

Effect of 15% Alcohol Dependence on Alveolar Bone Loss and TNF- α Secretion in Wistar Rats

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The aim of the present study was to evaluate the effect of 15% alcohol dependence on ligature-induced alveolar bone loss and TNF- α secretion in Wistar rats. Thirty-three male Wistar rats aged 45-60 days (mean weight=253 g) were randomly allocated test or control groups. Test group (n=18) received 15% alcohol as liquid intake and control group (n=15) received water during the experimental period. TNF- α was analyzed by ELISA assay in 11 animals per group. After 14 days of alcohol/water intake, alcohol dependency was assessed and silk ligatures were placed around the left second upper molars. Ligature presence and body weight were checked weekly. After 40 days, animals were sacrificed and the maxillae were defleshed for morphometric analysis using standardized images. All animals in the test group displayed signs of alcohol dependency at day 14. No statistically significant differences in final body weight (334.83 ± 21.38 vs. 322.48 ± 30.65 g, $p=0.20$) were observed between groups. In relation to alveolar bone loss, no statistically significant difference was observed among test and control groups both for ligated teeth (0.76 ± 0.06 vs. 0.74 ± 0.10 mm, $p=0.60$) and unligated teeth (0.41 ± 0.16 vs. 0.35 ± 0.05 mm, $p=0.22$). The TNF- α secretion also did not display statistically significant differences between test and control groups (10.78 ± 1.84 vs. 12.13 ± 2.11 pg/mL, $p=0.12$). It may be concluded that 15% alcohol dependency was not capable to alter alveolar bone loss and TNF- α secretion in Wistar rats.

Key Words: periodontitis,
rats, alcohol dependence,
alveolar bone loss.

Introduction

The relationship between alcohol consumption and several chronic diseases has been reported as a U- or J-shaped curve attributed to a dose-related combination of beneficial and harmful effects. While alcoholism or alcohol abuse can lead to cardiovascular, liver and pancreatic diseases, light to moderate consumption of alcohol may display a protective effect (1-3). Several biological mechanisms can be involved in the pathogenesis or prevention of these chronic diseases, such as the modulation of some pro-inflammatory markers (e.g. IL-6 and TNF- α) (4,5). The drinking pattern can also impair or repair the immunological function by modulation of neutrophils, macrophages and T-cells (6,7).

Periodontal diseases have an infectious-inflammatory nature and are associated mainly to gram-negative bacteria (8,9). The relationship between alcohol consumption and periodontal diseases remains unclear in the literature. Similarly to the relationship with other chronic diseases, results from animal and human studies seem to report a U- or J-shaped curve (4,10). However, there is no sense in the literature about what pattern of alcohol consumption is considered safe, beneficial or harmful. Studies in rats with high alcohol concentration (20%) suggest that alcohol can have a destructive effect on the ligature-induced

alveolar bone loss (11,12). On the other hand, low alcohol concentration (5%) seems to present a protective effect in spontaneous alveolar bone loss (13). Additionally, it has been demonstrated that moderate alcohol consumption is associated with lower levels of cytokines linked to periodontitis in humans (5).

Few studies have sought to clarify the relative role of alcohol dependence on periodontitis in humans and are scarce in animal models (14-16). Therefore, important questions remain regarding this issue. Among these, the role of alcohol dependence on pro-inflammatory cytokines and what is the concentration that could be attributed as beneficial or harmful to the periodontium. The aim of the present study is to evaluate the impact of alcohol consumption on the pathogenesis of periodontal diseases in rats. The hypothesis to be tested is that 15% alcohol dependence can alter both periodontal breakdown and TNF- α secretion in Wistar rats.

Material and Methods

Animals

Thirty-five male Wistar rats aged 45-60 days with mean weight of 253 ± 19.86 g were used in the present study. Animals were housed in cages with five animals at a temperature range from 18 °C to 20 °C and a 12 h light

and dark cycle. The Ethical Committee on Animals Use of the Federal University of Rio Grande do Sul, Brazil approved the experimental protocol (Protocol number 18979/10, September 14, 2010).

Sample Size Calculations

Sample size estimates were based on data from a previous study (11). A difference of 0.2 mm in alveolar bone loss was expected as significant. Considering alpha and beta errors of 0.05 and 0.10 respectively, a minimum number of 14 animals per group was considered necessary.

