UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

MILENA BASTOS MENDES

ANÁLISE DA PERDA ÓSSEA ALVEOLAR E DE PARÂMETROS DE ESTRESSE OXIDATIVO EM RATOS WISTAR EXPOSTOS OU NÃO À VITAMINA C

Porto Alegre
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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgião-Dentista.

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Porto Alegre, 13 de dezembro de 2019.

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RESUMO

Antecedentes: Estudos em animais têm observado a existência de uma relação entre a ingestão de vitamina C e melhores resultados do reparo periodontal. Entretanto, estudos avaliando os efeitos da vitamina C sobre marcadores de estresse oxidativo são escassos.

Objetivo: Verificar o efeito da utilização de vitamina C sobre a perda óssea alveolar e parâmetros de estresse oxidativo em ratos Wistar com e sem periodontite experimental induzida.

Metodologia: Sessenta e oito (68) ratos Wistar machos foram randomicamente divididos em 4 grupos com 17 animais em cada grupo: Controle, Periodontite (Perio), Vitamina C (VitC) e Vitamina C com doença periodontal (Perio+VitC). A doença periodontal foi induzida apenas nos grupos Perio e Perio+VitC por meio de ligaduras posicionadas no segundo molar superior direito. O dente contralateral foi considerado controle intragrupo. Nos grupos que receberam vitamina C, o mesmo foi feito na concentração de 1g/L. Peso corporal, consumo de sólidos e líquidos foram avaliados, além da perda óssea alveolar em fotografias padronizadas e níveis plasmáticos de FRAP (Ferric Reducing Ability of Plasma), sulfidril e carbonil por um examinador treinado e calibrado. O nível de significância estabelecido foi de 95%

Resultados: Observou-se um ganho de peso, consumo de líquidos e sólidos ao longo do tempo, sem diferenças significativas entre os grupos experimentais. Os animais do grupo Perio+VitC exibiram menores graus de perda óssea alveolar quando comparados aos animais do grupo Perio, apenas. Não foram observadas diferenças significativas na perda óssea entre os grupos nos lados contralaterais. Considerando os marcadores de estresse oxidativo, concentrações plasmáticas mais elevadas de sulfidril nos grupos Perio e VitC+Perio, nas análises de FRAP os grupos Perio e VitC+Perio apresentaram volumes plasmáticos menores em relação ao grupo Controle e nas análises do carbonil não houve diferença significativa entre os grupos.

Conclusão: A exposição à vitamina C potencialmente reduz a perda óssea alveolar, modulando parâmetros de estresse oxidativo em ratos Wistar.

Palavras-chave: Doença periodontal. Perda óssea alveolar. Vitamina C. Ácido ascórbico. Antioxidantes. Estresse oxidativo.

ABSTRACT

Background: Animal studies have evaluated the relationship between vitamin C intake and periodontal repair. However, studies evaluating the effects of vitamin C on oxidative stress markers are scarce.

Aim: To evaluate the effect of vitamin C on alveolar bone loss and oxidative stress markers in Wistar rats with and without experimental periodontitis.

Methods: Sixty eight (68) male Wistar rats were randomly divided into four groups with 17 rats in groups: Control, Periodontitis (Perio), Vitamin C (VitC) and Vitamin C plus Periodontal Disease (Perio+VitC). Periodontal disease was induced in the Perio and Perio+VitC groups by ligatures placed in the upper right second molar. The contralateral tooth was considered intragroup control. Rats were exposed to 1 g/L of vitamin C. Body weight, chow and fluid consumption were evaluated as well as alveolar bone loss in standardized photographs and plasma levels of FRAP (Ferric Reducing Ability of Plasma), sulphydryl and carbonyl by trained and calibrated examiner. The level of significance was set in 95%

Results: Significant weight gain, liquid and solid consumption were observed throughout time, with no significant differences between the experimental groups. Animals Perio+VitC groups exhibited lower degrees of alveolar bone loss when compared to animals with Perio groups. There were no significant differences in bone loss between groups on the contralateral sites. Considering oxidative stress markers, higher plasmatic sulphydryl concentrations were observed in the Perio and VitC+Perio groups, and in the FRAP analyzes the Perio and VitC + Perio groups had lower plasma volumes in relation to the Control group No statistical differences between the groups were observed for carbonyl.

