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Liver Histology after Chronic Use of Alcohol and Exercise Training in Rats

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Abstract: Objectives: To investigate the effects of physical training on the liver morphology and morphometry after chronic use of alcohol in rats. Methods: Twenty-four Wistar rats were housed in cages with controlled environment and randomly divided into four groups according to treatment received. In the initial treatment, alcohol was administered to SA (sedentary alcohol) and EA (exercise alcohol) groups. After four weeks, physical training program was held on a treadmill with EA and EC (exercise control) groups. Area, perimeter, maximum and minimum diameter and form factor of nucleus and cytoplasm of hepatocytes were analyzed. Key findings: Micro-vesicular fatty degeneration, predominantly pericentrolobular, of mild to moderate intensity, was found especially in animals treated with alcohol. EC group showed nucleus area greater than the nucleus area of EA and SA groups. The form factor was lower in the EC group than in the EA group. EA group showed maximum cytoplasm diameter is smaller than in SC (sedentary control) group. Conclusions: Physical training for two weeks was not enough to suppress histopathologic changes in the liver caused by chronic use of alcohol in rats. Chronic use of alcohol seems to have minimized the beneficial effect of physical training in the nucleus area of hepatocytes.

Key words: Alcohol, exercise, steatosis, alcoholic liver disease, animal model.

1. Introduction

ALD (alcoholic liver disease) includes a spectrum of diseases ranging from simple steatosis to hepatocellular carcinoma of which genetic and environmental factors interact to produce a disease phenotype and its progression [1-3]. This feature could explain why some individuals who make heavy use of alcohol [4] do not progress to steatohepatitis, while others who make moderate use do develop it [5, 6]. However, ALD is most often associated with high levels of alcohol consumption [7, 8].

Steatosis develops in approximately 90% of subjects which ingest more than 60 g/day ethanol, but this condition is completely reversible after 4 to 6 weeks of abstinence. Fibrosis and cirrhosis develops in 5 to 10% of individuals. Even in mild forms of alcoholic hepatitis, there is a high risk of developing progressive liver damage, with the development of cirrhosis in over 50% of cases. Alcohol abstinence is associated with histological normalization in 27% of patients with alcoholic hepatitis, with progression to cirrhosis in 18% and persistent hepatitis in the others [9, 10]. Although considered a benign and reversible histological abnormality, patients with steatosis that persist consuming alcohol may develop fibrosis and in some cases, cirrhosis, without prior development of steatohepatitis [11].

In principle, the entire global burden of ALD is preventable but difficult to achieve because it interferes with longstanding individual and cultural habits. The approach to minimize the global burden of ALD

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involves interventions particularly in the early stages of the disease [12, 13].

In this perspective, exercise can be an adjunctive therapy in ALD treatment because results have suggested that the action of aerobic exercise is inversely associated with the development of steatosis, either mediated by weight loss, either by direct effects. Regular aerobic exercises can reduce the levels of hepatic fat, and this benefit may occur, although to a lesser extent, with no weight loss. Studies generally address nonalcoholic fatty liver disease and little is known about the effect of exercise on ALD [14-19].

Animal models have been an important tool for understanding the progression mechanism of the various stages of this disease, signaling pathways that lead to injury and regeneration, allowing the identification of therapeutic targets [20]. Therefore, the aim of the present study was to investigate the effects of exercise training on liver morphology and morphometry after chronic use of alcohol in rats.

2. Material and Methods

2.1 Preparation of Animals

The study was approved (August 28, 2013; process number 42/2013) by the CEUA (Ethics Committee on Animal Use) of the UFV (Federal University of Viçosa). All experimental procedures were performed in accordance with the ethical principles of animal experimentation, proposed by the CONCEA (national council for animal experimentation control).

Twenty-four adult male Wistar rats aged 90 days, from the Central Animal Facility of the Laboratory of Biological Sciences and Health of UFV were used. The animals chosen were male, because alcohol related problems and alcohol dependence are significantly more prevalent among males [21]. The animals were randomly divided into four groups:

(1) Sedentary control group (no alcohol) (SC, n = 6): animals that received no alcohol and were not exercised.