Experimental Groups and Procedures

Animals were randomly assigned to two groups stratified by weight. The control group (n=15) was fed with a standard laboratory rat chow (Nuvilab CR1[®]; Nuvital Nutrientes SA, Colombo, PR, Brazil) and tap water *ad libitum* for 6 weeks. The test group (n=20) received the same diet except for the water, which was replaced by a 15% ethylic alcohol solution (Vetec[®]; Rio de Janeiro, RJ, Brazil) that was available *ad libitum* throughout the experiment. Prior to ligature placement, the test group was submitted to a 2-week period of 15% alcohol intake. Two animals of the test group were lost. The first died in the first week due to complications of alcohol intake and the second died in the day that the ligature was placed during the anesthesia. These deaths resulted in 15 animals for control and 18 animals for test group.

General anesthesia was performed by intramuscular administration of xylazine/ketamine (10 mg/kg - 1:1).

Alveolar bone loss was induced by placement of 4.0 silk ligatures (Ethicon[®], Johnson & Johnson[®], São Paulo, SP, Brazil) around the maxillary right second molars. The contralateral tooth remained as intra-group control (13,17). Animals were killed after 4 weeks of ligature placement. Figure 1 shows the flowchart of the study.

The liquid and solid intake was monitored during the study. Body weight and presence of ligature of the animals were evaluated weekly.

Alcohol Dependence Test

Prior to periodontal breakdown induction, animals from test and control groups were submitted to an alcohol dependence test (18,19). Animals were exposed to a 14 h of alcohol/water abstinence. Next, they were removed from their cages and then were gently manipulated and placed in another cage with minimum light and noise. Physical signs of alcohol withdrawal were observed and recorded. Animals from control group were used as negative controls. The examiner was blinded to the groups. The signs observed were trembles in tail and body, jaw grind and hyperactivity.

TNF- α Analysis

TNF- α analysis was performed in a randomly selected subgroup sample (11 animals per group). Animals were killed by decapitation and immediately trunk blood collected and centrifuged for 5 min in 5000 x g at room temperature. The levels of TNF- α in supernatants were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kit for rats TNF- α (Uscn Life Science Inc.,

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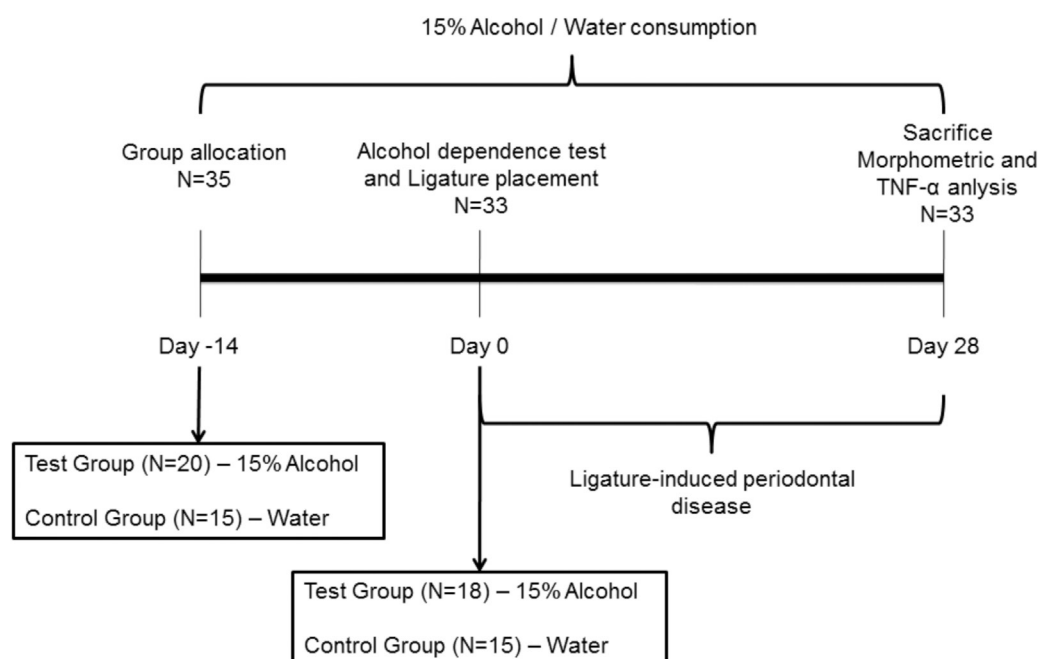


Figure 1. Study flowchart.