Conclusion: Vitamin C potentially reduces alveolar bone loss by modulating oxidative stress markers in Wistar Rats.

Keywords: Periodontal disease. Alveolar bone loss. Vitamin C. Ascorbic acid. Antioxidants. Oxidative stress.

SUMÁRIO

1 INTRODUÇÃO

A doença periodontal é causada pela infecção dos tecidos periodontais decorrente do acúmulo de biofilme dentário, que pode resultar na perda progressiva de inserção conjuntiva e osso alveolar (QUERIDO *et al.,* 2004). A periodontite caracteriza se como uma doença infecto-inflamatória que afeta os tecidos de sustentação e proteção do dente (BARIN *et al*., 2017). A gengivite, considerada a forma mais branda da doença periodontal, não afeta as estruturas de suporte dentário e não deixa sequelas, sendo extremamente prevalente na população mundial (OPPERMANN *et al.*, 2015). A periodontite, por sua vez, resulta na perda de tecido conjuntivo e de suporte ósseo, podendo acarretar perdas dentárias (GRAVES, 1999; PAGE; KORNMAN, 2000).

O acúmulo de placa bacteriana na superfície dentária não é único fator capaz de provocar periodontite no indivíduo (COCHRAN, 2008), sendo a resposta imunológica e a suscetibilidade do hospedeiro importantes na modulação da condição periodontal. A maior parte da destruição do tecido periodontal é causada por uma alteração da resposta do hospedeiro aos microrganismos que colonizam os tecidos periodontais na presença de biofilme dentário, mais especificamente, uma perda de equilíbrio homeostático entre enzimas proteolíticas e seus inibidores, espécies reativas de oxigênio e os sistemas de defesa antioxidante (CHAPPLE; MATTHEWS, 2007; CHAPPLE *et al*., 2002).

Uma célula inflamatória presente no tecido conjuntivo é o leucócito polimorfonuclear. Quando o tecido periodontal é agredido esses leucócitos são ativados e produzem maior quantidade de espécies reativas de oxigênio (CHAPPLE; MATTHEWS, 2000). A formação de radicais livres ocorre na mitocôndria dessas células através da cadeira transportadora de elétrons a qual faz a formação de energia em forma de ATP. Os radicais livres também são produzidos de forma fisiológica natural e tem importante papel em várias reações químicas intra e extracelulares, desde mediadores químicos em inflamações, a fecundação de óvulos (GENESTRA, 2007). Contudo, quando ocorre a liberação demasiada destes radicais livres, os mesmos podem sofrer oxidação através de metais como o ferro e o cobre formando espécies reativas de oxigênio as quais causam estresse oxidativo, que pode gerar danos às células circundantes. Essa liberação demasiada gera a perda do equilíbrio homeostático entre radicais livres de oxigênio e enzimas antioxidantes provocando

um acúmulo excessivo de radicais livres e influenciando a patogênese de algumas doenças, incluindo doenças vasculares, diabetes, isquemia renal, aterosclerose e, também as doenças periodontais (NONAKA *et al*., 2019). Os antioxidantes possuem a função no organismo de remover e converter em álcool e água os radicais livres em excesso, impedindo a oxidação desses radicais consequentemente impedindo o estresse oxidativo (KIYOSHIMA *et al*., 2012).

O organismo humano e animal possui enzimas e mecanismos antioxidantes enzimáticos e não enzimáticos para combater o estresse oxidativo neutralizando ou eliminando radicais livres ou quebrando as reações em cadeia (BISWAS, 2016). Alguns dos antioxidantes enzimáticos são a catalase, a superóxido-dismutase e a glutationa-peroxidase (BATTINO *et al*., 1999; CHAPPLE; MATTHEWS, 2007; KAKLAMANOS; TSALIKIS, 2002). Dentro dos marcadores enzimáticos que podem ser encontrados no plasma está o sulfidril, com grande importância no sistema antioxidante enzimático, pois o mesmo oferece suas ligações (S-H) para a glutationa reduzida (GSH) (CHAPPLE; MATTHEWS, 2007).

