Effect of red wine and its major components on periodontitis and systemic inflammation in rats

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Abstract

Objectives: to evaluate the influence of red wine exposure, alcohol, grape juice and resveratrol in the occurrence of spontaneous and ligature induced periodontitis as well as CRP, TNFα and IL-6 levels in Wistar rats.

Methodology: 50 male Wistar rats were randomly assigned to 5 groups (Control, Red Wine, Grape Juice, 12% Alcohol and 0.05mg/mL Resveratrol). All groups were fed with laboratory rat chow and liquid intake according to group allocation. After 8 weeks, ligatures were placed around the maxillary right second molars. The contra-lateral molars remained as intra-group controls. After 14 days, animals were killed, blood samples collected and specimens prepared for analysis. Group comparisons were performed by ANOVA. A cut-off point in the 75th percentile in the side without ligature was used for definition of spontaneous periodontitis.

Results: all animals completed the experiment. According to mean alveolar bone loss, no statistically significant differences were found. Animals exposed to red wine presented a lower occurrence of spontaneous periodontitis, lower levels of TNF-α (0.97 ng/mL) and CRP (0.29 mmol/μL) compared to controls (1.97 ng/mL, $p = 0.008$ and 0.45mmol/μL, $p \leq 0.05$ respectively).

Conclusion: Red wine exposure potentially affects the occurrence of spontaneous periodontitis, CRP and TNF-α levels in Wistar rats.

Keywords: Alveolar Bone, Animal Model, Cytokines, Periodontal Risk Factor

Introduction

Alcohol consumption represents a major health problem, resulting in approximately 2.5 millions of deaths worldwide according to the World Health Organization (2011). Studies demonstrate that heavy alcohol consumption (≥40g/day for men and ≥20g/day for women (according to the World Health Organization) (2000)) can lead to liver complications, cardiovascular diseases and behavioral handicap (Mukamal *et al.*, 2006; Meyerhoff *et al.*, 2005; McClain *et al.*, 1999). Not only the amount of alcohol consumed, but also the type of alcoholic beverage may be associated with different responses in the human organism. Moderate consumption of red wine is known to have a protective effect in chronic diseases such cardiovascular, ischemic, circulatory, blindness and periodontitis (Leifert *et al.*, 2008a; Mannari *et al.*, 2010; Sheu *et al.*, 2010; Natella *et al.*, 2011; Magrone *et al.*, 2010; Kongstad *et al.*, 2008; Susin *et al.*, 2014; Wagner *et al.*, 2017). Moreover, red wine consumption may lower the amount of plaque attached to tooth surfaces supra and subgingivally (Signoretto *et al.*, 2010) due to substances such as polyphenols which display anti-adhesive properties against *Streptococcus mutans*, as well as inhibition of substances involved in synthesis of an adherent, water-insoluble glucan from sucrose which evolves to dental biofilm (Signoretto *et al.*, 2006).

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The potential benefits observed from exposure to red wine are not completely understood in the literature. The anti-inflammatory potential might be either related to the presence of alcohol or to other substances in the composition of the wine. Resveratrol is one of the supposed beneficial substances present in wine. It has been demonstrated that resveratrol is an important antioxidant, anti-inflammatory, cardio-protector (Lin *et al.*, 2008) and assists in obesity loss and metabolic diseases associated to aging process (Baur *et al.*, 2006). It also has a potential to decrease the formation of viable colonies of *Agregatibacter actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg) *in vitro* (O'Connor *et al.*, 2011). Resveratrol reduces the expression of nitric oxide, IL-1β, IL-6, IL-8, IL-12 and TNF-α in the damaged tissue induced by Pg (Antonietta *et al.*, 2012).

