Seminiferous Epithelium of Rats with Food Restriction and Carbon Tetrachloride-Induced Cirrhosis

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ABSTRACT

Objective: Analyze the changes in the seminiferous epithelium in rats with carbon tetrachloride-induced cirrhosis (CCl_4).
Materials and Methods: Forty-eight male Wistar rats aged 45-50 days, weighing 150-180 grams were used. Twenty-two rats underwent CCl_4-induced cirrhosis with CCl_4 0.25 mL/Kg weekly intragastrically once a week, during 10 weeks. Additionally, they had a 44% food restriction diet (Group 1). The control group was divided in two subgroups: 13 rats had a 44% food restriction diet and no CCl_4 (Group 2) and 10 rats were not submitted to CCl_4 or food restriction (Group 3). After 10 weeks, the rats were sacrificed and liver sections were collected for histological analysis. The testicular analysis was carried out to evaluate the frequency of tubules in stages VIII and XIV.
Results: The mean rates of stage VIII in animals with food restriction plus CCl_4-induced cirrhosis and food restriction without CCl_4 were significantly different from animals without either food restriction or CCl_4 ($18.1 \pm 5.5\%$, $20.5 \pm 2.5\%$ and $13.4 \pm 3.5\%$, respectively, $p = 0.002$). The mean rate of stage VIII in rats with cirrhosis was not significantly different from rats without cirrhosis ($18.1 \pm 5.5\%$ and $17.4 \pm 4.6\%$ respectively). The mean frequency of stage XIV in rats with cirrhosis was significantly greater than rats without cirrhosis ($4.7 \pm 2.3\%$ and $6.8 \pm 1.9\%$ respectively, $p = 0.027$).
Conclusion: Animals with CCl_4-induced cirrhosis and food restriction have shown alterations in spermatogenic cycle that were not seen in rats without CCl_4-induced cirrhosis and food restriction.

Key words: rats; liver cirrhosis, experimental; carbon tetrachloride; food deprivation; spermatogenesis
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INTRODUCTION

The presence of hypogonadism in cirrhosis was first described in 1966 (1); however, its pathogenesis has not yet been well established. Some cytokines of anabolic function in the testicles, such as IGF-I (Insulin-like growth factor-I) are reduced in cirrhotic rats. This cytokine reduces the effects of cirrhosis in the testicle (2).

The gonadal dysfunction is common in chronic liver diseases, but most of the studies have been done in patients with cirrhosis induced by alcohol (3). Some studies have sought the explanation for the relationship between cirrhosis and hypogonadism. Patients with cirrhosis presented higher serum levels of 17 beta-estradiol and prolactin and lower FSH than controls (4). Hypogonadism has been correlated to the level of liver damage in cirrhosis caused by chronic hepatitis (5). On the other hand, it has been demonstrated that energy-restricted diet is responsible for deleterious effects on reproduction more than protein-restricted diet in rats (6). It is unclear if cirrhotic

patients could present any spermatogenesis dysfunction induced not only by liver disease but also induced by malnutrition secondary to liver disorder.

Spermatogenesis presents three important phases: a) proliferative phase (spermatogonia), in which cells undergo rapid successive divisions; b) meiotic phase (spermatocytes) in which genetic material is recombined and segregated; and c) differentiation or spermiogenic phase (spermatids) in which spermatids transform into specialized cells able to fertilize (7). The stages of spermatogenesis can be divided by morphological criteria into various development steps, based on the form and shape of the acrosome and the cells of a cell association (8).

The morphological features of the seminiferous epithelium in cirrhotic rats and restricted diet intake have not yet been described. Thus, the present study aimed to check, by means of the histological analysis of the testicles, the possible changes in the seminiferous epithelium in rats with cirrhosis induced by Carbon Tetrachloride (CCl_4) and food restriction.

MATERIALS AND METHODS

The study was conducted according to the guidelines for animal research (Guide for the Care and Use of Laboratory Animals) (9), and was approved by the Hospital Research Ethics Committee.

