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Effects of Alcohol and/or Tobacco Exposure on Spontaneous Alveolar Bone Loss in Rats

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The aim of the present study was to evaluate the effect of alcohol and/or tobacco exposure on spontaneous alveolar bone loss in Wistar rats. Twenty-four, male, 60 day-old, Wistar rats were assigned to 4 groups: Group 1 received 10 mL/kg of glucose solution (5%). Group 2 received 2 g/kg alcohol (20%). Group 3 was exposed to tobacco smoke (6 cigarettes/60 min). Group 4 received both interventions of groups 2 and 3. Alcohol was given by gastric gavage and cigarette exposure was performed using a forced ventilation chamber. After 30 days, animals were sacrificed and the upper maxillae removed and defleshed. Morphometric analysis of alveolar bone loss (ABL) around the second molar was performed in standardized digital photographs. Statistical analysis was conducted using paired t-test, one-way ANOVA and occurrence of spontaneous periodontal disease (ABL ≥ 0.39 mm) was analyzed by Fisher's exact test. Significant differences in body weight were observed between all groups. Group 2 presented higher body weight as compared to the 3 other groups at 4 weeks ($p \leq 0.05$). Mean ABL values were 0.31 mm (±0.08), 0.29 mm (±0.07), 0.33 mm (±0.10), and 0.33 mm (±0.08) for groups 1, 2, 3, and 4, respectively. No significant differences were found among groups. In the analysis of occurrence of periodontal breakdown, alcohol exposure decreased the occurrence of ABL and cigarette exposure increased ABL. The combination of alcohol and cigarette exposure did not differ from the control group. Alcohol consumption decreased the occurrence of periodontal breakdown, while tobacco increased this rate.

Key Words: alcohol, smoking, periodontitis, periodontal disease, rats.

Introduction

Alcohol and tobacco are drugs widely consumed throughout the world and have attracted human concernment for thousands of years. High degrees of consumption can place the health of the individual at risk for a series of diseases. According to the World Health Organization, these drugs are associated with different chronic diseases, like cardiovascular diseases (arterial hypertension, stroke, arrhythmia) and cancer (1). However, when the consumption of these substances is considered light/moderate, recent studies have shown different and intriguing results on health. While the relationship between the consumption of tobacco and chronic diseases is considered dose-dependent, light to moderate alcohol intake appears to offer a protective effect on immunity, besides strengthening the inflammatory system. Therefore, the consumption of alcohol seems to present a U- or J-shaped curve related to risk for related-diseases (2).

Microbial biofilms, mainly gram-negative bacteria, are foremost contributors to the development of periodontal diseases. Furthermore, emerging evidence supports a role of systemic factors in the progression of periodontal disease. Several studies suggest that smoking, glucose disorders, obesity and alcohol abuse might contribute to the development of periodontal diseases (3,4). However, studies in humans and in animals have shown that light to moderate alcohol consumption seems to have a beneficial impact on the immune response, as well as infectious and inflammatory processes (5,6). Nevertheless, the biological mechanisms between possible protective effects of alcohol on the periodontium remain not completely understood. On the other hand, clinical and epidemiologic studies consistently find that tobacco exposure is linked with imbalance in cytokine production and T-cell subsets, changes associated with poor periodontal health. The literature suggests that tobacco is a major risk factor for periodontal diseases and probably the most important (4).

Studies evaluating the occurrence of spontaneous alveolar bone loss by morphometric assessment related to alcohol and/or tobacco consumption are nonexistent. Therefore, the present study is warranted. The hypothesis to be tested in the present study is: (i) rats exposed to light to moderate alcohol consumption may present lower occurrence of periodontal breakdown than rats not exposed to alcohol; (ii) rats exposed to tobacco may present higher occurrence of periodontal breakdown than rats not exposed to tobacco; and (iii) rats exposed simultaneously to alcohol and tobacco may present a different pattern of occurrence

of periodontal breakdown than a control group. The aim of present study was to assess the occurrence of spontaneous periodontal breakdown in Wistar rats exposed to alcohol and/or tobacco.

Material and Methods

Study Design

This is a prospective, controlled, and blinded animal model study. The study protocol was approved by the Animal Research Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (Protocol #19566). The protocol complies with the regulations set down by the Universal Declaration of Animal Rights (UNESCO, January 27, 1978) and the International Ethical Guidelines for Biomedical Research Involving Animals (CIOMS - Council for International Organizations of Medical Sciences). All necessary procedures to minimize pain and discomfort were carried out by the researchers.