Houston, TX, USA), according to manufacturer protocols. Samples were incubated on 96-well flat bottom plates previously coated with anti-TNF- α antibody for 2 h at 37 °C. After this first incubation, a TNF biotin-conjugated antibody preparation specific for TNF- α was added to each well and incubated for 1 h and avidin-conjugated horseradish peroxidase for 30 min at 37 °C. The reactions were stopped with 150 mL of 1.0 M H₂SO₄ and ELISA reader measured the absorbencies at 490 nm. The standard TNF- α curve was performed on each plate, ranging from 0 to 1.000 pg/mL. The tests were performed in duplicate for each sample.

Morphometric Analysis

Following sacrifice, the right and left segments of the maxillae were defleshed in sodium hypochlorite with 8.5% active chlorine (Mazzarollo[®], Gravatai, RS, Brazil) for 5 h. After rinsing, the specimens were washed and dried. After that, maxillae were stained during 1 minute in 1% methylene blue to delineate the cemento-enamel junction (13,17).

Standardized digital pictures were taken from the buccal, palatal and proximal aspects of each specimen using a millimeter ruler and a Nikon D100 (Nikon[®], Ayuthaia, Thailand) camera coupled in a tripod and equipped with a 100 mm macro-lenses with minimal focal distance. Each specimen was placed with the occlusal surface parallel to the floor. Linear measurements were performed with Adobe Photoshop CS4[®] software (Adobe Systems Inc., San Jose, CA, USA). Alveolar bone loss was defined as the distance between the cemento-enamel junction and the alveolar bone crest. Buccal and palatal measurements were made at five points (13,17). Next, in order to perform proximal measurements, removal of the first and third molars was necessary. After these procedures, measurements were made at three points in each proximal surface (20). For

each surface, mean values were calculated and, following, a mean value for the tooth was generated. Figures 2 and 3 demonstrate the measurement points in teeth without (A) and with ligatures (B) in buccal and proximal areas, respectively.

The examiner was unaware of the group distribution as well as of the presence or absence of ligature. Measurements were converted into millimeters, utilizing a precision ruler as reference. Reproducibility was checked by re-analyzing the specimens within an one-week interval. Intraclass correlation coefficient (ICC) was 0.96 and 0.89 for buccal/palatal and proximal surfaces, respectively.

Statistical Analysis

For each evaluated parameter normality was tested by means of Shapiro-Wilk analysis and the appropriate statistical test was selected according to this assumption. The rat was considered the unit of analysis in this study. All tests were performed by SPSS[®] version 17.0 (SPSS Inc.,

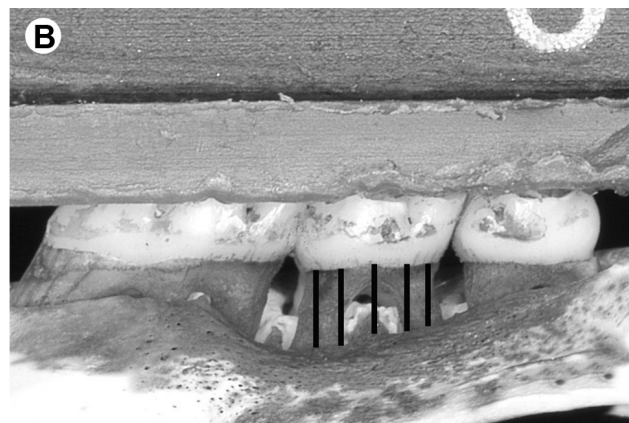
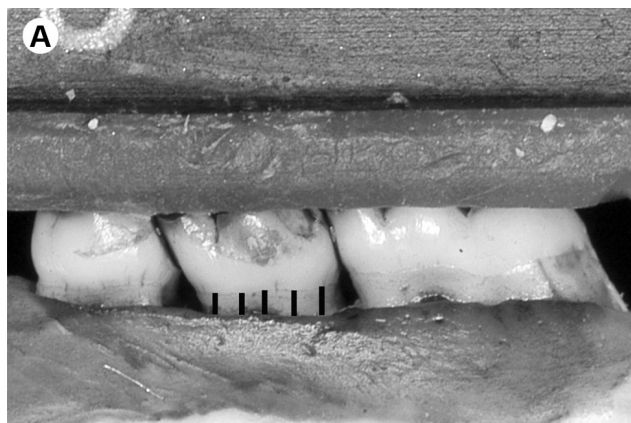


Figure 2. Demonstration of the measurement performed in teeth without (A) and with (B) ligatures in the buccal area.