Em contrapartida, os agentes antioxidantes não-enzimáticos, são tidos como agentes secundários no combate ao estresse oxidativo. Os mecanismos antioxidantes não enzimáticos incluem uma infinidade de componentes, como vitaminas C e E, glutationa e beta-caroteno (NORDBERG; ARNER, 2001; HALLIWELL, 2006). Geralmente eles são obtidos de maneira exógena, por meio de uma dieta balanceada contendo uma série de frutas e vegetais importantes, tais como cenoura (PLATEL; SRINIVASAN, 2015), espinafre (BOHLOOLI *et al*., 2015), tomate (CHANDRA *et al*., 2012), abacate (KOPEC *et al*., 2014), uva, morango (ZHAO *et al.*, 2015), goiaba e laranja (ALAGL; BHAT, 2015).

A vitamina C é um antioxidante eficaz que modula o estresse oxidativo e participa de uma variedade de reações bioquímicas. A vitamina C promove especialmente a proliferação de queratinócitos e a migração de fibroblastos, melhora a síntese de colágeno e estabiliza as estruturas terciárias da molécula de colágeno; isto parece crucial para o processo de cicatrização/regeneração e cicatrização de feridas. A vitamina C também pode diminuir as respostas de alguns biomarcadores inflamatórios, incluindo proteína C reativa, e várias citocinas pró-inflamatórias, como fator de necrose tumoral, interferons e interleucinas (LI *et al*., 2018). É possível ingerir vitamina C na dieta nos legumes, verduras, e frutas. Embora a laranja seja considerada uma fonte rica em vitamina C, outras frutas apresentam até mesmo

maiores concentrações desta vitamina, tais como goiaba, kiwi, melancia, mamão e frutas vermelhas (ALAGL; BHAT, 2015).

A resposta inflamatória do organismo frente à doença periodontal libera radicais livres que provocam o estresse oxidativo e induzem danos nos tecidos periodontais de proteção e sustentação (NOACK *et al*., 2000). A vitamina C, como um antioxidante não enzimático, tem como uma das suas funções diminuir o estresse oxidativo no organismo. Portanto é lícito supor que a ingestão de vitamina C poderia de alguma forma modular a expressão de marcadores de extresse oxidativo, modulando com isso a expressão da doença periodontal destrutiva. Assim, o objetivo do presente trabalho de conclusão de curso foi verificar o efeito da utilização de vitamina C sobre a periodontite experimental e parâmetros de estresse oxidativo em ratos Wistar.

2 ARTIGO CIENTÍFICO

O presente trabalho de conclusão de curso está estruturado no formato de um artigo científico intitulado “Análise da perda óssea alveolar e de parâmetros de estresse oxidativo em ratos Wistar expostos ou não à vitamina C”, que será submetido à periódico de circulação internacional.

ANALYSIS OF ALVEOLAR BONE LOSS AND OXIDATIVE STRESS PARAMETERS ON WISTAR RATS EXPOSED OR NOT TO VITAMIN C

Runing Title: Vitamin C and experimental periodontitis

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Key Words: periodontitis; alveolar bone loss; ascorbic acid; antioxidants; oxidative stress.

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INTRODUCTION

Recent studies have suggested a possible interaction between non-enzymatic antioxidants and periodontal disease, with the main outcome being the reduction of alveolar bone loss (ABL) (TOMOFUJI, 2006; SANBE, 2008; TOMOFUJI, 2009). Periodontal disease is an infecto-inflammatory disease that affects the teeth's protective and supportive tissues. It occurs from a complex inflammatory process, in which polymorphonuclear leukocytes, in addition to defending against periodontal pathogens, release free radicals that can undergo oxidation to generate reactive oxygen species, causing oxidative stress. This oxidative stress can trigger tissue damage through various mechanisms, such as damage to the proteins, deoxyribonucleic acid (DNA), lipid peroxidation, enzymatic oxidation, and stimulation of pro-inflammatory cytokines (CHAPPLE, 1997; CHAPPLE; MATTHEWS, 2007).

Non-enzymatic defenses can eliminate these reactive oxygen species, with vitamin C among the most studied non-enzymatic antioxidants in periodontal disease (GRAVES, 1999; PAGE; KORNMAN, 2000). Studies in animal models and humans have suggested that a higher intake of vitamin C can mitigate ABL. An inversely proportional relationship has been demonstrated between the ingestion of vitamin C and the number of teeth presenting progression of periodontal disease and ABL (AKMAN, 2013; IWASAKI, 2013).

This study aimed to verify the effect of vitamin C on ABL and oxidative stress parameters in Wistar rats.