Animals and Experimental Procedures

Twenty-four, male, Wistar rats (weighting approximately 290 g) were used in the present study. Animals were housed in groups of 3 under a light/dark cycle of 12 h and room temperature (22 ^{o}C ± 2 ^{o}C) with free access to water and rat chow (Nuvilab CR-1; Nuvital, Curitiba, PR, Brazil). The liquid and solid intake as well as body weight was monitored during the study. The animals were distributed in 4 groups and all experimental procedures lasted 4 weeks. Group 1 (Control Group; N = 6): animals received 10 mL/kg of glucose solution (5%) by gastric gavage, twice a day. Group 2 (Alcohol Group; N = 6): animals received 2 g/kg of alcohol solution (20%) by gastric gavage, twice a day. Group 3 (Tobacco Group; N = 6): animals receiving 6 cigarettes by inhalation during 60 min and 10 mL/kg of glucose solution (5%) by gastric gavage, twice a day. Group 4 (Alcohol+Tobacco Group; N = 6): animals received the same intervention of groups 2 and 3, twice a day. Alcohol solution was administrated before the start of the exposure to cigarette smoke.

In order to expose animals to the same effects of stress, groups 1 and 2 stayed during the identical time of groups 3 and 4 in the forced ventilation chamber, although without cigarette smoke. Daily, all experimental groups started experimental procedures at 09 AM and 14 PM.

Glucose and Alcohol Solution

The glucose solution (5%) was prepared by diluting 5 g of glucose in 100 mL of distilled water. Alcohol solution was prepared diluting ethylic alcohol (99%) in glucose solution (5%) in order to obtain a solution containing 20% alcohol (weight/volume). This concentration of glucose was successfully used in rats to increase the absorption of alcohol in relation to the self-administration is tested (7).

Forced Ventilation Chamber

A ventilation chamber was built to administrate cigarette smoke inhalation in groups 3 and 4. It consisted of a glass transparent cage (50x50x30 cm), completely closed except for two holes that allowed the air inlet and outlet that was kept in constant flow of 10 L/min (8). The entry hole of the box was connected to a small apparatus which allowed burning of cigarettes and drag of smoke inside the chamber by vacuum pump coupled to the exit hole. The burning of a cigarette took approximately 10 min. For purpose of no intoxication, 10-min intervals were used without burning cigarettes. Thus, the whole smoke inhalation period lasted for approximately 2 h. Groups 1 and 2 remained the same time, in a similar glass transparent cage, but without exposure to cigarette smoke.

Morphometric Analysis

The morphometric analysis was performed by standard digital photographs (11,12). Pictures were taken using a 6.1 megapixel digital camera (Nikon; Coolpix, Ayutthaya, Thailand) coupled to a tripod and equipped with 100 mm macro-lenses with minimal focal distance. Specimens were fixed to an endodontic ruler, parallel to the ground. Photographs of the buccal and palatal aspects of right and left hemimaxillae were taken.

A calibrated examiner performed the measurements of the linear distances from the cementoenamel junction to the bone crest, using Adobe Photoshop™ CS4 software (Adobe Systems Inc., San Jose, CA, USA). An external researcher kept group distribution in order to warrant blindness of the examiner. Five measurements were performed on each surface of the second molar both buccally and palatally (two on the distal root, two on the mesial root and one on the furcation). The measurements in pixels were then converted into mm using the markings of the endodontic ruler to which the hemimaxillae were attached as reference. All procedures for specimen preparation, photographs as well as morphometric analysis were performed at the Laboratory of Periodontology of the Federal University of Rio Grande do Sul and followed the methods described by Fernandes et al. (9).

Definition of Spontaneous Periodontal Breakdown

The primary outcome of the present study was the occurrence of spontaneous periodontal breakdown. For that, a cut-off point was established, in order to define the occurrence of periodontal breakdown. An analysis of data from the control group was performed and the 75th percentile was considered the cut-off point. Thus, measurements ≥ 0.39 mm were considered as spontaneous

periodontal breakdown.