(2) Exercise control group (no alcohol) (EC; n = 6): animals that received no alcohol but have been exercised.

(3) Exercise alcohol group (EA; n = 6): Animals that received alcohol and were exercised.

(4) Sedentary alcohol group (SA; n = 6): animals that received alcohol but were not exercised.

Animals from experimental groups were housed in cages and divided according to treatments, i.e., one cage for each group in an environment with controlled average temperature and light-dark cycle of 12 h (07:00 am to 07:00 pm). Animals received food and water *ad libitum* on free demand. Food and water were replenished every other day, as well as the cleaning of cages. Animals received food or water throughout the experiment.

Alcohol was administered at the dosage of 4 g/kg/day, considered as heavy use [20], to EA and SA animals for four weeks by gavage from 12:30 pm to 13:30 pm [7]. Control animals received water by gavage at the same time for about 10 days. The initial ethanol concentration was 5% (v/v), increased by 5% (v/v) every two days up to final concentration of 20% (v/v). Final concentration reached on the seventh day of gavage and maintained until completing the fourth week of treatment. Four to twelve weeks of alcohol consumption correspond to chronic use for Wistar rats [20].

The weight of animals was obtained every two days to readjust the volume of alcohol administered, keeping the proportion of 4 g/Kg/day.

After the fourth week, alcohol administration was interrupted to EA and SA groups. EA and EC animals started the physical training program 24 h after the cessation of alcohol consumption.

Physical training began with a period of adaptation of animals to run on the treadmill. During adaptation, the exercise groups walked 5 meters/minute for 10 minutes/day for 3 days (50 meters per day). After the adaptation phase, exhaustion test was conducted to determine the MRS (maximum running speed). This test was performed as follows: the animal started to run

at a speed of 5 meters/minute, with increments of 3 meters/minute every 3 minutes until each animal from experimental groups reached fatigue. The time of fatigue was set and the test was stopped when the animal failed to maintain the running speed on the treadmill.

The exercise was performed 5 days/week for 2 consecutive weeks [22]. On the first day, training duration was 30 minutes, and in the next four days, training duration was increased by 10 minutes/day up to 60 min and the intensity was maintained at 65% of MRS, which corresponds to physical activity of moderate intensity. Therefore, from the fifth day, animals exercised for 1 hour/day

A second exhaustion test was performed at the end of exercise training 24 h prior to sacrifice in order to assess the performance of the animals. The same protocol was followed, calculating 65% of the new of maximum average running speed value.

All 24 animals completed the study after 6 weeks of experiment (four weeks of alcohol consumption followed by two weeks of exercise training). Euthanasia was performed by increasing anesthesia by 4.0% isoflurane in 100% oxygen (1 $L \cdot min^{-1}$) and immediate exsanguination by cardiac puncture. This procedure started after the finding of loss of all reflexes including of hind limbs.

2.2 Histopathological Examination of the Liver

After euthanasia, the liver was collected for histological analysis. The organ was fixed in 10% formalin for 24 h to paralyze cellular metabolism and preserve tissue structures, followed by dehydration of tissues in 70, 80, 90 and 100% alcohol, followed by diafanization (whitening) with xylene and embedded in paraffin. Tissues were submitted to two dissolved paraffin exchanges (57 ± 2). Subsequently, the material was removed from the oven and left at room temperature to solidify.

The paraffin block with the tissue was taken to the microtome and sliced to a thickness of 5 μm. The paraffin sections were carefully separated by a scalpel and were placed in a water bath (warm water) so that the folds caused by cutting the tissue disappeared.

The inclusion paraffin was removed. The section adhered to the glass slide was washed in xylol to dissolve the paraffin, immersed in a series of decreasing ethyl alcohol concentrations to be hydrated and then placed in the dye. In the case of eosin hematoxylin, the tissue was immersed first in haematoxylin, washed with water to remove excess and then immersed in eosin, being washed again, passing through increasing alcohol concentrations for removal of water (dehydration). A cover slip was placed over the cut delicately, covering and completely protecting the section.