Animal studies are important to better understand the causal chain of periodontal diseases and, in this respect, studies have demonstrated that lower concentration of alcohol exposure protects against spontaneous alveolar bone loss (Liberman *et al.*, 2011; Oballe *et al.*, 2014); on the other hand, increase in alcohol concentration exposure seems to potentialize both induced (de Souza *et al.*, 2006; Souza *et al.*, 2009) and spontaneous alveolar bone loss (Surkin *et al.*, 2014; Bannach *et al.*, 2015). In addition, high concentrations of alcohol exposure seem to increase the levels of TNF-α (Irie *et al.*, 2008) and of the inflammatory signs in otherwise healthy gingiva (Surkin et al., 2014). To the best of our knowledge, animal models have been used only testing the amount and concentration of alcohol intake, but not a specific beverage.

Taking into consideration the positive effects of red wine consumption in humans and the lack of studies in animal model, the aim of this study was to evaluate the influence of red wine intake, alcohol intake at 12% (same as red wine), grape juice and resveratrol (a polyphenol found in Red Wine and Grape Juice) in the occurrence of spontaneous and ligature-induced periodontitis in Wistar rats. In addition, C-reactive protein (CRP), TNF-α and IL-6 levels were measured in the groups. The hypothesis to be tested is that the combination of different substances in red wine rather than each one isolated is responsible for the supposed beneficial effects both in periodontal breakdown as well as in systemic inflammatory markers.

Materials and methods

Ethical considerations

The present study was approved by Institutional Review Boards both of the Universidade Federal do Rio Grande do Sul (protocol # 26632) and Hospital de Clínicas de Porto Alegre (protocol # 14/0335). This study was funded by the Incentive Fund for Reaserch # 110051) and by the Coordenation for Enhacement of Higher Education Personnel, Brazil (CAPES – grant PROCAD NF – 2008).

Sample size calculation

In order to estimate the sample size, a difference of 0.7mm between groups submitted to alcohol at 10% and 20% in a previous study was utilized (Souza et al., 2009). Considering alpha and beta errors of 0.05 and 0.10 respectively, 8 animals per group were necessary. Considering a possible attrition rate of 20% (Galvao *et al.*, 2003), 10 animals per group were used.

Animals

Fifty male Wistar rats, 45 days old with a mean body weight of 308.75g (±25.26g) were randomly assigned to 5 groups of 10 animals each. Randomization was performed by draw, stratified by weight. The following groups constituted this study: Control (water), Red Wine, Grape Juice, 12% Alcohol (same concentration of red wine) and 0.05mg/L Resveratrol (the same concentration found in red wine). The Control group was fed with a standard laboratory rat chow (Nuvilab CR-1, Nuvital®, Curitiba, Brazil) and tap water *ad libitum*. Test groups received the same standard diet but water was replaced with the respective solutions of Red Wine (Almaden Cabernet Sauvignon®, Bento Gonçalves, Brazil), Grape Juice (Sunny Days®, Bento Gonçalves, Brazil), 12% Alcohol (Vetec, Rio de Janeiro, Brazil) and 0.05 mg/L Resveratrol (Equilibrium Pharmacy, Porto Alegre, Brazil). Liquid and food were available *ad libitum*. Animals were housed in boxes of 5 animals each in a controlled environment (temperature 22°C ± 2°C and dark/light cycle of 12 hours) during the entire experimental period. Food and liquid intake were measured daily and body weight was assessed once a week. Figure 1 shows the study flowchart.

Spontaneous and ligature-induced alveolar bone loss

General anesthesia was obtained by inhalation of Isoflurane in all animals. In all groups, alveolar bone loss was induced by placement of 4-0 silk ligatures (Ethicon®, Johnson & Johnson®, São Paulo, Brazil) around the right maxillary second molars. The contra-lateral tooth remained as intra-group control (Liberman et al., 2011) and was used for the analysis of spontaneous periodontal breakdown. After 14 days of the ligature placement, animals were killed, blood samples collected and specimens prepared for morphometric analysis.

