HEALTH SCIENCES

# Even without changing the bone mineral density, alcohol consumption decreases the percentage of collagen, the thickness of bone trabeculae, and increases bone fragility

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**Abstract:** Excessive alcohol consumption is considered a risk factor for bone health, as it causes a reduction in mass and increases the risk of fracture. However, the determination of bone mineral density (BMD) has not always been an adequate predictor of bone fragility. Thus, the hypothesis arises that chronic alcohol consumption interferes with collagen synthesis and the quality of bone trabeculae, with consequent bone fragility. Groups: Control (n = 6; water intake only during the entire study period); Ethanol (n = 6; ingestion of ethyl alcohol according to the protocol for inducing chronic alcohol consumption). The chronic alcohol consumption model did not cause a significant change in BMD, but there was a significant reduction of 20% in the thickness of the bone trabeculae and of 1.56% in the collagen located in the neck region of immature rat femurs. Although there was no significant change in the mineral matrix, the changes in the organic matrix were able to provide a significant reduction in bone strength. The results suggest harmful effects of alcohol intake on the bone quality of young adult animals and draw attention to the need to also consider methods for the diagnosis of collagen as an element of bone fragility.

**Key words:** alcohol, bone, collagen, rats.

## INTRODUCTION

Bone resistance to fractures results from the association of intrinsic and extrinsic properties of bone. The former deals with to bone as material and depend on the mass, architecture of the trabeculae, degree of mineralization, and metabolism of bone material. The latter, also called structural properties, consider bone as a whole and depend on its size, mass, geometry, and the intrinsic properties of bone tissue (Currey 2001).

Changes in bone turnover directly interfere with the arrangement and conditions of interaction of bone matrix components, providing effective changes in bone architecture, quality, and resistance. This process can be seen in diseases such as osteogenesis imperfecta and osteoporosis, in which metabolic changes in the remodeling rate occur (Viguet-Carrin et al. 2006). In these conditions, the negative bone balance, in addition to being related to bone loss, is associated with changes in microarchitecture, with reduced thickness of the trabeculae and cortex, leading to increased porosity and modification of collagen cross-links (Bala et al. 2015).

Under normal conditions, the collagen molecules line up precisely in fibers with overlap at their ends, providing a site for nucleation

mainly of apatite calcium crystals, deposited in parallel to the collagen fibers. The morphology and organizational characteristics of collagen fibers determine the orientation and size of the crystals (Yerramshetty & Akkus 2008). Thus, in osteogenesis imperfecta, extrafibrillary mineral crystals tend to be larger than crystals found in healthy individuals, while the crystals associated with collagen are smaller than in normal bones (Traub et al. 1994).

Therefore, evidence indicates that the disorganization of collagen fibers in the lamellar bone tissue can reduce the mechanical properties of bones, even in those with adequate bone mineral density (Marotti et al. 1994). Thus, it is evident that changes in the collagen matrix can negatively affect the mechanical strength of the bones.

So far, bone mineral density (BMD) is the main clinical parameter of fracture risk, as well as therapeutic efficacy. However, in some cases, bone mass is not an adequate predictor of bone fragility (Schuit et al. 2004, Ott 1993), as occurs in long-term treatment with bisphosphonates, where some patients are at increased risk of fracture, despite the increase in BMD (Sellmeyer 2010, Yoon et al. 2011, Ahlman et al. 2012).

It is known that dual energy X-ray absorptiometry (DEXA) is the main method of evaluation of BMD, although it is a limited technique for analyzing bone structure and matrix (Sroga & Vashishth 2012). Bone architecture, on the other hand, can be assessed using high resolution peripheral quantitative computed tomography (HR-pQCT) and magnetic resonance imaging (MRI). However, these exams are not widely used due to their high cost and poor accessibility (Melton et al. 2007).

Bone health can be affected by numerous factors such as genetics, hormones, and nutrition, or related to physical activity (Binkley et al. 2008, Mora & Gilsanz 2003). Sedentary lifestyle, excessive alcohol use, and smoking are among the main risk factors for osteoporosis and osteoporotic fractures in our society (Pisani et al. 2016).