MATERIAL AND METHODS

Study Design and Ethical Considerations

The study was a randomized and prospective trial in an animal model. **Figure 1** shows the process of the study in detail. The protocol was approved by the CEUA Institutional Review Board of the Hospital de Clínicas de Porto Alegre, Brazil (Protocol 170115). The protocol was conducted according to the ARRIVE Guidelines (KILKENNY, 2012).

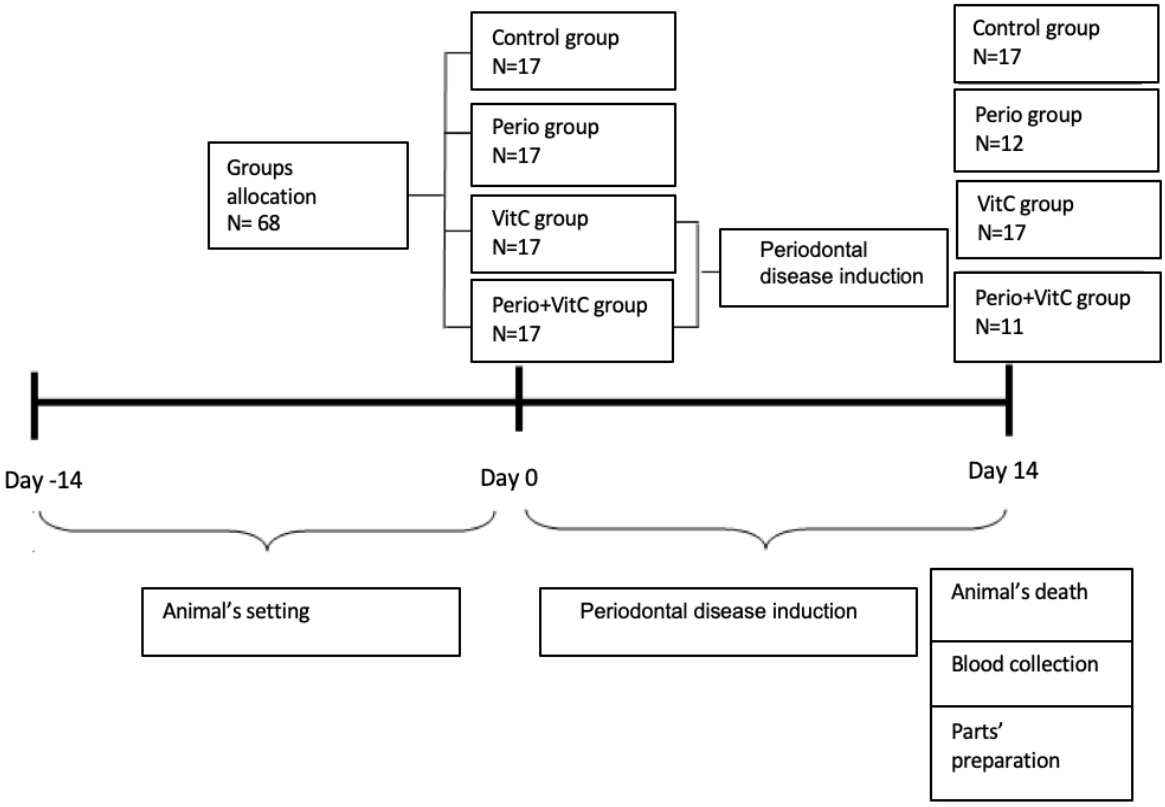

Figure 1. Detailed flowchart of the study.

Animals

Sixty-eight male Wistar rats aged 60 days were stored in polypropylene housing with sawdust bedding, distributed in groups of three to four animals. All boxes were kept in the Animal Experimentation Unit of the Hospital de Clínicas de Porto Alegre. A light–dark cycle of 12 hours, ambient temperature (22±2 °C), humidity of 65–75%, and an air exhaust system were used. Animals had free access to standard rat feed and liquid according to experimental group.

Animals were weighed on three occasions: after the 14 days of the adaptation period, seven days after the beginning of the experimental period, and on the last day of the experimental period. Weighing was performed on an electronic scale with a precision of 0.01 g.

