



Bone impairment in adolescent female rats chronically exposed to ethanol

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Ethanol consumption has increased among teenagers worldwide considerably, including females. Long-term ethanol consumption in women has been reported to cause bone metabolism imbalance. However, only few studies are available on the impact of long-term ethanol consumption on bone morphology during adolescence. Here, we report the effects of chronic ethanol consumption on bone structure in adolescent female rats. Twenty female Wistar rats (35 days old) received, by gavage, distilled water (control) or ethanol (6.5 g/kg/day, 22.5% w/v) once daily for 55 days. After ethanol administration, animals were perfused, and the femora were collected. Morphometric evaluations were performed by electron microscopy scanning. Femora length, cortical bone thickness and medullar bone diameter was measured. The results demonstrated that ethanol exposure during adolescence reduced the length of femurs, with a decrease of the anterior thickness, posterior thickness, and mid-lateral diameter ($P < 0.05$). Thus, long-term ethanol intake may lead to alterations on bone morphometry, reducing the thickness of compact bone and femur length in adolescent females.

Keywords: Femur, Scanning Electron Microscopy

Worldwide, alcohol consumption has increased in many forms of ingestion. In the Americas, 56.7 percent of adults (aged 15 and over) consume at least one standard alcoholic beverage per year, with 25% of this group considered heavy drinkers (consuming more than 60 grams of pure alcohol)¹. It's worthy to note that problems concerning the links between alcohol use disorders and self-harm, interpersonal aggression, digestive diseases, cancer, and death have increased substantially¹.

Among alcohol use disorders, binge drinking pattern is one of most damaging to general health². Teenagers and children are most vulnerable to damages associated to ethanol than other groups². One survey showed that underage drinking is about 29% of high school students, and around 14% of them binge drank. Female adolescents were more likely to binge drink than males with highest prevalence of alcohol abuse disorders in students³.

Among younger and postmenopausal women, low to moderate alcohol intake may reduce bone

resorption, which reflects a beneficial effect⁴. On the other hand, heavy chronic ethanol exposure on skeletal health has discrepant results, which effects on decreased mineral bone density⁴. However, the effects of heavy alcohol exposure during adolescence in female rats on bone loss has not been adequately investigated yet.

Chronic ethanol consumption can increase fracture risk in adults⁵, this fact is supported by experimental studies which show that alcohol impairs bone remodeling, replacement of old bone areas by organic matrix that is mineralized subsequently turning into a young bone, in order to preserve the integrity and optimize the function of the tissue⁴. Besides, the ethanol elicits an imbalance between bone formation and resorption, and ultimately bone resorption becomes more prevalent.

Earlier, we demonstrated that heavy chronic ethanol exposure, from the adolescence to adulthood, induces alveolar bone loss in female rats⁶. Here, we investigated whether ethanol exposure during adolescence also display femoral bone loss, a kind of secondary bone with a more complex structure.

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Materials and Methods

Experimental animals

A total of 20 adolescent female (35 days old, weight range from 50 to 180 (g) Wistar rats were obtained from the Federal University of Pará (UFPA) and maintained in collective cages (five animals per cage) under controlled conditions (25°C), a 12h reverse light/dark cycle (lights on 7:00 a.m.), with food and water *ad libitum*. All procedures followed the guidelines suggested by the NIH Guide for the Care and Use of Laboratory Animals⁷. Moreover, the Ethics Committee on Experimental Animals of the Federal University of Pará approved the present work under license number BIO-042-12. At 35th days old, animals received orally (intragastric gavage) distilled water (N=10) or ethanol (6.5 g/kg/day, 22.5% w/v, N=10) for 55 days (until the 90th day of life). Such alcoholic protocol has been developed and investigated by our group in order to observe heavy alcohol intake in the several systems in a rodent model^{8,9}. Besides, such ethanol dose reflects heavy alcohol exposure (i.e. among alcoholics) that reaches BAC ~ 422 mg/dL¹⁰.

To control possible effects of daily exposure to ethanol on the nutrition of animals, food amount as well as body weight were controlled (animals were weighted in the beginning (35th days of life), middle (67th days of life), and final of the ethanol protocol (90th days of life).

Perfusion and morphometric analysis

After 55 days of treatment, all animals were anaesthetized with a mixture ketamine hydrochloride (72 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.) and perfused through the left ventricle of the heart with saline solution 0.9% heparinized, followed by formaldehyde 4%. After perfusion, the right femurs were collected, cleaned of soft tissue and post-fixed in formaldehyde 10%.