Statistical Analysis and Reproducibility

Shapiro-Wilk test for normality was used and a normal distribution was detected in all continuous variables. Mean body weight was calculated and compared by paired t-test (intragroup analysis) and ANOVA (intergroup analysis). Mean and standard deviation of the distance from the CEJ to the alveolar bone crest and consumption of food for different groups were calculated and compared by ANOVA. Sites classified as positive for periodontal disease occurrence were compared between groups by Fisher's test and interpreted by adjusted residuals. All analyses conducted in the present study were performed using the animal as the unit of analysis, except for the occurrence of periodontal breakdown, in which the site was analyzed. Statistical analyses were performed in Stata 10.1 for Macintosh (Stata; StataCorp, College Station, TX, USA). The level of significance was set as 0.05.

Twelve pictures for the morphometric analysis were randomly selected to be double-measured with a one-week interval. The intra-class correlation coefficient (ICC) between measurements was 0.93.

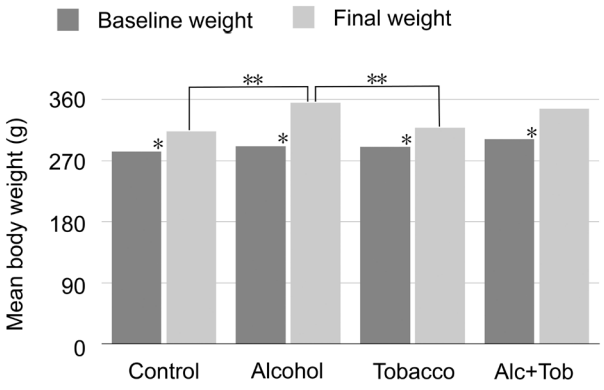

Figure 1. Mean body weight (g) for each experimental group.

Results

Figure 1 shows the mean body weight for the 4 experimental groups at baseline and after 4 weeks. Body weight of animals at baseline was of approximately 290 g, with no statistically significant difference between groups. After 4 weeks of exposure to regular diet, alcohol and/or tobacco a statistically significant difference in body weight was observed in all groups. Furthermore, the animals exposed only to alcohol presented statistically significant higher mean body weight as compared to control and tobacco groups at 4 weeks. No statistically significant difference was observed between alcohol+tobacco exposure and the other groups.

Throughout the study, it can be observed that there was a significant decrease in food consumption in all groups, except for rats in the tobacco group (Fig. 2). However, there were no significant differences between experimental groups in food consumption (g/day/rat) at any time of the experiment. Mean (±SD) alveolar bone loss was 0.31 mm (±0.08), 0.29 mm (±0.07), 0.33 mm (±0.10), and 0.33 mm (±0.08) for control, alcohol, tobacco and alcohol+tobacco groups, respectively (ANOVA). No significant differences were observed between groups (p=0.32).

The main outcome of the present study is demonstrated in Figure 3. In animals submitted to alcohol, only 1 site was classified as experiencing spontaneous periodontal breakdown. However, tobacco and alcohol+tobacco groups exhibited 9 and 4 sites with spontaneous periodontal breakdown, according to the present cut-off point, respectively. A statistically significant difference between groups was detected (p=0.03). The interpretation of the analysis by adjusted residuals demonstrated that there is a positive association between alcohol and absence of

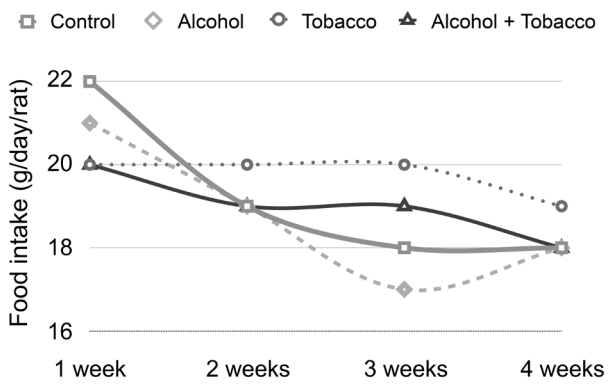

Figure 2. Mean food intake (g/day/rat) throughout the study according to the experimental groups.

Figure 3. Occurrence of alveolar bone loss (mm) according to experimental groups after use of a cut- off point ≥ 0.39 mm (75th percentile).

spontaneous periodontal disease. On the other hand, there is a positive association between tobacco and presence of spontaneous periodontal breakdown. Furthermore, there is no association between alcohol+tobacco with spontaneous periodontal breakdown.

