm. i) Cirrhotic liver showing hemosiderin deposit (arrow) on the fibrous septum. PAS-Hematoxilin staining. Bar: 40 µm. j) Cirrhotic liver showing nuclear lesions. Hematoxilin–eosin staining. Bar: 40 µm

Collagen measurement. In 20 animals randomly selected from the TAA groups we could observe an increase of 8.7% (11.4 ± 2.4% to 12.4 ± 3.5%) of the volumetric proportion of liver collagen compared with their own biopsy 30 days after the end of cirrhosis induction. In control group the volumetric proportion of liver collagen was 0%.

Quantification of the number of regenerating nodules. The average of regenerating nodules per mm² in 10 animals in the TAA group was 3.6 ± 1.5 at biopsy time, increasing to 5.5 ± 1.9 after 20 days from biopsy, representing a statistically significant increase of 51.9% (*p* = 0.003).

Scanning Electron Microscope (SEM)

The liver of control rats presented a flat and smooth surface (Figure 3A), whereas in the TAA group the liver showed an irregular surface with macronodules and micronodules, with predominance of the first type (Figure 3B). In freeze-fractures images of control rat liver it was possible to observe the liver sinusoids and centrolobular veins (Figure 3C). The parenchyma has a spongy aspect whereas in the cirrhotic liver the parenchyma is more compact with no evident confluence of sinusoids (Figure 3D). The corrosion procedure for electron microscopy showed in normal livers that the collagen fibers formed a scaffold of fine fibers with no presence of nodules (Figures 3E and 3G). In the cirrhotic livers, the fibers were thick and formed regenerating nodules (Figures 3F and 3H). These thicker fibers most likely are type I collagen. In some nodules only the thick fibers could be observed.
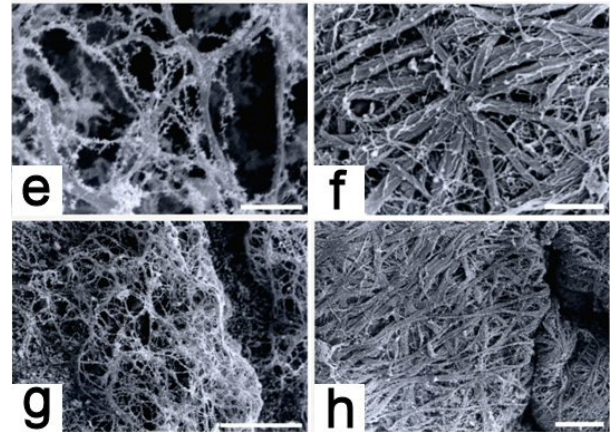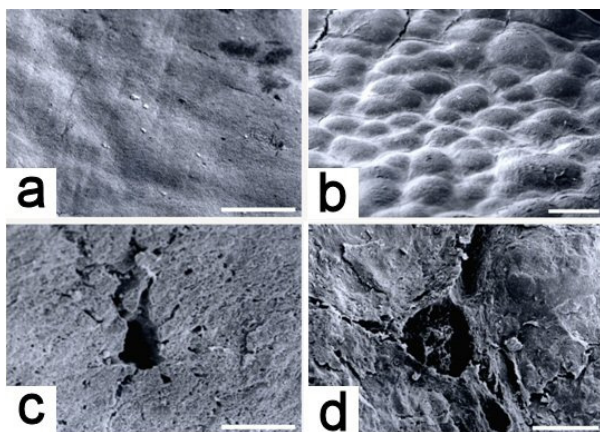

Figure 3. Scanning electron microscopy of livers from control group C and cirrhotic rats. a) Smooth surface of normal liver. Bar: 300 µm. b) Nodular surface of cirrhotic liver. Bar: 500 µm. c) Centrolobular vein of lobular sinusoids after freeze-fracture. Bar: 50 µm. d) Cirrhotic liver submitted to freeze-fracture, showing the centrolobular vein (asterisk) after freeze-fracture. Presence of sinusoids are less evident due to collagenization. Bar: 50 µm. e and g) Healthy livers submitted to corrosion with NaOH, showing collagen fibers and proteoglycans. Bar: 20 and 50 µm. f and h) Cirrhotic livers showing regenerating nodules after corrosion with NaOH. The collagen fibers of the stroma skeleton are more numerous and thicker if compared with control livers. Bar 10 µm.