Histological analysis was performed at the Laboratory of Pathology, Department of Medicine and Nursing, Federal University of Viçosa, in Olympus® BX 41 light microscope Olympus BX 41. Images were obtained in Olympus C31 photomicroscope, which were morphometrically analyzed using the Image-Pro Plus® software (Media Cybernetics). A veterinarian experienced in pathological analysis performed the blind reading of 48 hepatic tissue fragments (2 fragments per animal).

In addition to the qualitative analysis, the following parameters were analyzed for the nucleus and cytoplasm of hepatocytes: area, perimeter, maximum and minimum diameter and form factor. Six photos were taken of each fragment in 400× fields, 3 photos of the portal region and 3 photos of the central region, since fat accumulation initially occur in zone 3 (perivenular) of hepatocytes and, with the progression of steatosis, it reaches zones 2 and 1 (periportal) [1, 2, 11-13]. In each photo, 10 nuclei and 10 cytoplasm tissues were analyzed. The analysis of parameters was performed by three different researchers and the value considered was the average of the measurements obtained.

For the morphometric analysis of the cytoplasm, the Image-Pro Plus® software was used (Media

Cybernetics). Variables "area" and "Perimeter" were directly obtained by manual measurement. Since the morphology of the cytoplasm of hepatocytes is heterogeneous, dysmorphic and not circumferential, the cytoplasmic perimeter of hepatocytes obtained was transformed into circumference in order to obtain the same diameter. For this, parameters "maximum diameter" and "minimum diameter" were indirectly obtained using variables "maximum perimeter" and "minimal perimeter" (provided by manual measurement) and the mathematical formula $C = 2\pi R$, where C = length (perimeter) R = radius and diameter = 2R.

The form factor of nucleus and cytoplasm was calculated using the mathematical formula [(perimeter) $2/4 \cdot \pi \cdot$area]. The smallest value of this factor is equal to one, which means that the shape of the cytoplasm and/or nucleus resembles the shape of a circle. When this factor is greater than one, it is understood that the shape of the cytoplasm and/or nucleus is not circular [23].

2.3 Statistical Analysis

Initially, all data were submitted to Komolgorov-Smirnov test to verify the normality of data. Then, analysis of variance (ANOVA) followed by Tukey test was used for parameters with normal distribution. Nonparametric data were analyzed by the Kruskal-Wallis test. The T-Student paired test was used to compare the parameters of the initial and final exhaustion test. Sigma Stat software version 1.0 (San Jose, California, United States of America) was used to perform the analyses. The significance level was 0.05. Regarding animal experimentation, the minimum sample size was defined according to an estimated variance previously described in literature [20, 24-26]. The formula proposed by Callegari-Jacques and Cochran was used for the calculation. It was possible to observe significant differences with 5% significance, with a minimum of five animals in each group.

3. Results

3.1 Characteristics of Animals

The four groups of animal studies showed no statistically significant differences between them with respect to initial weight, weight after 4 weeks of alcohol use and weight at the end of two weeks of exercise (Table 1). The final weight was significantly increased in relation to the initial weight of SC, EC and SA groups, a fact that was not observed in the EA group. There was significant weight gain in EC and SA groups after oral use of alcohol for 4 weeks. This finding was not found in SC and EA groups (Table 1).

Before starting the experiment, the exhaustion time (when the animal reaches fatigue) and the maximum running speed did not differ significantly between groups (Table 2). Exercise groups (EC and EA) showed significant increases in the exhaustion time and maximum running speed compared to SA group at the end of the experiment. However, only EA group showed increased exhaustion time compared to SC group (Table 2).

Table 1 Average weight of animals during the experimental procedure.

Groups	Parameters			
	Initial weight (g)	Weight after 4 weeks of the experiment (g)	Final weight (g)	*p*
SC (n = 6)	325 ± 22	364 ± 25	376 ± 30[a]	0.011
EC (n = 6)	325 ± 17	379 ± 20[a1]	375 ± 27[a2]	< 0.01
EA (n = 6)	324 ± 31	357 ± 38	362 ± 44	0.206
SA (n = 6)	328 ± 29	382 ± 32[a3]	389 ± 35[a4]	< 0.05

SC = sedentary control (no alcohol), EC = exercise control (no alcohol), EA = exercise alcohol, SA = sedentary alcohol. Area: Data presented as mean and standard deviation ($p < 0.05$). Significant differences are represented by letter: a. vs. Initial weight (a1 $p = 0.002$; a2 $p = 0.003$; a3 $p = 0.012$; a4 $p = 0.026$, ANOVA followed by Tukey test).