In the macroscopic morphometric analysis, the right femurs length was measured through a digital pachymeter (LEE Tools 0.05×150 mm, Santo André, SP Brazil). Then, the femurs were transversely sectioned on the epicenter region, to obtain a slice of bone piece with 3 mm of thickness, and prepared for analysis by scanning electron microscopy following previous protocol⁶.

The histological bone samples were observed in a scanning electron microscope (LEO-1430; Carl Zeiss, Oberkochen, Germany). Thereafter, micrographs were obtained, and measurements of the anteroposterior diameter and mid-lateral of the medullary space and the anterior and posterior cortical thickness of the bone were measured. The methodology of the histological bone section is systematized in Fig. 1.

Statistical analysis

All values are expressed as means + S.E.M. (n = 10 animals/group). The distribution of data was tested by the Shapiro-Wilk method, adopting the alpha level of significance. Statistical comparison was performed using Student-t test. The accepted level of significance was $P < 0.05$. Statistical comparison of body weight gain between control and ethanol groups was performed using repeated measure one-way analysis of variance (ANOVA) followed by Unpaired Student-t test. All analyzes were performed using the GraphPad Prism 5.0 software package (San Diego, CA, USA).

Results

Chronic exposure to ethanol causes a decrease in body weight of the animals

The group intoxicated with ethanol (6.5 g / kg / day – n=10) for 55 days showed significant reduction in body gain weight in the 63rd days of life ($P < 0.01$). However, the final body weight showed no statistical difference (Fig. 2).

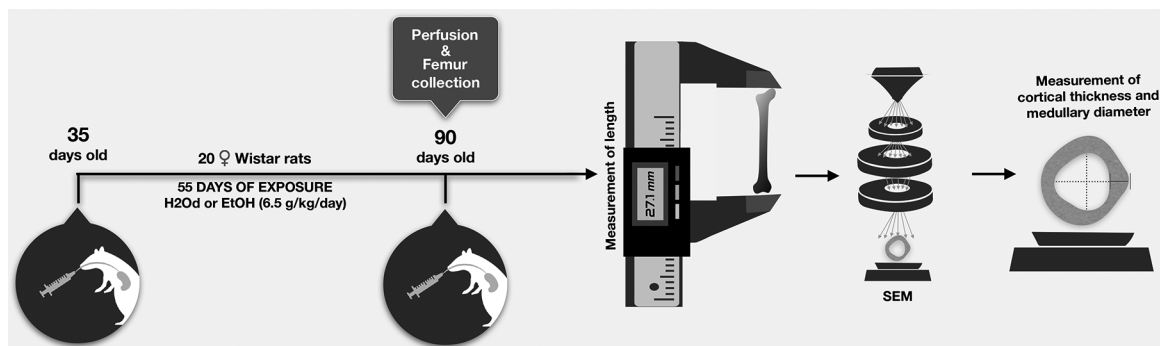


Fig. 1 — Methodology figure of the femora bone section.

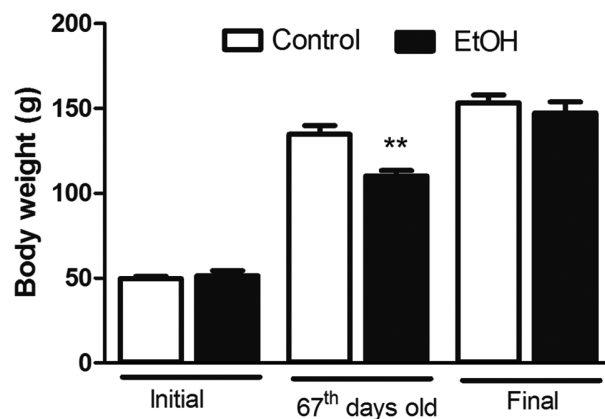


Fig. 2 — Effects of chronic ethanol (EtOH) (6.5 g/kg/day) administration from adolescence (35 days-old) to adulthood (90 days-old) on body wt. gain of female Wistar rats evaluated in the beginning (35th days of life), middle (67th days of life), and final of the ethanol protocol (90th days of life). [The results are expressed as mean \pm standard deviation of body weight (g) (n = 10 animals per group) ** P <0.01 compared to control group (Repeated measure one-way ANOVA, Student-t test)]