Thus, excessive alcohol consumption is considered a risk factor for many organs and tissues, including bone health (Maurel et al. 2012), as it reduces BMD and increases the risk of fracture (Kanis et al. 2005). Recently, experimental findings have pointed out that chronic alcohol consumption negatively affects the bones of young rats, making them weaker and osteopenic, suggesting interference with bone growth. Thus, the excessive use of alcohol by young people becomes increasingly worrisome (Rosa et al. 2019).

In this context, we hypothesize that chronic alcohol consumption negatively affects bone quality, by causing a decrease in collagen, the thickness of bone trabeculae, and the mechanical resistance of female rats. Taking into account the worrying levels of alcohol consumption by young people and the fact that BMD assessment has not always been shown to be adequate in assessing bone fragility, the aim of this study was to investigate the effect of chronic experimental alcohol consumption on BMD, collagen percentage, bone trabeculae thickness, and the mechanical resistance of the femurs of growing rats.

## MATERIALS AND METHODS

The research was conducted in accordance with international guidelines as well as the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocol was approved by the Ethical Animal Committee of Federal University of Triângulo Mineiro - Brazil (CEUA / UFTM - nº 323/2014).

Twelve female albino rats (*Rattus Norvegicus*) of the Wistar strain were used, with

an average initial body mass of 250 g (± 10 g), housed in standard cages, in a bioterium with room temperature set at 22 ± 2 ° C, air humidity at 55 ± 5%, light-dark cycles of 12 h, and free access to water and chow (Nuvilab CR-1, Colombo, PR, Brazil).

The volume of liquid diet was measured daily, with an estimate of the average weekly consumption of alcohol per standard cages, within a 24-hour interval.

In order to induce chronic ethanol consumption, the animals received 5% ethyl alcohol diluted in water in the first week. In the subsequent week, they received 10% ethyl alcohol, and in the third and fourth weeks, 20% ethyl alcohol. The animals were randomly assigned to two groups, according to the type of liquid diet: Control Group (CG - n = 6) water intake only during the entire period of the experiment and Ethanol Group (GE - n = 6) ingestion of ethyl alcohol at the concentrations established in the protocol for inducing chronic alcohol consumption (PIA). After the PIA, the animals in the GE group continued to receive a liquid diet with 20% ethyl alcohol for another eight weeks, totaling 90 days. At the end of the experiment, an average weekly consumption of 56.14 ± 10.92 ml of pure alcohol per animal was determined.

The euthanasia procedures were performed with an excessive dose of sodium thiopentate, applied intraperitoneally. Then, the two femurs of each animal were collected, soft tissue removed and stored in a cold saline solution.

Bone mineral density was assessed using the dual-energy X-ray absorptiometry method (DXA), and performed on a Lunar DPX-IQ densitometer (Lunar; software version 4.7 and, GE Healthcare, Chalfont St. Giles, United Kingdom). For this, the femurs were positioned parallel to inside of a vat and scanned in series. The region of

interest was established as an area of 0.90 cm² centralized on the neck of all femurs.

Then, a femur from each pair was selected at random for histomorphometric analysis. The contralateral femur was reserved for mechanical testing and kept in a freezer wrapped in moist gauze. For histological processing, the whole bone was fixed in 10% formaldehyde for 48 hours, decalcified for six days in a 20% sodium citrate solution and 30% formic acid, with the solution being changed every 48 hours. After decalcification, femurs were washed in running water for 24 h and had their size reduced to select only the proximal region of the femur. There was dehydration in ethyl alcohol concentration solutions, xylol clearing, and inclusion in paraffin blocks. Serial sections were obtained in the 4.0 µm thick coronal plane, and hematoxylin and eosin (HE) and picro sirius red staining were performed.