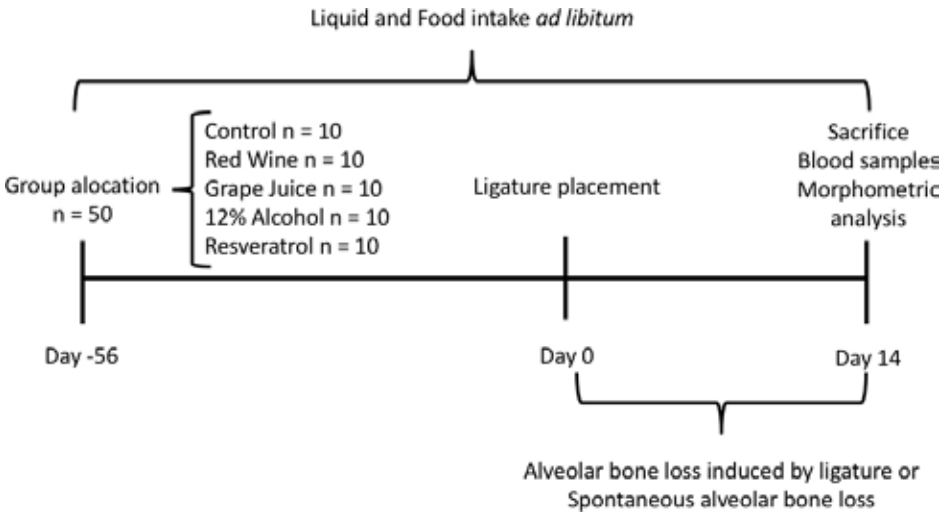

Figure 1: ***study flowchart***

Morphometric analysis

Following sacrifice, the right and left segments of the maxillae were defleshed in sodium hypochlorite with 9% active chlorine (Mazzarollo®, Gravataí, Brazil) for 2 hours. After rinsing, the specimens were stained with methylene blue 1% (Quinta Essência, Porto Alegre, Brazil) to delineate the cemento-enamel junction (Liberman et al., 2011).

Standardized digital pictures were taken from the buccal and palatal aspects of each specimen using a millimetric ruler and a Nikon D5100® camera coupled with medical lenses and minimal focal distance. Each specimen was placed with the occlusal surface parallel to the floor. Linear measurements were performed with Adobe Photoshop CS6 (Adobe Systems Software Ireland Ltd). These measurements resulted in a pixel value, which was converted into millimeters. Periodontal bone loss was defined as the distance between the cemento-enamel junction (CEJ) and the alveolar bone crest. Buccal and palatal measurements were made at five points and a mean of these values was considered as bone loss.

The examiner was unaware of the group distribution as well as of the ligature presence or absence. A trained and calibrated examiner performed all the measurements. Prior to measurements, calibration was performed by double measurement of randomly chosen pictures (n=20) with one-week interval. The intra-class correlation coefficient (ICC) between measurements was 0.99. During the experiment a new calibration was performed with 10% of the sample and the ICC between measurements was also 0.99.

For definition of spontaneous periodontitis, a cut-off point was established, in order to define the occurrence of destruction. An analysis of data from the contra-lateral tooth (without ligature) was performed and the 75th percentile was considered the cut-off point (Oballe et al., 2014). Thus, teeth that presented a mean alveolar bone loss $\geq$ 0.39mm were considered as having spontaneous periodontitis.