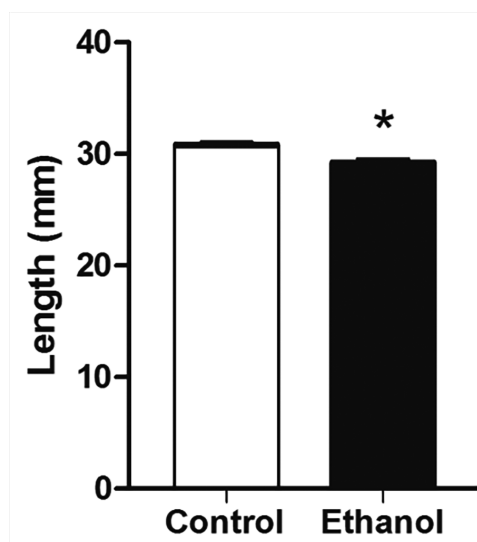


Fig. 3 — Effects of chronic exposure to EtOH (6.5 g/kg/day) from adolescence (35th days old) till adulthood (90th days old) on the length of the femur of female rats. Panel A represents the reduction on femoral length of EtOH exposed animals when compared to control group. [The results were expressed as mean \pm standard deviation length (mm) of rat femurs * P <0.05 compared to control group (t-student test)]

Intoxication by ethanol causes a decrease in the length of the femurs

The chronic administration of ethanol for a period of 55 days led to a significant reduction in the length of rat femurs ($p=0.0002$; Control group $n=10$; EtOH group $n=10$). Ethanol group showed shorter length of the femur (29,19 mm; \pm 1,190) than the control group (30,80 mm; \pm 0,8113) (Fig. 3).

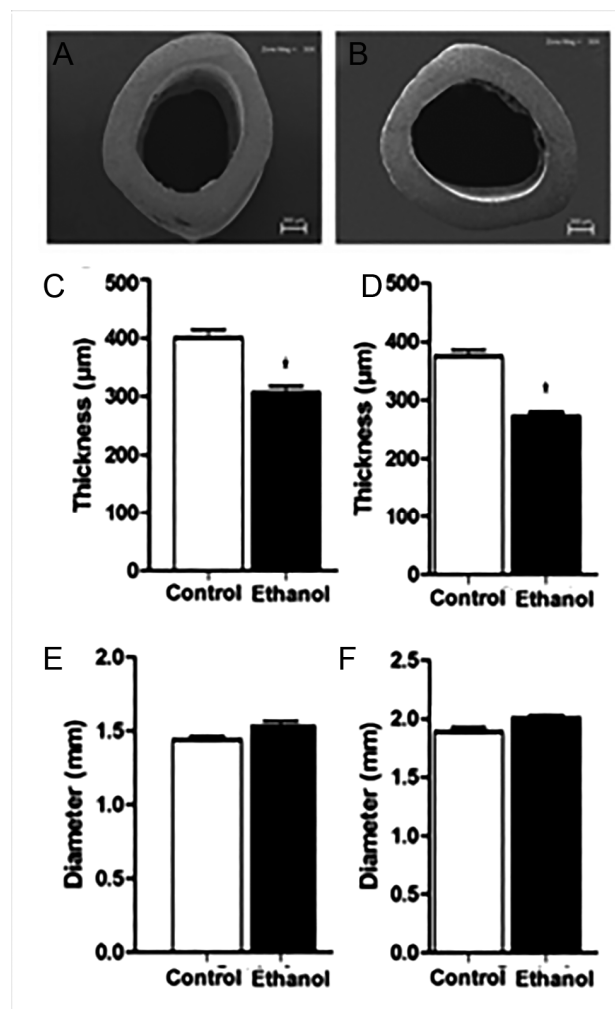


Fig. 4 — Effects of EtOH exposure (6.5 g/kg/day, 22.5% w/v) from adolescence (35 days old) till adulthood (90 days old) on thickness of compact bone and marrow diameter of female rats femurs. (A & B) scanning micrographs representing the control and experimental groups, respectively; (C & D) Graphs illustrating the loss of the anterior and posterior thickness of compact bone in the group intoxicated; and (E & F) Graphs showing a higher medial-lateral diameter of the medullary bone area and not change the antero-posterior diameter

Ethanol leads to reduction in anterior and posterior thickness of compact bone

Intoxication with ethanol provokes impairment in anterior thickness (control - $n=10$: 400.1 μ m; \pm 53.09 / ethanol - $n=10$: 306.1 μ m; \pm 44.57; P <0.0001) and posterior thickness (control: 374.7 μ m; \pm 42.99 / ethanol: 271.5 μ m; \pm 29.30; P <0.0001) of compact bone (Fig. 4).

Ethanol administration promoted an increase in medial-lateral diameter of femurs

The intoxicated group ($n=10$) showed higher medial-lateral diameter (2.008 mm; \pm 0.07352; $p=0.0095$ -

n=10) than control (1.887 mm; \pm 0.1513; $p=0.0095$ – n=10). Although, in the anteroposterior diameter analyses, no statistical difference between groups was found (control – n=10: 1.439 mm; \pm 0.1167 / ethanol – n=10: 1.529; \pm 0.1481; $p=0.0752$) (Fig. 4).