The HE-stained slides were used to analyze the thickness of the bone trabeculae, expressed in µm. For obtaining the measurements, the fragment in each slide was divided into four fields, and in each field was selected, so random five bony trabeculae. For each bone trabecula, five measurements were taken, extending from the smallest to the largest axle. Thus totaling 100 measures per blade (histological section). A video camera coupled with a common light microscope, with a 40x objective, and a Zeiss / Zen Primostars® image analyzer system (Axiocam 105 color®) were used.

The total collagen was analyzed in the sections stained by picro sirius red, under polarized light, with 400x magnification, and a video camera coupled to a microscope with an automatic analysis system, LeicaQWin Plus® (Leica Microsystems, Inc., Wetzlar, Germany). The birefringent areas, usually green, orange, or reddish, were marked by the same observer to

quantify an analysis area in each field (Amaral et al. 2020, Bertoldo et al. 2019).

The mechanical test was static, destructive, and three-point flexion. After defrosting the bone to the refrigerator temperature and after reaching equilibrium with the environment, each femur was rested on two metallic supports that were 25 mm apart, and a progressive load was vertically applied at the center of the posterior surface of the diaphysis at a constant displacement rate of 1 mm/min, until bone fracture (Rosa et al. 2019). The EMIC® test device (São José dos Pinhais, PR, Brazil) was equipped with a 50 N load cell, and the load-deflection curve was obtained in real time. The maximum load and stiffness were calculated using the TESC® software (version 13.4, São José dos Pinhais, PR, Brazil).

A statistical analysis of data was performed using the GraphPad Prism® program, version 5.0. The normal distribution of the data was verified with the Shapiro-Wilk test. The *Student test* was used for parametric data, and the Mann-Whitney U test for nonparametric data. Differences were considered statistically relevant when p values were less that 5% (p <0.05).

## RESULTS

Table I shows the mean and standard deviation of the final body mass data of the animals in the control and ethanol group, collected in the last week and in euthanasia. There was no statistical difference in the comparison of body mass gain between the control group and the ethanol group, although with borderline results (p = 0.532).

There was a 23% reduction in bone mineral density (BMD) of femurs in the ethanol group, compared to the control group. In the control group, the average was 0.153 ± 0.080 g / cm³, and in the ethanol group 0.131 ± 0.035 g / cm³. There

**Table I. Mean ± standard deviation of body mass of rats in grams (g).**

| Rats | Control Group (g) | Ethanol Group (g) |
|:---:|:---:|:---:|
| 1 | 562,17 ± 25,68 | 503,75 ± 17,75 |
| 2 | 590,92 ± 14,72 | 524,50 ± 18,32 |
| 3 | 513,33 ± 17,57 | 531,17 ± 23,30 |
| 4 | 533,67 ± 16,16 | 537,58 ±18,23 |
| 5 | 542,17 ± 16,45 | 547,25 ± 14,68 |
| 6 | 515,29 ± 18,08 | 557,67 ± 16,93 |
| **Average** | **542,93 ± 29,66** | **533,65 ± 18,77** |

*Student t test* **(p=0.532).**

was no significant difference in the comparison between groups (p = 0.507) (Figure 1).

There was a significant reduction of 20% in the thickness of the trabeculae in the metaphysis of the neck of the femurs of the rats in the ethanol group (60.04 ± 19.63 µm), compared to the control group (94.92 ± 29.97 µm) (p < 0.0001) (Figures 2 and 3).

The ethanol group had a lower percentage of collagen in the femoral neck metaphysis (1.55%), compared to the control group (2.15%), with a significant difference of -1.56% (p <0.0001) (Figures 4 and 5).
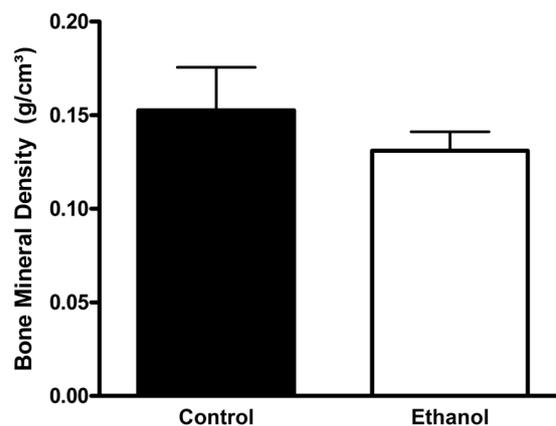