C-reactive protein (CRP)

C-reactive protein was measured in the liver where it is synthesized. For RNA extraction, tissue samples were fragmented and mixed with Trizol reagent (Invitrogen) using a tissue homogenizer (MA102, Marconi). The concentration and quality of RNA was estimated by reading on a spectrophotometer at 260 nm and calculating the ratio 260/280, respectively. RNA samples were treated with Dnase (Deoxyribonuclease I, Invitrogen) for removal of possible genomic DNA residues. Next, reverse transcription reaction was performed using 2mg of total RNA, reverse transcriptase (Superscript III, Invitrogen) and Oligo dT primer (Invitrogen) in the presence of Rnase inhibitor (RNase OUT, Invitrogen) in a final volume of 20 ml. PCR amplifications were performed in duplicate and using 50ng cDNA per reaction. The reactions were prepared with standardized reagents for Real Time PCR (TaqMan Universal PCR Master Mix, Applied Biosystems) plus the primer sets (Mm00432680_g1 - C-reactive protein and Mm99999915_g1 - internal control GAPDH) and specific probe for each gene. The reaction conditions were 50°C for 2 minutes, 95 °C for 10 minutes and 40 times of 95 °C for 15 seconds and 60 °C for 1 minute. The fluorescence readings were performed by 7500 Real-Time PCR equipment (Applied Biosystems) to each amplification cycle and then analyzed by the Sequence Detection Software (SDS) v1.3 (Applied Biosystems).

All reactions were submitted to the same conditions of analysis and normalized by dye signal of ROX passive reference for correction of fluctuations in reading due to changes in volume and evaporation throughout the reaction. The result, expressed in TC value, refers to the number of PCR cycles required to fluorescent signal reach the detection threshold.

Individual results expressed in CT values were assembled according to the experimental group for statistical analysis.

TNF-α and IL-6 analysis

TNF-α and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) using serum samples (Mini ELISA Development Kit, 900-M21, PeproTech Inc, New Jersey, USA). The intra-assay coefficient of variation was <7.5% for all cytokines. Briefly, plates of 96 wells were incubated in overnight with capture antibody anti-IL-6 or TNF-α diluted in 1x PBS buffer. After blocking for 1 hour to avoid non-specific binding, 100µL of standard IL-6 or TNF-α and serum samples were placed. The cytokines were detected by horseradish peroxidase-labeled monoclonal antibody to each target after addition of 100µL anti-human IL-6 or TNF-α biotinylated antibodies were placed in each well and incubated for 2 hours at room temperature. The microplate was washed to remove unbound enzyme-labeled antibodies. The amount of horseradish peroxidase bound to each well was determined by the addition of 100µL substrate solution. The reaction was stopped by the addition of 100 µl of 1 M sulfuric acid, and the plates (ThermoPlate, São Paulo, Brazil) were read at 405 nm. The concentrations of cytokines were determined by interpolation from standard curve and presented as pg/mL, and converted into ng/mL.

Statistical analysis

Shapiro-Wilk test for normality was used and a normal distribution was detected in all continuous variables. Means and standard deviations were calculated and intergroup analyses were performed by ANOVA followed by Bonferroni. All analyses were performed using IBM SPSS Statistics 20.0 for Windows.

Results

All animals completed the experimental protocol. Body weight throughout the study is show in Figure 2. No statistically significant differences were observed in body weight between groups during the study.

Table 1 shows the average alveolar bone loss (mm ± SD) for all groups. No statistically significant differences were observed between groups both in sides with and without ligature.

Figure 3 demonstrates the occurrence of periodontitis according to the established cut-off point. In sides without ligature, animals which consumed red wine, presented a lower occurrence of periodontitis compared to the other groups (Red Wine: 1 case; Control, 12% Alcohol and 0.05mg/mL Resveratrol: 2 cases; Grape Juice: 6 cases).

Table 2 demonstrates the cytokine profile obtained after sacrifice. For IL-6, no statistically significant differences were observed between groups. However, animals which were exposed to red wine presented lower levels of TNF-α (0.97ng/mL ± 0.49) and CRP (0.29 mmol/µL ± 0.04) compared to controls (1.97ng/mL ± 1.06, $p = 0.008$ and 0.45 mmol/µL ± 0.03, $p \leq 0.05$ respectively).