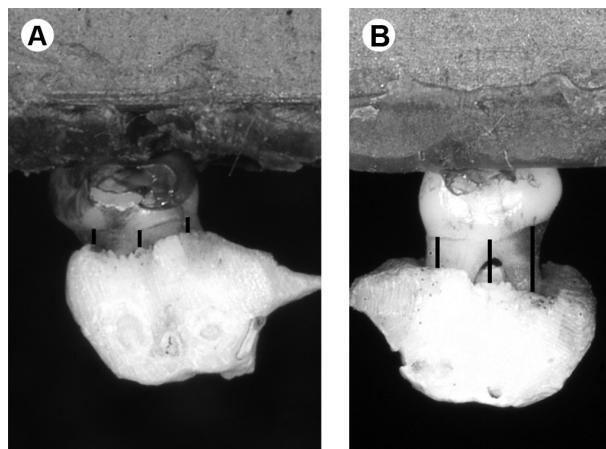
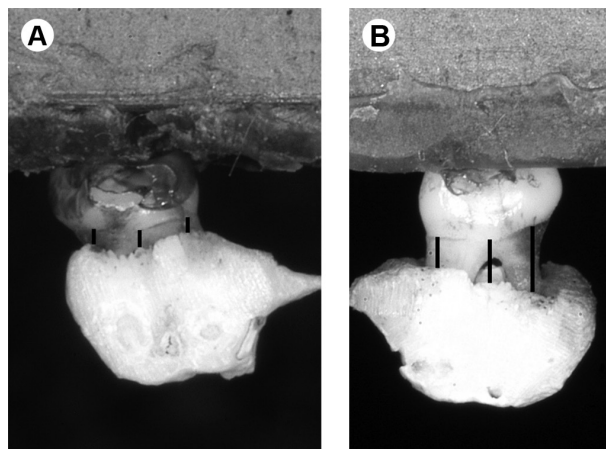


Figure 3. Demonstration of the measurement performed in teeth without (A) and with (B) ligatures in proximal area.

Chicago, IL, USA). Mean body weight throughout the study was calculated and compared by repeated measurements ANOVA. Intragroup mean body weight, alveolar bone loss and the TNF- α secretion were compared by independent samples *t*-test.

Results

At day 0, animals were tested for alcohol dependence. All animals from the test group demonstrated signs of alcohol dependence (trembles in tail and body, jaw grind and hyperactivity) whereas this characteristic was not detected in animals from control group.

The body weight of the animals was monitored throughout the study. Statistically significant differences in mean body weight were observed between control and test groups at days 7 ($p=0.01$) and 28 ($p=0.04$). During the entire study, higher values of body weight were observed in the control group (Fig. 4). Body weight increased both in test and control groups, with statistically significant differences being detected by repeated measures ANOVA within each group along the study when compared to day -14 ($p<0.00$).

During the experimental period, animals from the test group had a mean daily alcohol consumption of 21.69 mL. Figure 5 shows the evolution of alcohol consumption for test group from day 0 to day 28 (repeated measures ANOVA). Animals from test group presented less daily alcohol consumption in day 14 ($p=0.01$) and day 21 ($p=0.03$)

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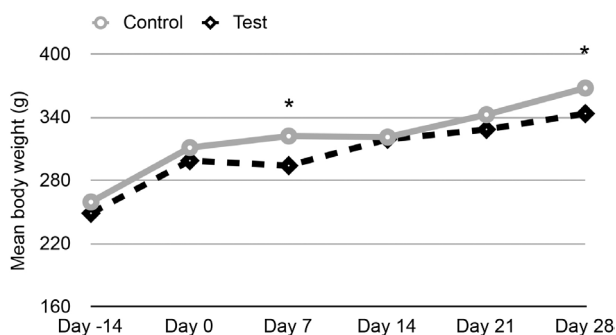


Figure 4. Mean body weight (grams) for control and test groups throughout the study. *Independent samples *t*-test ($p < 0.05$).

compared to day 0. At day 28, no differences were observed compared to day 0 ($p=0.08$).