During a quarantine period of observation, the animals received a standard rat chow (Nuvilab CR-1®, Nuvital S.A., Colombo - PR, Brazil), based on recommendations from the National Research Council and National Institute of Health - USA - providing 290 KcaL/100g, which composition was 22% protein, 4% fat and 4% crude fiber. Based on our previous data, the ad libitum food intake was established as 22g/rat/day. In order to have CCl_4 full toxicity (10), a 44% food restriction intake (12 grams/rat/day) was used.

Forty-five male Wistar rats aged 45-50 days and weighing 150-180g were used. The rats were kept in groups of five per cage, at a room temperature between 18-22ºC with cycles of light-darkness of 12 hours.

Cirrhotic animals (Group 1): Twenty-two rats were used in this group. Cirrhosis was obtained by the administration of CCl_4 (Merck p.a., Germany), 0.25mL/kg, diluted in 1mL of olive oil. The CCl_4 was given once a week, intragastrically by gavage using a 6F polyethylene catheter for tracheal aspiration (MarkMed Ltd., São Paulo, Brazil) during 10 weeks. All rats received Phenobarbital, 350 mg/L, added to the ad libitum drinking water (10).

Control animals: The main control group consisted of two subgroups. Thirteen rats (Group 2) were submitted to a 44% food restriction diet and received once a week 1mL of olive oil by gavage in the same way as the animals of group 1, treated with CCl_4. The other group consisted of 10 rats that were not submitted to any kind of procedure (Group 3).

After 10 weeks, all animals were sacrificed and liver sections were stained with hematoxylin-eosin (HE) and Sirius red. A semi-quantitative score was adapted to categorize liver damage: 0 = no fibrosis; 1 = stellate enlargement of portal tract but without septa formation; 2 = enlargement of portal tract with rare septa formation; 3 = numerous septa without cirrhosis; 4 = cirrhosis (10). The testicles were fixed in Bouin during 12 hours to be analyzed with HE stain.

Two hundred transversal sections of seminiferous tubules were analyzed in each testicle from all animals. The percentages of tubules in stage VIII (elongated spermatids that moved to the luminal aspect of the seminiferous epithelium and lined to the lumen) and stage XIV (meiotic anaphase or telophase of meiosis I, secondary spermatocytes, or any of the phases of meiosis) were checked (11).

A cell association or stage is a defined grouping of germ cell types at particular phases of development in cross-sectioned tubules (8). This classification divides the seminiferous epithelium cycle of rats in 14 stages according to changes in cell associations (stages) arranged in a logical sequence of developmental progression from spermatogonia through spermatozoa.

Before the analysis of different stages, a general evaluation of the sample slide was done searching for any cross-sectioned tubule with degeneration. This evaluation is important because tubules with

marked degeneration usually loose their normal association cells, and were not included in the classification described above. A ratio of stage XIV/stage VIII was also calculated, as the proportion of cells in each of these stages seems to be dependent on each other.

Results were analyzed by two-way analysis of variance (ANOVA) by using SPSS version 12.0 - USA. In all cases, p = 0.05 was established as statistically significant.

RESULTS

All 22 animals treated with CCl_4 presented cirrhosis. The rats that were not submitted to CCl_4 (with or without food restriction) did not show histological changes of the liver.

Rats without cirrhosis and food restriction (group 3) presented all tubules without degeneration. A few number of degenerated tubules was observed in both food restricted and cirrhotic animals (Table-1).

The majority of sections of seminiferous tubules of all animals was normal, and among them, a few number of degenerated seminiferous tubules were seen. The latter were characterized by loss of germ cells, vacuolization of germinative epithelium, interruption in meiosis, or presence of Sertoli cells only, as shown in Figure-1.