**Figure 1. Comparison of bone mineral density (g/cm³) between the control group (CG) and the ethanol group (GE). Mann-Whitney test; p=0.507.**
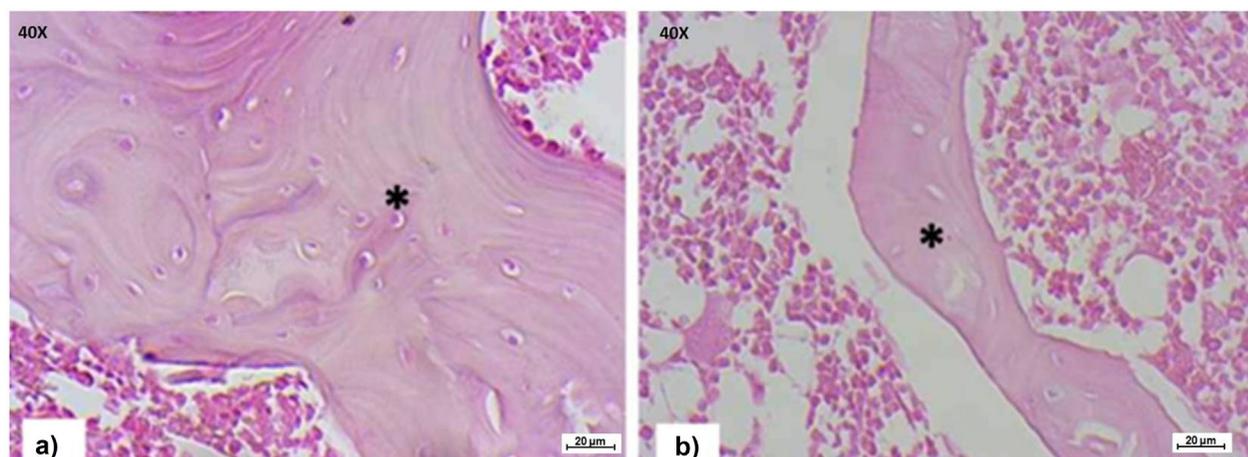
**Figure 2. Bone trabeculae\* from the femoral neck of rats a) not submitted to alcohol intake and b) submitted to ethanol intake. Hematoxylin and eosin (HE), original magnification 400x.**

In three-point mechanical flexion tests, the mean maximum force was 17.4% lower in the ethanol group (90.6 ± 15.2 N) than in the control group (109.7 ± 20.3 N) (p <0.001). The femurs of animals in the ethanol group (166.85 ± 30.50 N / mm) had a mean stiffness rate 25.6% lower than that of animals in the control group (132.82 ± 22.12 N / mm) (p <0.001) (Figure 6).

## DISCUSSION

The findings of the present study confirm the hypothesis that chronic experimental alcohol consumption interferes with bone trabeculae structure and the percentage of collagen. The mechanical resistance of femurs was also significantly decreased, showing the structural weakening of the bone, represented by the decrease in maximum load and less stiffness. These results indicate that the model adopted was adequate to identify some deleterious effects of chronic alcohol consumption. Histological examination contributed to show the important role of collagen in bone mechanical resistance. In effect, this parameter is the result of the quality of the trabeculae, as well as the mineralization. Although we have only performed a test with the whole bone,

which gives us only the structural mechanical properties, the material properties of the bone as a tissue (performed on standardized samples) must also have been affected, as there is a correlation between both.