The main outcome of the present study is periodontal breakdown. Table 1 shows the mean alveolar bone loss in teeth with and without ligature in control and test groups. Data are demonstrated for each tooth surface and also combining all tooth surfaces. Statistically significant differences between groups were observed only in the distal aspect of teeth with ligatures ($p=0.02$). It can be observed that teeth without ligature present approximately 40% less periodontal breakdown than teeth with ligatures.

TNF- α secretion analysis (mean \pm SD) showed no statistically significant differences between control and test group (12.13 ± 2.11 and 10.78 ± 1.84 pg/mL) respectively, ($p=0.12$).

Discussion

This study analyzed the influence of 15% alcohol dependence on alveolar bone destruction and TNF- α secretion in male Wistar rats. No differences in the mean alveolar bone loss were found in this study, when the whole tooth is considered. Previously, studies showed that a low concentration of alcohol consumption (5%) had a protective effect on spontaneous periodontitis (13) while a higher concentration of alcohol consumption (20%) had a destructive effect on the periodontium (11). Similarly, in epidemiologic studies, a moderate consumption of alcohol can have a protective effect for periodontal disease while a

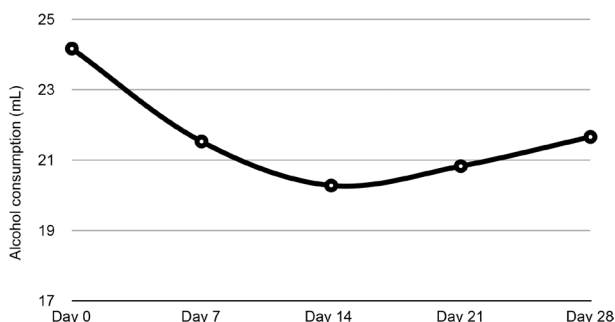


Figure 5. Mean daily alcohol consumption (milliliters) per animal in the test group from day 0 to 28.

Table 1. Mean alveolar bone loss (mm (\pm SD)) in teeth with and without ligatures in control and test groups

Group	Teeth with ligature					Teeth without ligature				
	Mesial	Distal	Palatal	Buccal	Total	Mesial	Distal	Palatal	Buccal	Total
Control	0.86 (± 0.15)	0.81 (± 0.10)	0.69 (± 0.13)	0.89 (± 0.15)	0.76 (± 0.06)	0.50 (± 0.16)	0.49 (± 0.56)	0.46 (± 0.10)	0.29 (± 0.05)	0.41 (± 0.16)
Test	0.89 (± 0.16)	0.69 (± 0.16)	0.69 (± 0.12)	0.84 (± 0.20)	0.74 (± 0.10)	0.48 (± 0.12)	0.31 (± 0.12)	0.42 (± 0.04)	0.26 (± 0.03)	0.35 (± 0.05)
p*	0.58	0.02	0.96	0.44	0.60	0.80	0.19	0.17	0.10	0.22

*Independent samples *t*-test.

heavy consumption is associated with a destructive pattern (10). These evidences seem to confirm a U- or J-shaped curve relationship between alcohol consumption and periodontal disease, similarly to what happens in relationship with cardiovascular diseases and the consumption of alcohol. In this sense, it would be interesting to determine the cut-off point of the curve in order to better understand this relationship.

One interesting finding of the present study is that when surfaces are analyzed separately, considering the distal aspects, a statistically significant difference was detected among groups, demonstrating, for these surfaces, a protective effect of alcohol. This is in line with other studies, and this result should not be ruled out. Therefore, a potential protective effect was demonstrated in this study. The findings of the present study seem mimic low/moderate alcohol consumption.

Low/moderate alcohol consumption has been considered a preventive factor by reducing the risk for myocardial infarction, stroke and other systemic disorders. However, high alcohol consumption poses a higher risk of myocardial infarction. It is not clear which kind of beverage or what is the concentration of alcohol that would have a preventive effect and why low/moderate regular alcohol intake should be beneficial to humans (3,21). A drinking pattern of 3-4 times per week has been found inversely associated with the risk of myocardial infarction in men (1).