The percentages of stages VIII and XIV were analyzed in 100 non-degenerated tubules (with normal cell association) from each animal. Rats with cirrhosis presented lower number of cells in meiosis (XIV) than the group without food restriction. Animals submitted only to food restriction presented intermediate frequency of cells in meiosis (Table-2). Animals with food restriction, with or without cirrhosis showed approximately the same ratio of 1:4 (0.27 ± 0.1). On the other hand, rats without food restriction showed an approximate ratio of 1:2 (0.53 ± 0.2).

Table 1 – *Frequency of rats with cirrhosis, with and without food restriction, presenting cross-sections of degenerated seminiferous tubules.*

Groups	N of Rats with Seminiferous Tubules Degeneration	N of Rats without Seminiferous Tubules Degeneration	Total
Group 1	6/22 (27.3%)	16/22 (72.7%)	22
Group 2	2/13 (8.7%)	11/13 (91.3%)	13
Group 3	0/10 (0%)	10/10 (100%)	10

Chi-square test; p = 0.111. Group 1 = cirrhosis induced by CCl_4 + 44% food restriction; Group 2 = 44% food restriction without CCl_4; Group 3 = without food restriction and without CCl_4.

Table 2 – *Frequencies and ratios of stages VIII and XIV of seminiferous epithelium in animals with CCl_4-induced cirrhosis, food restriction and without food restriction.*

	Group 1	Group 2	Group 3	P *
Stage VIII	18.1 ± 5.5	20.5 ± 2.5	13.4 ± 3.5	0.002
Stage XIV	4.7 ± 2.3	5.5 ± 1.4	6.8 ± 1.9	0.027
Ratio Stage XIV / VIII	0.27 ± 0.1	0.27 ± 0.1	0.53 ± 0.2	< 0.001

*ANOVA and post-hoc test; Group 1 = cirrhosis induced by CCl_4 + 44% food restriction; Group 2 = 44% food restriction without CCl_4; Group 3 = without food restriction and without CCl_4.
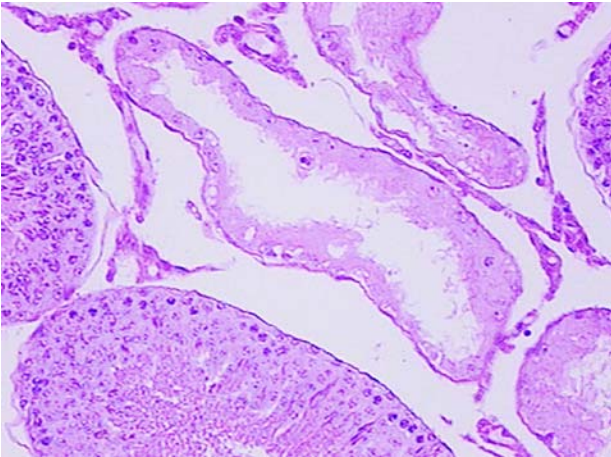
Figure 1 – *Photomicrography of a transversal section of the testis, showing degenerated seminiferous tubules (HE, X 100).*

COMMENTS

In this study, abnormal frequencies of stages VIII (related to liberation of elongated mature spermatids) and XIV (related to meiosis) of the spermatogenic cycle were observed in rats with food restriction CCl_4-induced cirrhosis. Differences in animals submitted only to food restriction were also observed.

Changes in other features of seminiferous epithelium disorder beyond the interruption of cell division (mitosis and meiosis), such as cell degeneration and phagocytosis of germ cells, lack of maturation, and loss of germs cells. A generalized degeneration of the seminiferous epithelium was not observed, only focal alterations. These results partially agree with other studies (2), which did not find a diffused damage to the seminiferous epithelium in rats with CCl_4-induced cirrhosis.

Gonadal dysfunction is common in patients with chronic liver diseases, especially in cirrhosis induced by alcohol (3). However, there is a lack of experimental studies concerning seminiferous epithelium disorders that used alcohol to induce cirrhosis.