Discussion

Our results demonstrated that long-term exposure to ethanol from the adolescence to adulthood induced damage in the bone morphometry, affecting the thickness of compact bone, as well as the diameter and length of the femurs of female Wistar rats.

As cited above, such ethanol administration protocol was established in previous studies by our group, in which we found damage in the central nervous system⁸, salivary glands⁹ and alveolar bone⁶. Furthermore, the life period chosen (from the adolescence to adult stage) reflects the time where happens an increase in ethanol consumption², period more vulnerable to the damage caused by alcohol when compared to older age periods¹¹.

Although men seem to submit themselves to a heavy drinker profile during the life more than women, females in the adolescence period have been exposed to ethanol intake as far as males¹. Moreover, have highlighted the alcohol-induced bone impairment related to younger and sex specific effects require special consideration¹². In this sense, we hypothesize that prolonged ethanol exposure, at a heavy paradigm, from adolescence till adulthood, may develop bone alteration in female rats¹³.

The bone maturation is completed in adulthood (around 20 years old)¹⁴. Therefore, studies suggest that late adolescence is a time for construction of more than 95% of the adult skeleton, thus, interferences as ethanol consumption can affect directly the period of intensive bone remodeling¹⁵.

Several techniques have been used in morphological studies for evaluation of mineralized tissues, such as computed tomography and X-ray¹⁶. The scanning electron microscopy (SEM) has been demonstrated a precise, accurate and reliable technique for analysis of solid biological tissues⁶. Besides, it has also been used in recent studies for the evaluation of long bones of rats in order to assess various morphological parameters and fracture lines⁴.

The SEM showed that the animals after chronic exposure to ethanol presented deleterious changes in femurs morphometry. In fact, chronic alcohol consumption has been reported as a risk factor for the development of osteoporosis⁴. Thus, characteristics

such as low bone mineral density, low bone mineral volume and higher incidence of fractures are detected in chronic ethanol consumers¹.

It is necessary differentiate the alcohol-induced osteoporosis and the osteoporosis related to gonadal insufficiency¹⁴. The main characteristic is related to the age, since the former has been reported in young subjects¹³. In this sense, considering that the animals of the present study were 90 days-old at the time of perfusion, we can conclude that bone tissue damage was induced by heavy alcohol exposure. Besides, alcohol-induced osteoporosis generally affects trabecular and cortical bone tissue whereas gonadal insufficiency impairs trabecular bone⁴. Experimental studies have found that ethanol primarily affects the bone remodeling process in the skeletal system and may act directly or indirectly on bone⁴.

In the bone direct effects, ethanol displays both cellular proliferation and osteoblasts activity inhibition, that results in the bone formation process deficiency¹⁶. It is noteworthy that osteoblasts consist of the main cells responsible for organic matrix production¹⁷. Besides, ethanol also stimulates the proliferation and activity of osteoclasts, which consists of cells responsible for bone resorption, resulting in enhancement of bone resorption¹⁸. In this sense, ethanol promotes directly an imbalance between bone formation and resorption^{17,18}. In addition, ethanol displays indirect effects on bone tissue by changes in regulatory hormones of bone metabolism (vitamin D metabolites, parathyroid hormones and calcitonin), nutritional deficiencies¹⁹, and the presence of liver diseases¹⁹.

Considering these effects, a worrying factor in chronic alcohol consumption is the fact that bone metabolism is not fully restored even after stopping the intake of ethanol, which may perpetuate deleterious effects promoted during consumption period until the end of the life¹⁰. In fact, extensive alcohol consumption has been associated to a macroscopic overestimation of age (i.e., the presence of light-weight bones). However, microscopically alcohol consumption results in bone cortical thinning and decelerated bone turnover, which are similar to younger subjects. Thus, our data demonstrated that such bone alterations also occur in adolescent female rats¹⁰.

Conclusion

Our findings provide new evidence that heavy consumption of ethanol, during the adolescence, impairs the bone morphometry affecting the thickness

of compact bone, mid-lateral diameter, and length of female rat femurs. The exact mechanisms involved in the deleterious effects observed at this dose should be investigated in further research, but our results highlight the intake of alcohol in early adolescence may be a risk for bone metabolism disorders in adulthood. We also suggest to further research, periods of abstinence analysis to evaluate potential repairs of bone after chronic exposure.

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Conflict of interest

Authors declare no competing interests.

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