Discussion

The objective of this study was to evaluate the effect of red wine, 12% alcohol, grape juice and resveratrol exposure in the occurrence of spontaneous and ligature induced periodontitis as well as CRP, TNF-α and IL-6 levels in Wistar rats. The main results demonstrated that rats which were exposed to red wine, presented lower levels of CRP and TNF-α and potentially affects the occurrence of spontaneous periodontitis compared to the other groups.

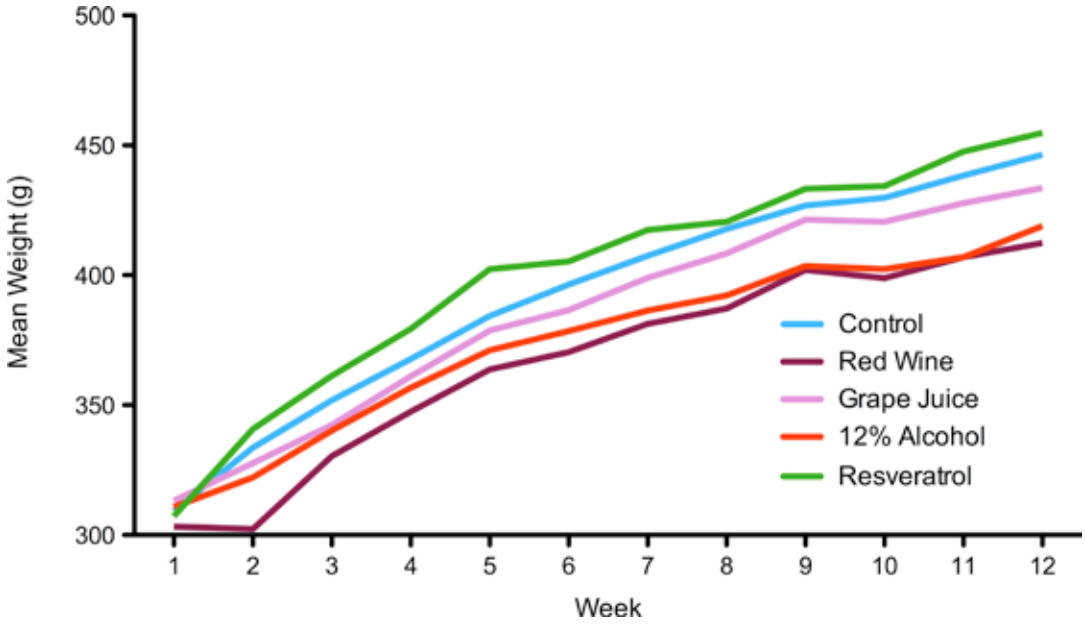

Figure 2: ***Mean body weight (g) throughout study. No statistically differences were observed between groups along the study (ANOVA, p>0.05).***

Table 1: Mean alveolar bone loss (mm, ±SD) in teeth with and without ligature according the experimental group.

	Control	Red Wine	Grape Juice	Alcohol 12%	Resveratrol	*p**
With ligature	0.60 (±0.07)	0.59 (±0.04)	0.64 (±0.10)	0.59 (±0.09)	0.67 (±0.08)	0.12
Without ligature	0.36 (±0.04)	0.35 (±0.04)	0.39 (±0.05)	0.35 (±0.04)	0.36 (±0.03)	0.17