Randomization

After a two-week adaptation period, the animals were randomly distributed by raffle to their respective experimental groups, taking into consideration the body weight from extracts represented by quartiles of weight. Each experimental group initially constituted 17 animals, distributed as follows.

Control Group (Control): Periodontal disease was not induced; vitamin C exposure occurred. The animals received water and rat feed *ad libitum*.

Vitamin C Group (VitC): Periodontal disease was not induced; diet comprised feed and distilled water containing vitamin C at a dilution of 1 g/L *ad libitum*.

Periodontal Disease Group (Perio): Experimental periodontitis was induced by ligatures for two weeks; feed and water *ad libitum* were provided.

Periodontal Disease and Vitamin C Group (Perio+VitC): Periodontal disease was induced in the same way as in the Perio group; feed and distilled water containing vitamin C at a dilution of 1 g/L were offered *ad libitum*.

Experimental Procedures

Vitamin C Administration

Oral administration of vitamin C was through addition to distilled water at a concentration of 1 g/L. This water was prepared daily for the VitC and Perio+VitC groups over 14 days. To maintain the properties of the vitamin, all containers for ingestion were protected from light.

Periodontal Disease Induction

Induction of experimental periodontitis in the relevant experimental groups was by placing ligature on the upper right second molar. This procedure was performed under general anesthesia by inhalation of 5% isoflurane with the supervision of a veterinarian. The Control and VitC groups were anesthetized so that they were subjected to stress similar to the others. Ligature used a silk 4-0 Ethicon® placed with two Castro Viejo needle holders in the interproximal area with a knot in the buccal site. The contralateral region was considered the intra-group control (GRAVES, 1999; CHAPPLE, 2000; CAVAGNI, 2016).

Liquid Consumption and Diet

Measurement of consumption of liquids and diet was performed daily in all experimental groups. The mean values of liquid and solid consumption considered the number of animals in each box and an average was generated for each.

Euthanasia and Specimen Preparation

After 14 days, the animals were euthanized by exsanguination through cardiac

puncture under general anesthesia. Approximately 5 mL of blood was collected at the moment of cardiac puncture. Immediately after collection, the blood was centrifuged in a refrigerated centrifuge. Plastic tubes were used for 15 minutes at 3000 x g, at a temperature of 4 ºC. Then, the supernatant plasma was pipetted and aliquoted into Eppendorf tubes, on a frozen rack to maintain the temperature of the centrifuge, and immediately stored in a -80 ºC freezer. The jaws were removed and stored in a 10% buffered formalin solution. The soft tissues were chemically and mechanically removed with 10% sodium hypochlorite and chisels, respectively.

Laboratory Tests

Morphometric Analysis of Bone Loss

Photographs were taken with a digital camera, 18.1 megapixels, model T5i with macro lens 100 (Canon® EOS Rebel T5i, Japan), coupled to a tripod with minimal focal length, so that the cone was as parallel as possible to the ground. An apparatus made with silicone was used for fixation to an endodontic ruler perpendicular to the floor. The pieces were fixed to the ruler with a 07 wax blade, with the occlusal plane parallel to the ground.

In each hemi-maxillae, buccal and palatal sites were photographed, and the distance from the cementoenamel junction to the bone crest was measured with the Image J 3.0 program, by a blinded and calibrated examiner. Measurements were performed in pixels and converted to mm using the endodontic ruler as reference. For calibration, 20 specimens were randomly chosen to be twice measured, with a one-week interval. The intraclass correlation coefficient observed was 0.96.

Five linear measurements were taken of the distance from the cemento-enamel junction to the bone crest in the buccal and palatal aspects. For each site, two measurements were made in the mesial root, two in the distal, and one in the furcation region. The bone loss of the teeth was generated from the mean of these 10 linear measurements (FERNANDES *et al.,* 2007).

Analysis of Total Plasmatic Sulfhydryl

To analyze the total plasmatic sulfhydryl, plasma (45 uL) was mixed with 120 mL of PBS and 35 mL of Tris (30 mM)/EDTA 3mM (pH 8) buffer in a microplate. After the basal absorbance was read (412 nm), the samples were regulated with 10 mL of 5.5'-dithiobis-(2-nitrobenzoic acid) (10 mM in ethanol) for one hour. Then, the samples

were read again, and the basal reading was discounted. These values were compared with the values obtained in a standard curve, sulfhydryl. The results were expressed as nmol SH/mg protein, as previously described (RIENER; KADA GRUBER, 2002).