Table 2 Mean exhaustion test and maximum speed values at the beginning and end of the experiment.

Parameters	Groups				*p*
	SC (n = 6)	EC (n = 6)	EA (n = 6)	SA (n = 6)	
Exhaustion time (minutes)—Beginning	13 ± 3	14 ± 4	16 ± 4	11 ± 2	0.092
Exhaustion time (minutes)—end	15 ± 2	21 ± 6[b1]	22 ± 5[a]	13 ± 2	< 0.05
Maximum average speed (m/min)—Beginning	17 ± 3	17 ± 4	20 ± 4	15 ± 2	0.090
Maximum average speed (m/min)—End	19 ± 2	25 ± 7[b2]	26 ± 6[b3]	17 ± 2	< 0.05

SC = sedentary control (no alcohol), EC = exercise control (no alcohol), EA = exercise alcohol, SA = sedentary alcohol. Area: Data presented as mean and standard deviation ($p < 0.05$). Significant differences are represented by letter: a. vs. SA ($p = 0.006$) and SC ($p = 0.031$) groups, b. vs. SA group (b1 $p = 0.017$; b2 $p = 0.020$; b3 $p = 0.014$; ANOVA followed by Tukey test).

3.2 Qualitative Histological Analysis (Morphology of Hepatocytes)

Qualitative analysis of the blades defined the following diagnostic impressions:

- SC and EC groups: very mild micro-vesicular and pericentrolobular fatty degeneration of normal aspect and without alterations.
- EA group: mild micro-vesicular and pericentrolobular fatty degeneration with small amount of Mallory bodies.
- SA group: mild micro-vesicular and pericentrolobular fatty degeneration with rare Mallory bodies.

Therefore, there was micro-vesicular fatty liver degeneration, predominantly pericentrolobular of mild to moderate intensity, especially in animals fed with diet with alcohol.

3.3 Quantitative Histological Analysis (Morphometry of Hepatocytes)

Tables 3 and 4 show the results observed for the analysis of nucleus and cytoplasm of hepatocytes, respectively, in the different groups.

EC group showed nucleus area significantly greater than the nucleus area of EA and SA groups. However, the form factor observed was significantly lower in the EC group compared with the EA group. For the other nucleus parameters, significant differences were observed among groups.

EA group exhibited maximum cytoplasm diameter smaller than the maximum diameter for SC group. For the other parameters, no significant differences were observed among groups.

4. Discussion

This study evaluated morphometric and histopathologic changes of hepatocytes induced by chronic alcohol use and the effect of physical activity on them. The main finding was the larger nuclear area of hepatocytes in control exercise group compared to both groups treated with alcohol. Initial fatty liver degeneration was also observed in animals treated with alcohol, with no improvement of the histopathological pattern in animals submitted to physical activity.

4.1 Morphometry of Hepatocyte Nucleus

The hepatocyte nucleus morphometric analysis showed that the group of animals that were not treated with alcohol and participated in physical training (EC) had a significantly higher nuclear area than the nuclear area of groups treated with alcohol (SA and EA) and form factor significantly smaller than in the alcohol and exercise group (EA) (Table 3). The explanation for a smaller nuclear area observed in the alcohol groups could be that the direct damage to cellular DNA is among the dangers of alcohol consumption [27]. Moderate physical activity can help reduce the hepatic oxidative stress through modulation of reactive oxygen species, increase the synthesis of antioxidant enzymes, up-regulation of protective systems against gene mutation and thermal shock, and increase growth

Table 3 Parameters assessed in the nucleus of hepatocytes.