*ANOVA

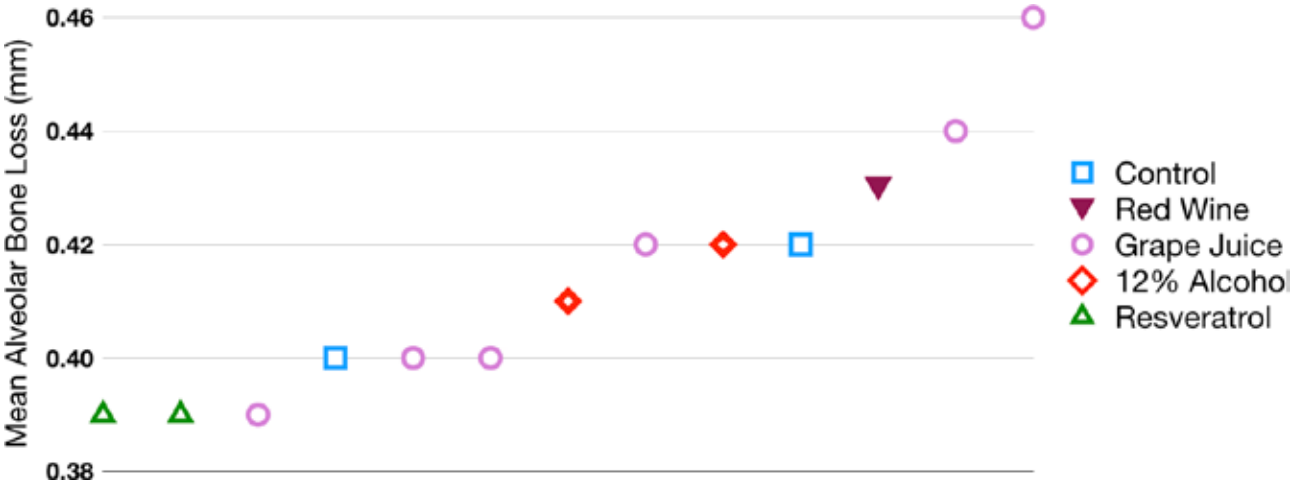

Figure 3: ***Distribution of spontaneous cases of periodontitis according to study groups***

Table 2: Levels of CRP (mean ± SD in mmol/μL), IL-6 and TNF-α (mean ± SD in ng/mL) according to test groups.

	Control	Red Wine	Grape Juice	Alcohol 12%	Resveratrol	P
CRP	0.45 (±0.03)	0.29* (±0.04)	0.36 (±0.02)	0.53 (±0.05)	0.32 (±0.08)	< 0.001*
IL-6	0.77 (±0.38)	0.66 (±0.28)	0.54 (±0.23)	0.97 (±0.47)	0.67 (±0.20)	0.06
TNF-α	1.97 (±1.06)	0.97* (±0.49)	1.63 (±0.38)	2.05 (±0.57)	1.72 (±0.30)	0.008*

* Significant difference compared to control group

The hypothesis behind the study was that the exposure of red wine could be a protective factor for the occurrence of periodontal breakdown. This hypothesis rises from the studies demonstrating the anti-inflammatory properties of red wine, as well as the benefits it seems to cause in non-communicable chronic diseases (Leifert and Abeywardena, 2008a; Leifert *et al.*, 2008b; Mannari et al., 2010; Magrone and Jirillo, 2010; Sheu et al., 2010). In fact, the actual mechanisms by which red wine benefits in such cases has not been completely understood. Some studies infer that the presence of alcohol in such concentration is responsible for the outcomes and others report that components of the red wine, such as polyphenols are the main source of benefit (Coimbra *et al.*, 2005; Davalos *et al.*, 2009; Lin et al., 2008; Baur et al., 2006).

The present study follows a line of research by our group, in which we previously demonstrated in animals that alcohol in low/moderate concentrations could decrease spontaneous alveolar bone loss in Wistar rats (Liberman et al., 2011; Oballe et al., 2014). The experimental groups include the main objective of test (red wine), as well as three groups which have parts of the composition of the red wine: grape juice, alcohol and resveratrol. The comparison of these groups with a control group should allow for the understanding if and what part of the components of red wine would be beneficial for periodontal tissues.