One aspect that deserves to be highlighted is the fact that, in this model, exposure to alcohol consumption did not significantly interfered with the body mass gain of our animals, differently from what was observed by other authors, in which there was a reduction in body mass, consumption of chow, and poor nutrient uptake (Faustino & Stipp 2003, Maddalozzo et al. 2009). However, our body mass results were
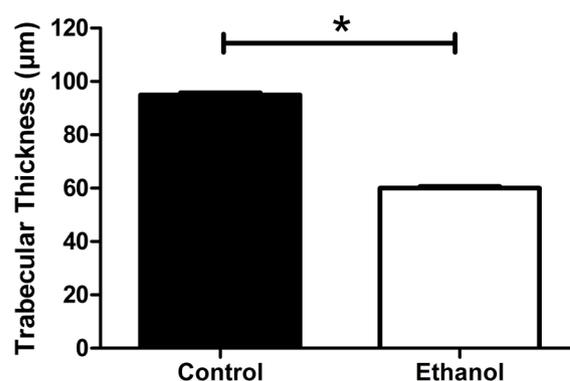


**Figure 3. Thickness of the trabeculae of the proximal metaphysis of the femurs of rats in the control group (GC) and the alcohol intake group (GA); *Student t test*; \*asterisk indicates significant difference (p<0.0001).**
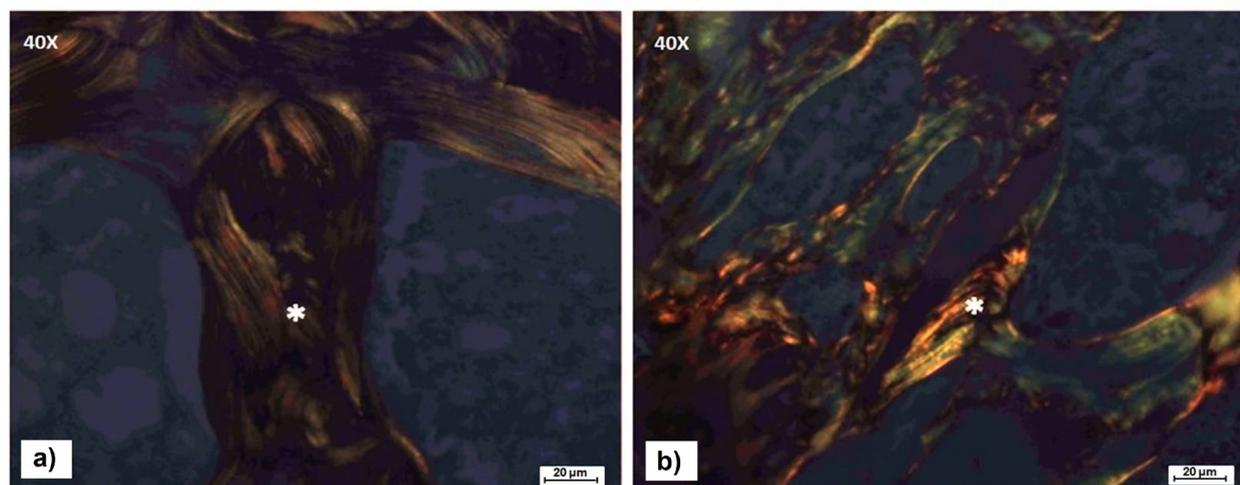
**Figure 4.** Collagen images from the neck of the femurs analyzed under polarized light microscopy. Bone trabeculae*. In a) total collagen percentage in the control group; in b) total collagen percentage in the ethanol group. The collagen fibers appear in red, orange, and green color (picro sirius red, original magnification 400 x).

borderline; perhaps if there had been a longer time of exposure to alcohol consumption, this effect would have been observed.

Our results did not show a significant change in BMD associated with chronic alcohol consumption, but there was a significant reduction of 20% in the thickness of bone trabeculae and a reduction of 1.56% in the content of collagen in the metaphysis of the femur neck of immature rats. Despite this, the changes in the organic matrix were able to decrease bone strength, with a reduction of 25.6% in stiffness and 17.4% in the maximum strength of the diaphysis of the femurs, as shown by the mechanical test.