Analysis of Ferric Reducing Ability of Plasma

Detecting the total non-enzymatic antioxidant capacity of plasma used a technique capable of evaluating the plasma ability to reduce Fe^{3+} in Fe^{2+} by means of color generation. For this purpose, 7 uL of the plasma diluted with 18 ul of H_2O was distilled and subsequently reacted with 175 ul of ferric reducing ability of plasma (FRAP) working reagent. This reagent consists of 10 parts acetate buffer (300 mM, pH 3.6), one part 2,4,6-Tris(2-pyridyl)-S-triazine (10 mM in 40 mM HCl), and one part $FeCl_3$ (20 mm). A standard $FeSO_4$ curve was performed in parallel and the absorbances were read after 10 minutes of reaction (593 nm). Finally, the absorbances of the samples were compared with those of the standard curve and the results were expressed in mM/L of plasma, as previously described (BENZIE; STRAIN, 1996).

Analysis of Carbonyl

For carbonyl analysis, all samples (1 mg protein) were reacted with 10 mM 2,4-dinitrophenylhydrazine for 30 minutes and subjected to protein precipitation with 10% TCA and centrifugation (11,000 x g for three minutes at 4 °C). The precipitate was then washed with ethanol and ethyl acetate (1:1) and centrifuged (11,000 x g for 3 minutes at 4°C). Afterwards, the precipitates were resuspended in 6 M guanidine hydrochloride (in 20 mM KH_2PO_4, pH 2.4). In parallel, samples reacted with 2 M HCl in place of 10 mM 2,4-dinitrophenylhydrazine were used to discount basal absorbance (white). Results were normalized to protein amount and expressed as nmol carbonyl/mg protein as described in Levine *et al*. (1994).

Statistical Analysis

The normality was checked using the Shapiro–Wilk test. Distribution curves were generated to evaluate symmetry and kurtosis. Body weight, food intake, and liquids were evaluated by repeated measures ANOVA with post hoc Sidak correction. Mean and standard deviation of ABL converted to mm and oxidative stress markers were evaluated by one-way ANOVA followed by Bonferroni's multiple comparisons test. The rat was considered the unit of analysis and the level of significance

established was 95%.

RESULTS

The results presented refer to a sample number of 57 animals. Five animals from the Perio group and six animals from the Perio+VitC group were excluded. No animal was lost during the study for reasons related to the protocol of use of vitamin C.

Figure 2 shows the animals' body weight during the experimental period. An increase in weight was observed during the three weeks in all experimental groups. Analyzed separately, there was a significant difference only between the first and third weeks, as well as between the second and third weeks when all the experimental groups are considered. Regarding the time–group interaction, the analysis did not indicate statistically significant differences between the experimental groups.