Discussion

There are similarities between the TAA experimental model in rats and the animal and human cirrhosis, according to the histology and hemodynamics analysis, are greater than those induced by CC14 (9). Although the induction time with TAA is longer when compared to CC14 and DMN, TAA is eliminated through the urine in the first 24 hours after administration and causes lower mortality rates (1).

The increased number of regenerating nodules found in the liver of animals in the TAA group 20 days after biopsy indicates that we were able to produce a chronic cirrhosis in all animals undergoing TAA induction and that it was characterized by low mortality and by the presence of hepatic insufficiency. This chronic cirrhosis has not been previously described in rat TAA model. It is important to emphasize that the biopsies were done 10 days after the end of the TAA-cirrhosis induction period and that we waited 20 days more before euthanasia. Thus, the final results correspond to the progression of cirrhosis for a total of 30 days after the end the TAA intraperitoneal administration. In our model, the lesions were advanced and well established, with the presence of nuclear lesions, hemosiderin deposition, bile ducts proliferation, and an increase in hepatic enzymes, besides the great deposition of collagen in the hepatic parenchyma. Splenomegaly was also observed by others in TAA-induced cirrhosis (15).

The increase of liver weight and volume in the TAA group may be explained by the increase in

collagen content, which is characteristic of the cirrhotic process, and not a consequence of an increase in the parenchyma tissue, as the collagen showed a proportional volumetric increase when compared to that observed in the parenchyma. The parenchyma may have suffered a decrease in volume, as the increased serum levels of AST and ALT indicate low hepatic integrity.

The 4% death rate observed during cirrhosis induction may be explained by a great deficiency of the hepatic functions. Nevertheless, this mortality rate is still lower than that reported by others, in which mortality rates may reach 100% when DMN is used (10), 30-50% with CCl4 (14, 15, 20) or higher (10). It is interesting to note that most studies on induced cirrhosis do not draw conclusions about mortality rates. The low mortality rate found in our study indicates that the TAA may be a good alternative for studies on induced cirrhosis. The low mortality rate may be directly related to controlling the animals' weight and adjusting the doses of TAA accordingly, which is not a usual procedure adopted by others. As an exception, Li et al. (15) adopted a strategy of controlling the animals' weight and doing adjustments of the dosage accordingly and had 0% mortality. However, these authors were able to induce cirrhosis in only 90% of the animals after giving TAA for 12 weeks at a concentration of 0.03% added to the drinking water (lack of mortality can be explained by a lack of increase in the ALT, in this experiment). When TAA changes were made without taking in account the animals' weight by the same authors, the mortality rate increased to 30%.

Macroscopic analysis, including the animals' weight and their general condition, done in previous studies revealed a problem in the TAA-induction model. We observed that the animals apparently adapted themselves to the effects of TAA, because after an initial period losing weight, hair fall and jaundice, the animals started to regain weight indicating adaptation to the drug. In order to counter this effect, the TAA dose was increased 20% after the seventh week in order to obtain a scenario of stable cirrhosis, which we succeeded to obtain in the end of the experimental period.

The fact that the blood markers of hepatic lesion were increased, characterizes the occurrence of hepatic insufficiency, showing that the model is not limited to morphologic lesions but that functional changes also occurred. However, although changes were observed in the serum levels of AST, ALT, GGT, AP, albumin and bilirrubins, the production of creatinine or triglycerides was not compromised. Others did not observe a difference in ALT levels between the test and the control groups (15).

We recommend the use of TAA in experimental models of cirrhosis in rats and suggest that some tests are done to make sure that the best rat strain is used for the study. We recommend that increases in TAA dosages are done when animals start to regain weight and that the weight of each animal is taken in account and dosages changed accordingly.

These measures should be adopted to obtain low mortality rates and a cirrhosis that is more homogeneous and which is present in all animals included in the study.

Acknowledgement

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