Discussion

The present study assessed the occurrence of spontaneous periodontal breakdown in Wistar rats exposed to alcohol and/or tobacco. The results showed that light to moderate alcohol consumption was associated with a decrease in the occurrence of periodontal breakdown, while tobacco was related with an increase in this rate. The main novelty of this study is to assess the combined effect of two known risk factors to periodontal diseases on spontaneous alveolar bone loss.

In order to put the results of the present study into perspective, some methodological considerations are warranted. The present study evaluated spontaneous periodontal breakdown in Wistar rats. The emerging literature has started to pay closer attention to the spontaneous periodontal destruction both looking at the control sides of animals (10), as well as performing studies without induction of periodontal disease (11,12). This is related to the fact that the placement of ligatures or the induction by bacterial strains (e.g., *Porphyromonas gingivalis*) combined or not with ligatures may promote an acute inflammation model which might not be equivalent to the process of chronic disease development in humans (13). Therefore, it could be supposed that looking at spontaneous periodontal breakdown could increase the translational potential of the study. Additionally, this kind of study gives supplementary information to the previously published articles with induced-breakdown, since the extra challenge is not present.

Additionally, the present study tried to follow best-laboratory research practice, especially concerning blindness of the examiner, reliability and consistency of the assessments, trying to avoid bias (14). Moreover, in the present study, similarly to different approaches that have been performed in clinical and epidemiological research, the establishment of a cut-off point for definition of periodontal breakdown occurrence appears to be an attractive way to consider the data. In this approach, by means of data distribution and considering the results of the control group, the 75th percentile was used to determine a dichotomic way of looking at disease occurrence (10). We chose a 4-week period to expose rats to cigarette smoking and/or alcohol because we wanted to inquire a prolonged, rather than an acute, exposure. However, the length of cigarette smoking/alcohol exposure is evidently shorter than happen in humans. Even so, it is clear the influence of tobacco smoke and alcohol exposure on the occurrence of spontaneous alveolar bone loss.

In the present study, two risk factors for periodontal diseases are considered *per se* and combined: alcohol and smoking. If in one hand, the literature is virtually unanimous in demonstrating the deleterious effect of smoking on the periodontium (4), in the other, the literature concerning alcohol exposure is still controversial (11,15,16). Therefore, animal models are one of the most adequate ways of understanding these facts. The inconsistent results concerning the effects of alcohol intake might be explained by some facts. The way the experiments are planned, especially time of administration of alcohol and dose may account for the contradictory results. Souza et al. (15) reported that a self-administration of 20% ethanol during 8 weeks did not alter alveolar bone loss morphometrically in female Wistar rats without ligature, but increased significantly alveolar bone loss in rats submitted to ligature-induced periodontitis. Recently, Dantas et al. (16) showed that rats that received 2 g/kg of alcohol (25%) by gastric gavage twice a day during 7 days, presented a statistically significant difference in the histological ligament height (furcation area) as compared to rats that received water. On the other hand, significant differences between groups were not observed when the alveolar bone volume was analyzed. However, a study performed by our group reported that low concentration alcohol intake (5%) during 9 weeks inhibited spontaneous alveolar bone loss in rats, and did not affect the alveolar bone loss in ligature-induced periodontal breakdown (11).

Epidemiological studies also found controversial results. Lages et al. (17) demonstrated that the occurrence of periodontitis amongst alcoholics was high and the frequency of alcohol consumption increased the odds of periodontitis. However, other epidemiological studies with representative samples also observed a protective effect against clinical attachment loss in light to moderate alcohol drinkers compared to non-drinkers and heavy drinkers (18).

In the present study, the dose daily of alcohol used mimics the amount of alcohol used by light-moderate drinkers (19,20). There are some plausible pathways by which light to moderate amounts of alcohol can protect the alveolar bone from destruction. One of the hypotheses relates to a reduction of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and C-reactive protein (21). In a recent review, Diaz et al. (2) stated that moderate alcohol consumption seems to have a better impact on the immune system than excessive or absence of consumption. Additionally to the anti-inflammatory effect and the possible benefits on host immune-competence, the eventual antimicrobial effect in dental plaque could also explain the better periodontal status observed in light to moderate drinkers.

Another important aspect of the present study is the observation of the effect of smoking on periodontal breakdown. Epidemiological studies consistently demonstrate the association between smoking and periodontal diseases (3,4). Also, animal studies clearly demonstrate the effect of smoking on periodontal diseases (22). The mechanism by which this effect is observed relates to neutrophil dysfunction causing reduction on phagocytic capacity, vasoconstriction, reduced levels of immunoglobulins, cytokines, enzymes, fibroblast dysfunction, among others (23). The results found in our study confirm the knowledge present in the literature, reinforcing that the model is adequate to study periodontal breakdown.