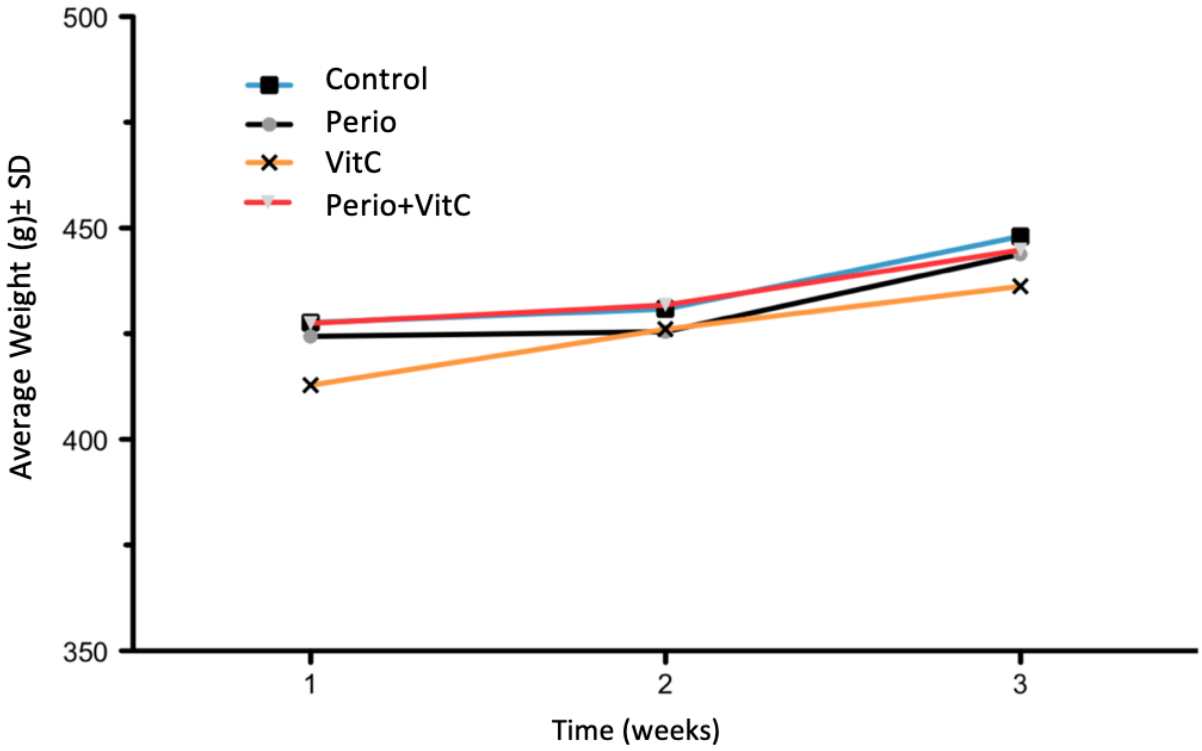

Figure 2. The weight (in g) over the experimental period according to the different experimental groups ANOVA repeated measures (Sidak).

Food and liquid intake are shown in **Figures 3 and 4**, respectively. Repeated measures ANOVA. The analysis did not allow a distinction in the consumption pattern, being the variable value throughout the study. Thus an interaction between the time and group for food intake could not be observed. Therefore, when experimental groups were compared in different experimental times, no significant differences in food intake

were observed. The analysis did not reveal any interaction between the consumption of liquids and the time of follow-up of the animals, nor a time–group interaction, demonstrating that the consumption of liquids was similar between the experimental groups over time.

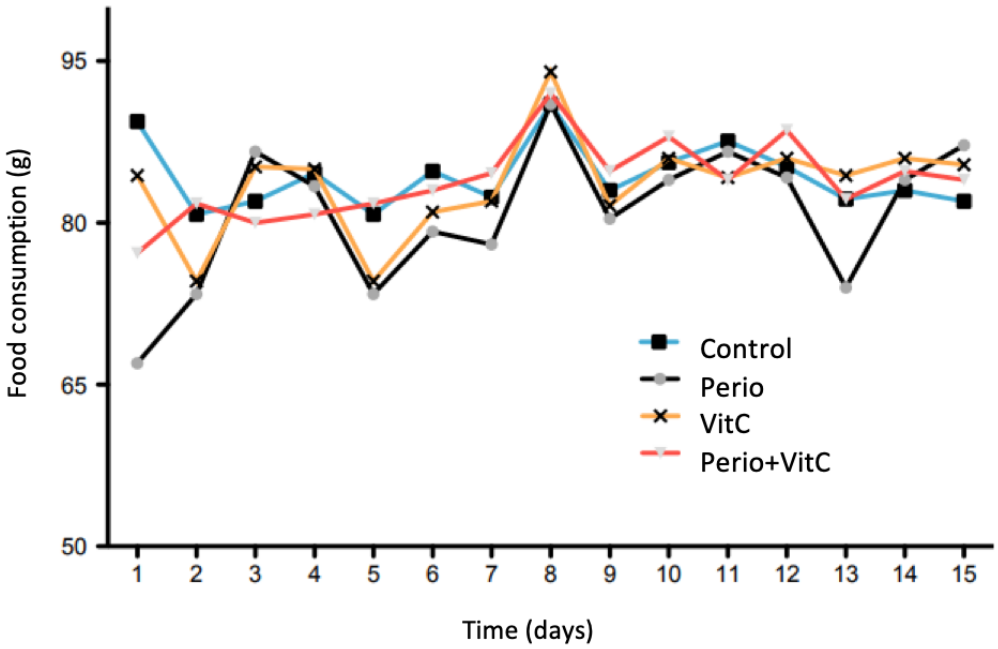

Figure 3. Food intake (in g) during the experimental period according to the different experimental groups-ANOVA repeated measures (Sidak)