The main results of the present study are in line with those found in humans where red wine exposure decreased levels of inflammatory markers such TNF-α (Wang *et al.*, 2013; Antonietta et al., 2012) and had a protective effect over periodontal tissues (Kongstad *et al.*, 2008; Bouchard *et al.*, 2006; Susin *et al.*, 2014). This study also found lower levels of CRP in rats which were exposed to red wine. It has been shown that CRP levels are related to smoking, obesity, triglycerides, diabetes and periodontal diseases and these levels increase in response to infection, inflammation and injury, being one of the markers of choice in control of this response (Tüter *et al.*, 2007; Gomes-Filho *et al.*, 2011). The understanding of the present study should be contextualized in terms of its methodological characteristics. The methodology applied for the present study includes all contemporary research principles for animal studies, including randomization, blindness, comparison and sample size calculation. This increases the validity of the results, allowing for the highest degree of translation, within the limitations of rat studies for periodontal diseases (Klausen, 1991).

For experimental periodontitis, rats have been used due to some similarities with humans such as anatomical, immunological and microbiological characteristics. In addition, rat studies have demonstrated the occurrence of both spontaneous and induced alveolar bone loss, with shifts in the periodontal microbiota and concomitant increase in inflammatory markers (Klausen, 1991). To the best of our knowledge, this was the first study in the literature which used a specific alcoholic beverage in an animal model for spontaneous and ligature-induced alveolar bone loss. In addition, red wine was chosen due to the fact that it is an alcoholic beverage with mild alcohol concentration, as compared to beer (low concentration) and distilled beverages (high concentration).

The published literature has used the red wine components (alcohol, resveratrol, red grape juice) isolated in studies for other diseases with varying concentrations (Leifert and Abeywardena, 2008b; Elkind *et al.*, 2006; Natella et al., 2011; Anselm *et al.*, 2007; Kasdallah-Grissa *et al.*, 2007). For example, it seems that there is a relationship between the amount and concentration of alcohol consumed and the severity of disease. This has been referred as a J-shaped curve and it has been extensively studied for hypertension (Moreira *et al.*, 2014; Tanna *et al.*, 2015). Lower concentrations of alcohol (5%) represent a protective effect on spontaneous alveolar bone loss (Liberman et al., 2011); on the other hand, higher concentrations ($\geq$ 20%) represent a risk factor both for ligature-induced (de Souza et al., 2006; Irie et al., 2008; Souza et al., 2009) as well as spontaneous alveolar bone loss (Surkin et al., 2014; Bannach et al., 2015).

The fact that higher concentrations of alcohol intake represent a risk may be explained because alcohol decreases neutrophil function (Patel *et al.*, 1996) and increases production of pro-inflammatory cytokines such IL-1, IL-6 and TNF-α (Szabo, 1999), and the presence of these markers in gingiva is associated with periodontitis (Offenbacher, 1996).

When rats that have a chronic alcohol intake are submitted to resveratrol, the side effects of alcohol (weight loss, hepatoxicity, anti-oxidant agents) decreases and there is an improvement of general health of these animals (Kasdallah-Grissa et al., 2007). Resveratrol also decreases TNF-α expression (Wang et al., 2013) and IL-17 in rats submitted to alveolar bone loss induced by ligature (Casati *et al.*, 2013). Despite of the positive results of the compounds isolated, interestingly we demonstrated that only the combination of the components (red wine) was capable of reducing the occurrence of spontaneous periodontitis, CRP and TNF-α levels in animal model.

It is important to consider that the differences in periodontal breakdown could be detected in the present study are restricted to spontaneous alveolar bone loss. This has also been the case in other studies (Liberman et al., 2011; Oballe et al., 2014). A possible explanation for this fact is that when a ligature is placed in order to induce periodontal breakdown, this agent may promote an acute inflammation which is not equivalent to the human process (Li *et al.*, 2007). Also, the challenge represented by the ligature could be of such magnitude that it could mask subtle effects. In order to evaluate the side without ligature, our research group has used the concept of spontaneous periodontitis where a cut-off point in the 75th percentile of the distribution is used. Animals which present losses above this point are considered as cases of spontaneous periodontitis (Oballe et al., 2014; Cavagni *et al.*, 2013).