Alcohol can have an anti-bacterial role, however the potential effect of regular low/moderate alcohol intake on the oral microbiota has not been fully demonstrated. Drinking patterns have been assessed and seem to alter the oral bacterial profile. Consumption for at least two years of coffee or red wine is associated with a reduction of the frequency of anaerobes in supra- and subgingival plaque samples compared to water drinkers (22). Studies on alcohol dependence and alcohol consumption on periodontal diseases are controversial. While some studies reveal increased risk with increasing daily consumption of alcohol, other studies verified no association (10,16,23). If alcohol consumption reduces the bacterial load in the oral cavity, this effect would be observed on the prevalence of infectious diseases in the mouth.

Pathogenesis of periodontal breakdown has been widely studied in rat models. Previous studies evaluated alveolar bone loss only in buccal and palatal surfaces (11,13,17). In the present study a new approach was conducted in order to measure proximal surfaces. Therefore, first and third molars were gently removed followed by standardized pictures of the mesial and distal surfaces, allowing the same as the measurements performed in buccal and palatal surfaces. It is important to emphasize that a positive correlation

between alveolar bone loss in buccal/palatal and proximal surfaces has been shown, which improves the use of this new approach to quantify alveolar bone loss in Wistar rats (20).

Several mechanisms have been suggested by which alcohol intake could lower the risk of inflammatory diseases, such as beneficial effects on C-reactive protein, IL-6, and TNF- α (5,24,25). The present study showed no differences in TNF- α levels between groups, although there is a reduction of approximately 18% in production of TNF- α in the test group. This mean percent reduction is very similar to human studies that found significant results between groups exposed or not to alcohol (5,24).

Adverse effects of high consumption of alcohol on human health are well known described in the literature. One possible explanation is that this seems to occur because the episodic consumption of large amounts of alcohol has been associated with increase of the host susceptibility to infections. This effect has biological plausibility to clarify its influence on periodontal diseases, since the high consumption of alcohol can cause damage in neutrophils, macrophages and function of T cells, increasing the likelihood of infections (6).

The findings of the present study are challenging, with possibilities of better understanding the relationship between alcohol consumption and periodontal breakdown. The possible biological mechanisms should be further understood and longitudinal follow-up is required. It can be concluded that 15% alcohol consumption generated dependence, however was not able to consistently modify alveolar bone loss and TNF- α secretion in male Wistar rats.

Resumo

O objetivo do presente estudo foi avaliar o efeito da dependência de álcool a 15% sobre a perda óssea alveolar induzida e secreção de TNF- α em ratos Wistar. Trinta e três ratos wistar com idade entre 45 e 60 dias (peso médio=253 g) foram alocados aleatoriamente para o grupo teste ou controle. O grupo teste (n=18) recebeu álcool a 15% como ingestão líquida e o grupo controle (n=15) recebeu água durante o período experimental. TNF- α foi analisado por meio de ELISA em 11 animais por grupo. Após 14 dias de ingestão de álcool/água a dependência do álcool foi determinada e ligaduras de seda foram colocadas ao redor dos segundos molares superiores esquerdos. A presença das ligaduras e o peso corporal foram verificadas semanalmente. Depois de 40 dias os animais foram sacrificados e as maxilas foram preparadas para análise morfométrica em fotografias standardizadas. Todos os animais do grupo teste apresentaram sinais de dependência de álcool no dia 14. Não foram observadas diferenças estatisticamente significativas no peso corporal final entre os grupos (334,83 \pm 21,38 vs. 322,48 \pm 30,65 gramas, p=0,20) Em relação a perda óssea alveolar, não foram observadas diferenças estatisticamente significativas entre os grupos teste e controle tanto para dentes com (0,76 \pm 0,06 vs. 0,74 \pm 0,10 mm, p=0,60) como para dentes sem ligadura (0,41 \pm 0,16 vs. 0,35 \pm 0,05 mm, p=0,22). A secreção de TNF- α também não demonstrou diferenças estatisticamente significativas entre os grupos teste e controle (10,78 \pm 1,84 vs. 12,13 \pm 2,11 pg/mL, p=0,12). Pode-se concluir que a dependência de álcool a 15% não foi capaz de alterar a perda óssea alveolar e a secreção de TNF- α em ratos Wistar.

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