It is common for young people aged between 28 and 30 to participate in at least one episode of alcohol use per month, in which 20% of women and 25% of men consume six or more drinks per occasion (Grossberg et al. 2004). Reports point out that the habit related to alcohol intake by young adults becomes a behavioral deviation that begins in adolescence (16 to 19 years old) and tends to persist in early adulthood (30 to 31 years old) (McCarty et al. 2004), an age group that covers the most critical period of peak bone

growth and maturation (Abrams 2003). Thus, it is important to note that the animals used in the study remained in experimentation from six to twelve weeks of age, which corresponds approximately to the period of 18 to 30 years of human age (Andreollo et al. 2012).

In this context, it is evident that our analyses were carried out exactly at the most critical period of bone growth and maturation, allowing us to highlight the effect of chronic experimental alcohol consumption on the organic matrix, hitherto not described in the literature. Another important point is that these changes occurred in the region of the metaphysis of the bones, which corroborates the findings of Rosa et al. (2019), in which chronic alcohol consumption decreased, providing a 5.3% reduction in length and mechanical resistance of the tibia in immature rats, suggesting interference with bone growth.

Alcohol consumption is detrimental to the integrity of bone tissue, with direct interference in bone repair (Lima et al. 2011), and is an important risk factor for osteoporosis (Santori et al. 2008). Consumption does not only harm bone structure, it leads to metabolic changes, vitamin
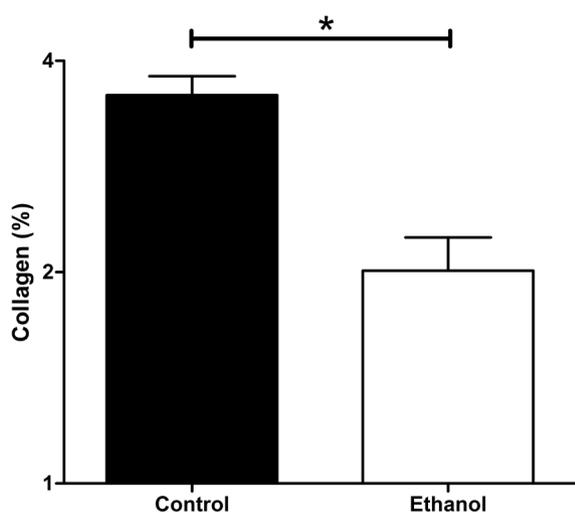
**Figure 5. Percentage of collagen in the proximal metaphysis of the femoral neck in the control group and in the alcohol group. Mann Whitney test; *asterisk indicates significant difference (p<0.0001).**

D deficiency, and the adoption of inappropriate eating habits, increasingly compromising bone integrity (González-Reimers et al. 2011).

Experimental studies have shown that chronic alcohol consumption in different concentrations (5 to 20%) causes morphological and biomechanical changes in the lamellar trabeculae bone, leading to changes in the volume and bone repair process (Lima et al. 2011, Horvath et al. 2010, Soares & Garcia 2011). Thus, orthopedic surgeons and dentists have discussed the need to establish preoperative guidelines to prohibit or reduce the consumption of alcoholic beverages (Budworth et al. 2019), considering that the adoption of such a measure has been shown to be a determining factor in the repair process and reduce the risk of bone infections.

Systemic metabolic changes and bone turnover, attributed to alcohol consumption, are well described in the literature, especially related to microarchitecture and bone mineral quality, in which the high remodeling rate is associated with reduced bone mass and stiffness (Lalor et al. 1986, Maurel et al. 2012).

However, in some cases BMD is not an adequate predictor of bone fragility (Ott 1993, Schuit et al. 2004), thus highlighting the role of collagen in bone resistance (Sroga & Vashishth 2012, Viguet-Carrin et al. 2006).

Although evidence indicates that an organic matrix, as well as mineral matrix, contributes strongly to bone strength and health (Bala et al. 2015), the main bone quality assessment technique remains DEXA, limiting the analysis to BMD only. However, deterioration in bone quality and fragility cannot be attributed to just one component of the bone, thus being multifactorial. The changes are mainly related to changes in the mineral matrix and collagen, with non-collagen proteins that regulate the arrangement of the bone matrix undergoing changes in the aging process, diseases, and antiosteoporosis treatments (Saito et al. 2006, Tang et al. 2009).