Parameters	Groups				*p*
	SC (n = 6)	EC (n = 6)	EA (n = 6)	SA (n = 6)	
Area (μm²)	42.2 ± 4.1	45.7 ± 3.1[a]	41.8 ± 4.5	41.5 ± 5.2	< 0.05
Perimeter (μm)	23.0 (22.0 – 23.9)	22.6 (21.9 – 23.3)	22.4 (22.0 – 23.7)	22.1 (21.4 – 23.6)	0.537
Maximum diameter (μm)	9.3 (8.8 – 9.9)	8.9 (8.6 – 9.4)	8.9 (8.3 – 9.4)	9.4 (8.9 – 9.4)	0.878
Minimum diameter (μm)	5.6 (5.1 – 5.7)	5.7 (5.2 – 5.8)	5.6 (5.2 – 5.8)	5.7 (5.2 – 6.0)	0.613
Form factor	0.983 (0.982 – 0.988)	0.981 (0.783 – 0.984)[b]	0.985 (0.983 – 0.989)	0.983 (0.977 – 0.987)	0.044

SC = sedentary control (no alcohol), EC = exercise control (no alcohol), EA = exercise alcohol, SA = sedentary alcohol. Area: Data presented as mean and standard deviation, the form factor data presented as median and interquartile difference ($p < 0.05$). Significant differences are represented by the letters: a. vs. EA ($p = 0.026$) and SA groups ($p = 0.047$), b. vs. EA group (ANOVA followed by Tukey test).

Table 4 Parameters evaluated in the cytoplasm of hepatocytes.

Parameters	Groups				*p*
	SC (n = 6)	EC (n = 6)	EA (n = 6)	SA (n = 6)	
Area (μm²)	327 ± 41	326 ± 33	288 ± 25	320 ± 29	0.152
Perimeter (μm)	76.2 ± 1.9	79.0 ± 3.9	74.9 ± 4.3	77.6 ± 3.6	0.263
Maximum diameter (μm)	29.8 ± 2.3	27.7 ± 1.2	26.6 ± 1.8[a]	28.0 ± 1.6	0.026
Minimum diameter (μm)	21.2 ± 1.8	22.7 ± 1.3	21.4 ± 1.3	21.7 ± 0.8	0.264
Form factor	1.44 (1.34 – 1.57)	1.53 (1.49 – 1.57)	1.56 (1.49 – 1.58)	1.49 (1.48 – 1.52)	0.331

SC = sedentary control (no alcohol), EC = exercise control (no alcohol), EA = exercise alcohol, SA = sedentary alcohol. Area: Data presented as mean and standard deviation, the form factor data presented as median and interquartile difference ($p < 0.05$). Significant differences are represented by the letters: a. vs. EA group ($p = 0.026$; ANOVA followed by Tukey test).

factors such as follistatin [19, 28]. The increase of these substances requires larger nuclear activity, since it requires that the DNA is mostly in the form of euchromatin and devoid of spiral form to allow RNA transcription and hence with increased nuclear area. This could explain the greater nuclear area observed in animals that received no alcohol and underwent exercise. In the group submitted to exercise and alcohol consumption, this beneficial effect of exercise may have been minimized by previous changes caused by alcohol. Regarding the shape factor of the nucleus, closer to 1, the more circular its shape is, possibly meaning that less deformation is, minor damage, which would be expected in the SC group.

4.2 Morphometry of Hepatocyte Cytoplasm

In cytoplasmic morphometry, EA group had maximum cytoplasmic diameter significantly lower than the maximum cytoplasmic diameter of the sedentary control group (no alcohol intake) (Table 4). Despite this lower value, other studies have shown similar values for the cytoplasm of control groups [20]. Therefore, it could not be concluded that physical exercise in the EA group is associated with cytoplasm reduction. Regarding the form factor of the cytoplasm, the average of groups was between 1.44 and 1.56 (not so close to value 1), not differing significantly, showing that its polyhedral shape with 6 or more surfaces should have kept, even though the EA group has remained with more histological changes.