Carbon Tetrachloride is a well-established hepatotoxic agent, which causes steatosis, necrosis and cirrhosis in animals. It has been extensively used as a model compound for inducing free radicals damage. It is bioactivated by cytochrome-P4502EI (12) into free radicals, leading to deleterious effects on liver due to lipid peroxidation (13). Carbon Tetrachloride was used in this study accordingly to previous classic experimental models of CCl_4-induced cirrhosis in rats (14). Phenobarbital can enhance the toxicity of this substance (6), as its main action is to induce the secretion of cytochrome-P4502EI (15).

The magnitude of liver injury can be influenced by food restriction. It has been suggested that food restriction can aggravate the toxicity of repeated oral administration of CCl_4 in rats through the enhanced metabolic activation of CCl_4 by food restriction (10). Nevertheless, the effect of food restriction on CCl_4 toxicity is controversial as food restriction could minimize drug-related increases in peroxidation and protect the system against drug toxicity, presumably by induction of antioxidant potential (16,17). The expected effect of food restriction in this study was to induce more severe liver injury (18).

Seminiferous epithelium in rats is classified in 14 stages, and some tubules can present cellular characteristics of more than one stage. The classification of stage VIII followed the orientation of Leblond & Clermont (11), and is characterized by the presence of elongated spermatids aligned in the lumen of the tubule to be liberated. In this study, for the animals without food restriction and without CCl_4, the average frequency of stage VIII was 13.4 ± 3.5. For the animals with food restriction and without CCl_4, it was 20.5 ± 2.5, and for the rats with CCl_4-induced cirrhosis and food restriction, it was 18.1 ± 5.5. No difference was seen between the group with cirrhosis and the group with food restriction. The greater frequency of stages VIII in the animals with cirrhosis and with food restriction seems to be due to an accumulation of this stage in relation to the others. This could also be explained since stage VIII is not immediately affected by the changes in the testicular function, as cells that no longer undergo cell division characterize it. As in this study, Lue et al. (19) observed that despite the accentuated loss of germ cells, after the application of heat on the testicles of rats, the elongated spermatids were still present in most of the seminiferous tubules. Other examples can be mentioned (20), which using a model of testicular degeneration, by means of the implant of testosterone in rats, a fourfold reduction was observed in the conversion of sper-

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matogonia to spermatocyte compared to the conversion of round spermatids to elongated ones. This clearly illustrates the effect only upon germ cells in division (mitosis and meiosis).

In this study, the frequency of stage XIV (meiosis) was lower in the group with cirrhosis indicating a reduction in the cell division rate. However, this difference was also observed when animals with normal liver with and without food restriction are analyzed (Table-2). These results indicate that starving may also have an influence on the meiosis rate, since both groups with food restriction had similar results independent of cirrhosis. Another study (7) demonstrated, in rats, that diet restriction caused reduction of testicles and epididymis weight, lower testosterone levels and copulatory efficiency. Moreover, it was observed that these effects were due to energy deficiency and not by reduced protein intake. Similar results were also observed in mice (21).

In conclusion, animals with CCl_4-induced cirrhosis and food restriction have shown alterations in spermatogenic cycle that were not seen in rats without CCl_4-induced cirrhosis and food restriction. Other studies are needed to better clarify the real role of CCl_4-toxicity and food restriction on the pathogenesis of seminiferous epithelium disorder.

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CONFLICT OF INTEREST

None declared.

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EDITORIAL COMMENT

The authors present an interesting study focusing on the evaluation, by means of histology, of the seminiferous epithelium in rats with carbon tetrachloride-induced cirrhosis.

It was observed that the animals with CCl_4-induced cirrhosis and food restriction have shown alterations in the spermatogenic cycle that had not been observed in rats without CCl_4-induced cirrhosis and food restriction. However, alterations in the frequency and ratios of meiosis stages were similar between cirrhosis and diet-restriction groups. Therefore, one may speculate that the effects seen in the seminiferous epithelium may be due to starvation rather than CCl_4. An additional experimental group, i.e., CCl_4-induced cirrhosis without food restriction, would be very interesting to evaluate the role of starvation on the seminiferous epithelium in rats.

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