In the present study, differences among groups could not be detected with the mean values. This could be explained by a regression towards the mean, as well as that, contemporarily other forms of analysis have been used, in addition to averages. Studies have used this model of analyzing the occurrence of periodontal breakdown with this cut-off point in the 75th percentile of the control group (Oballe et al., 2014; Cavagni et al., 2013). This allows for the understanding of the highest impacting effects, since in the simple mean analysis, the ones with no impact are considered together. Instead of using one site for statistical analysis, we used the tooth for analysis. In such an analysis, a clear impact of red wine intake was detected in the occurrence of a more impacting alveolar bone loss. This outcome should be compared with the concomitant pattern of cytokines, in other to see if there is a possible explanation.

Experimental periodontal breakdown has been extensively studied in the literature and it has been demonstrated that 15 days of ligature is enough to demonstrate a statistically significant bone loss by different techniques for alveolar bone loss determination. After the fifteenth day, the alveolar bone loss can be stabilized (Kuhr et al., 2004; Vargas-Sanchez et al., 2017). In this sense, when the objective of the study is to understand the role of an exposure in periodontal breakdown, such time can be enough. Additional time would only be interesting to understand the role of continuous inflammation in a different outcome. Moreover, exposure to the beverages for 70 days was sufficient for development of spontaneous periodontitis (Cavagni et al.; 2013).

In the present study, red wine intake decreased CRP levels in liver and TNF-α levels in the serum, demonstrating a possible anti-inflammatory effect. The differences in levels of interleukin-6 did not reach a statistical significance. However, TNF-α is clearly a marker associated with periodontal breakdown in different studies (Irie et al., 2008; Hienz *et al.*, 2015; da Costa *et al.*, 2015). The fact that no statistically significant differences had been observed in IL-6 in the serum, does not mean that there are not in the periodontal site, which was not object of evaluation in the present study. Rats are animals which an immune system with a significant adaptive capacity.

The present study has strengths and limitations. Among the strengths is the fact that a highly controlled environment is achievable in animal studies, giving the possibility of isolating the effect of an exposure, minimizing potential confounders. This is true both for sides with induced periodontal breakdown as well as spontaneous alveolar bone loss. However, it should be highlighted as limitation that animal studies are not a source of direct translation. Also, the amount of liquid intake is not equal in all animals in the present study.

The results of the present study also support the epidemiological data in which moderate alcohol consumption seems to have a beneficial effect in males (Susin et al., 2014; Kongstad et al., 2008; Wagner et al., 2017). High consumption, on the other hand, is demonstrated to be detrimental (Tezal *et al*., 2004; Pitiphat *et al*., 2003). The information from such studies should be included both in preventive as well as in therapeutic approaches. This is a challenge, since alcohol consumption should never be promoted to a level that alcoholism is inducedt. The literature is still controversial in this respect and despite of the positive results both in human and animal models, additional studies are needed to explain the mechanisms of red wine and its components in the development or inhibition of periodontitis.

Conclusion

In conclusion, red wine exposure decreases CRP and TNF-α levels and potentially affects the occurrence of spontaneous alveolar bone loss in Wistar rats.

Acknowledgements and Conflict of interest

The authors would like to thank to Animal Experimentation Unit staff of the Hospital de Clinicas de Porto Alegre for all support and to Miolo Wine Group (Bento Gonçalves, Brazil) which provided the Red Wine and the Grape Juice used in this study and to Equilibrium Pharmacy which provided the resveratrol used in this study. The authors declare not to have conflicts of interest. This study was supported by the Coordination for Enhancement of Higher Education Personnel, Brazil (CAPES – Grant PROCAD NF – 2008) and Incentive Fund for Research and Events of the Hospital de Clínicas de Porto Alegre, Brazil (FIPE – Grant number 110051).

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