One of the important aspects of the present study is that the combined effect of alcohol and smoking has not been extensively studied in relation to periodontal diseases. It is well known that this combination leads to higher chances of oral cancer (24). The pre-stated hypothesis of the present study leads to the expectation of a different pattern of periodontal destruction in rats exposed both to moderate concentration of alcohol and smoke. To our surprise, the results point out for a different situation. Rats exposed to both risk factors did not present higher degrees of periodontal breakdown. The possible explanation for this finding is that the well-known negative effects of smoking were at least partially neutralized by the anti-inflammatory properties of the used concentration of alcohol. The interpretation of this result should be performed with extreme caution, since it should not be conceivable to translate that alcohol would diminish the deleterious effect of smoking. It should be remembered that both exposures are related to other health problems and this is the most important information to be given.

Another important fact that should be given importance relates to the general health status of the animals. Body weight was used as a proxy of general health and no important differences were detected among groups. It should be remembered that alcohol was given together with glucose to increase the absorption of alcohol. It has been demonstrated that the presence of glucose increases alcohol absorption in relation to when sole administration is tested (7). Glucose solution was also used in the other groups to balance for caloric intake. The food ingestion, although varied, did not differ in the beginning and end of the experiment, suggesting that this would not be related to any of the findings. Also, the time of the experiment is probably not sufficient to generate the most impacting health problems that are known to be present both for alcohol and smoke.

The present study has some limitations. No data were collected with regard to the blood level of pro-inflammatory cytokines, such as tumor necrosis factor-α and Interleukins. However, this could only be used as explanatory variables. The main outcome chosen for the study, which is meant to have a meaning related to disease occurrence clearly demonstrated that smoking is deleterious and that moderate consumption of alcohol might be protective. Clinical studies suggest that periodontitis increases the blood level of these cytokines (25), and it is possible that a similar effect may also be present in the spontaneous periodontitis rat model. Another limitation of the present study concerns the way of alcohol was administrated (gavage). It is possible that the topical effect on periodontal tissue and/or on biofilm bacteria could result in an additional protective effect on the periodontium than only systemic effect. However, this has not been tested in the literature. In summary, the analysis demonstrated that there is a positive association between alcohol and absence of spontaneous periodontal disease. In contrast, there is a positive association between tobacco and presence of spontaneous periodontal breakdown.

Resumo

O objetivo do presente estudo foi avaliar o efeito da exposição do álcool e/ou tabaco sobre a perda óssea alveolar (POA) espontânea em ratos Wistar. Vinte e quatro ratos, machos, com 60 dias de vida foram divididos em 4 grupos: Grupo 1 recebeu 10 mL/kg de solução de glicose (5%). Grupo 2 recebeu 2 g/kg de álcool (20%). Grupo 3 foi exposto a fumaça do tabaco (6 cigarros/60 min). Grupo 4 recebeu a mesma intervenção dos grupos 2 e 3. A solução de álcool foi dada por meio de gavagem e a exposição ao tabaco foi realizada por meio de câmara de ventilação forçada. Após 30 dias de experimento, os animais foram sacrificados e as maxilas removidas. Análise morfométrica da POA ao redor do segundo molar superior foi realizada de modo padronizada. A análise estatística dos dados foi realizada por meio de teste t pareado e ANOVA. Ocorrência de doença periodontal espontânea (POA ≥ 0,39 mm) foi realizada pelo teste exato de Fisher. Diferenças significativas no peso corporal médio foram observadas em todos os grupos. Grupo 2 apresentou maior peso corporal médio quando comparado aos outros 3 grupos ao fim do experimento ($p \leq 0,05$). A média de POA foi 0,31 mm (±0,08); 0,29 mm (±0,07); 0,33 mm (±0,10) e 0,33 mm (±0,08) para os grupos 1, 2, 3 e 4, respectivamente. Não houve diferenças significativas entre os grupos. Na análise de ocorrência de destruição periodontal, a exposição de álcool diminuiu sua ocorrência, enquanto que exposição ao tabaco aumentou a POA espontânea. A combinação de álcool e tabaco não diferiu do grupo controle.

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