Despite the fact that reducing BMO is considered the main cause of bone fractures in some patients, there is no incidence of serious traumatic fractures, even among those diagnosed with osteoporosis (Sornay-Rendu et al. 2007). Thus, the evidence points to the importance of also considering the effects of the organic matrix during clinical practice, to understand the complex changes in macro, micro, and nanoscale, which occur in bone tissue and contribute to making it more fragile and osteoporotic. However, it is essential to develop new methods of diagnosis of the organic matrix and/or less costly actions that allow access to tests such as high resolution peripheral quantitative computed tomography (HR-pQCT) and magnetic resonance imaging (MRI), which allows us to also evaluate bone architecture (Melton et al. 2007).

The significance of this study is the suggestion that the organic matrix of bone may represent an important element in bone
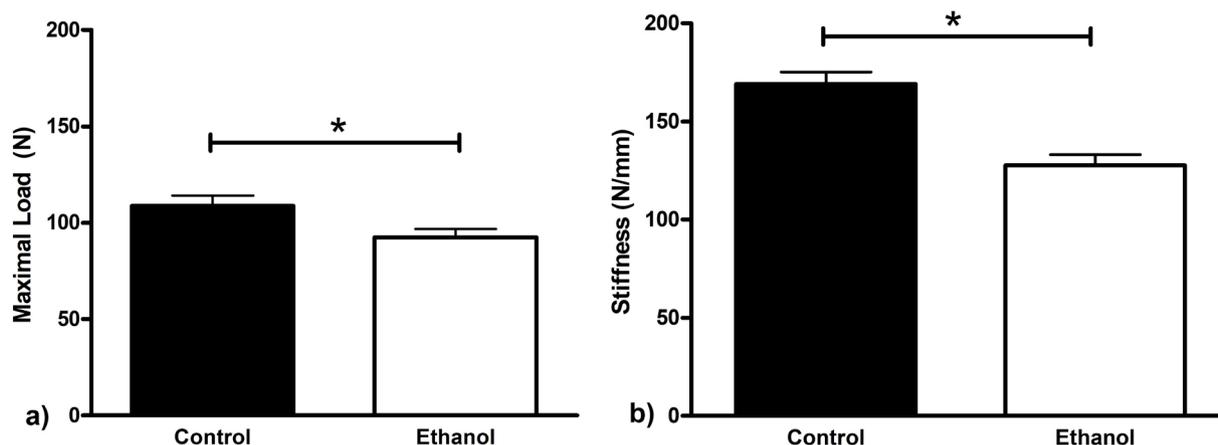
**Figure 6.** Analysis of mechanical resistance. a) maximal load; b) stiffness. The difference is significant (Mann Whitney test); *asterisk indicates significant difference (p<0.001).

resistance and not only in mineralization itself, which indicates that these aspects require more in-depth studies.

Limitations of this study include the fact that the lamellar structure of the femur compact (diaphyseal region) was not investigated and that other types of mechanical tests (flexocompression, torsion) were not used, which would certainly allow a better characterization of the bone and carrying out the study only in female rats.

Therefore, we conclude that, even without altering bone mineral density, alcohol consumption caused changes in the morphology and percentage of collagen in bone trabeculae in the region of the growing neck of the rat femur, making the bone more fragile. Thus, our results suggest harmful effects of alcohol intake on the bone quality of young adult animals and warn of the need to also consider methods for the diagnosis of collagen as an element of bone fragility.

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Odival Seabra, Vandair G. Pereira, Ana Paula Espindula and Fabrizio A.G. Cardoso were responsible for the experimental activities and data analysis. José B. Volpon, Sanívia A.L. Pereira and Rodrigo César Rosa were responsible for designing and supervising the project and for writing the manuscript with the collaboration of all authors.