4.3 Morphology of Hepatocytes

Histological analysis has shown micro-vesicular fatty liver degeneration, predominantly pericentrolobular, of mild to moderate intensity, especially in animals fed with diet containing alcohol. ALD appears from oxidative stress, from the toxic effects of the conversion of ethanol into acetaldehyde and increased lipogenic activity and decreased removal of liver triglycerides [1, 2, 9, 29]. The presence of more pronounced steatosis in animals receiving alcohol by gavage compared to those who did not use this

substance confirms the effects already described of alcohol on liver [6-9, 11]. In these two groups, rare MBs (Mallory bodies) were observed, whose presence occurs in approximately 65% to 75% of cases of alcoholic hepatitis [30, 31]. MBs are intracytoplasmic hyaline corpuscles that although unspecific, are primarily associated with ALD. The pathophysiology and pathological significance are not entirely clear; however, it is known that these bodies are aggregates of intermediate filaments and polypeptides of cytokeratin and other proteins, precipitates and insoluble, resulting from hepatocyte injury. Cytokeratins are products of toxic liver damage, and can contribute to the perpetuation or inflammatory injury [31-33].

The analysis of groups receiving alcohol, when morphologically compared to each other, showed that the exercise group persisted with more MB and pericentrolobular fatty vesicles than the sedentary group, contrary to our initial hypothesis that physical training associated with the cessation of the alcohol use would intensify the regression of alterations caused by alcohol [34-36]. One factor that may have contributed to this finding may be the fact that when stopping using alcohol, exercised rats increased consumption of *ad libitum* diet to compensate for the calorie loss due to exercise. Increased food intake, even without significant weight gain in exercise alcohol group (Table 1), may have contributed to the alterations observed. Controlling caloric intake and liver weighing of the groups in further studies can help in this differentiation. Another possibility is that acute series of exercises transiently reduce hepatic blood flow and may reduce even more if held for long periods or hot environments, which could increase oxidative stress and favors the permanence of histological changes found [37]. It appears that the blood flow progressively decreases as the exercise intensity increases. Vigorous and/or prolonged exercises also reduce the clearance of substances that depends on the blood flow to their purification. Although the intensity of exercises in our study has been moderate (Table 2), it was not possible to observe the liver benefits on hepatic histology described in literature with physical activity in animals evaluated. A longer period of physical activity may be required to make such changes meaningfully.

On the other hand, the AS group had higher plasma levels of the enzymes ALT (alanine aminotransferase) and ALP (alkaline phosphatase), when compared to other groups. These data allow us to infer that the association of sedentary lifestyle and alcohol consumption may have promoted a greater release of these enzymes into the plasma. And physical exercise was able to avoid a greater plasma release of ALP in rats that consumed alcohol [26].

Aerobic and resistance exercises are also effective in reducing liver fat in patients with nonalcoholic fatty liver disease [38]. However, there is no data to support that exercise alone without weight loss can improve or reverse nonalcoholic steatohepatitis. Stages of the disease that progressed beyond simple steatosis may require more than exercise alone to achieve histological improvement. Results for non-fatty liver disease have shown that interventions such as changing lifestyle using exercise and calorie restriction inducing weight loss (loss of about 5-10% body weight) are required for improvement of nonalcoholic steatohepatitis [38]. The results observed in this study support the finding with respect to ALD, but it was not possible to demonstrate the benefit of physical exercise in the regression of liver histological changes caused by alcohol.

5. Limitations

Some limitations of this study were the lack of control of the amount of water and diet consumed by animals; the lack of liver biopsy of animals at the end of alcohol consumption to evaluate histology prior to physical training, which would allow a comparison of the magnitude of changes caused by alcohol; the lack of serial biopsies during physical training to observe changes over time; control animals received water by gavage at the same time in view to avoid placebo effect,

but it was stopped after 10 days to avoid unnecessary injury and suffering; others trials used longer exercise protocols: 6.5 to 8 weeks [24, 25, 36].

6. Conclusions

The results have indicated that aerobic physical training carried out for two weeks was not enough to suppress the histopathological changes in the liver caused by chronic alcohol use in rats. However, these data do not exclude the hepatic benefits of aerobic physical activity, since chronic alcohol use seems to have minimized the beneficial effect of physical training in the nuclear area of hepatocytes. Future researches involving liver biopsies may elucidate and supplement the findings of this study.

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Conflict of Interest

None of the authors have any connection to alcohol, pharmaceutical or neither sport industries, nor have the present work been funded by any of these organizations. None of the authors have any financial conflict of interest with organizations that seek to provide help with or promote recovery from addiction.

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