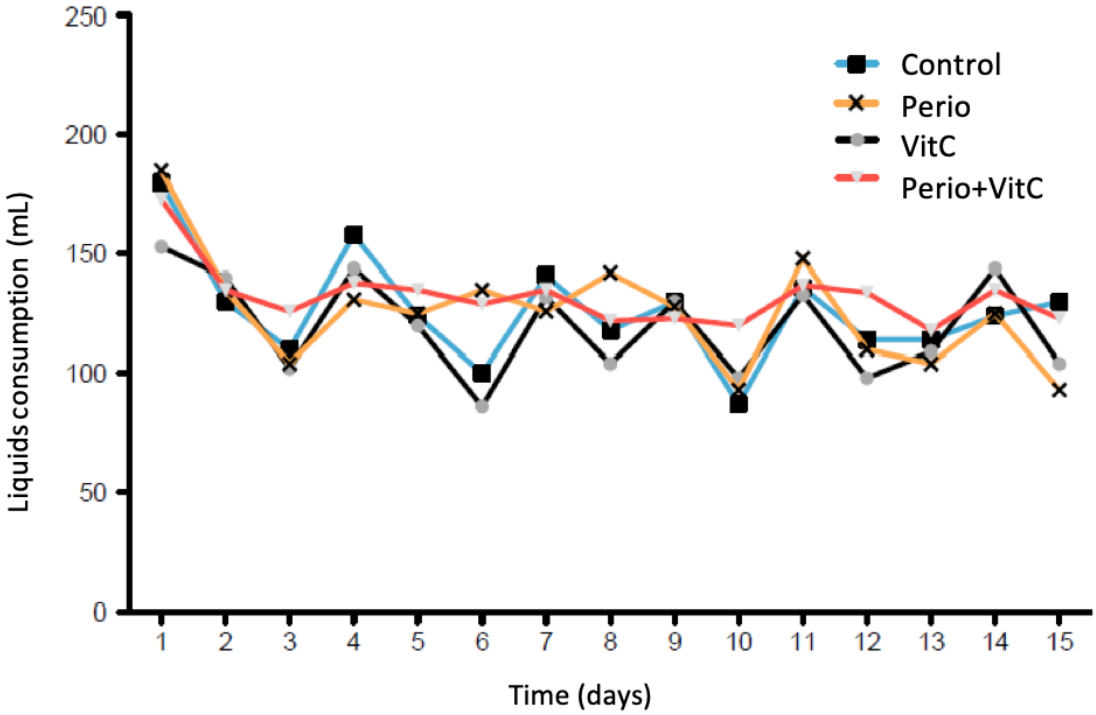

Figure 4. Consumption of liquids (in mL) during the experimental period according to the different experimental groups-ANOVA repeated measures (Sidak)

The ABL was evaluated separately between the right and left sites according to the presence of ligature. **Table 1** shows the mean ABL (in mm) for the different experimental groups on the right and left sites. All the animals included in the analysis exhibited some degree of ABL. Considering the left side, in which none of the animals from any of the experimental groups received induction of periodontitis, no statistically significant differences were observed between groups at the end of the experimental period. This means that the exposure of animals to VitC did not produce significant effects on spontaneous ABL.

In contrast, in the sites with induction of periodontitis, the animals exhibited pronounced ABL (0.715±0.13 and 0.609±0.12, for the Perio and Perio+VitC groups, respectively), statistically significant compared to the groups without periodontitis (0.243±0.27 and 0.254±0.22 for the Control and VitC groups, respectively). Additionally, exposure to vitamin C significantly reduced ABL induced by ligature by approximately 15%, with averages of 0.715±0.13 and 0.609±0.12 for the Perio and Perio+VitC groups, respectively.

Table 1**.** Mean (± SD) of the alveolar bone loss (in millimeters) to the right and left sides according to the experimental groups.

	side (with ligature in Perio and VitC + Perio)	(all groups without bandages)
Control	0.243 ± 0.27 **A**	0.242 ± 0.02 **A**
Perio	0.715 ± 0.13 **B**	0.245 ± 0.04 **A**
VitC	0.254 ± 0.22 **A**	0.253 ± 0.03 **A**
Perio+VitC	0.609 ± 0.12 **C**	0.257 ± 0.02 **A**

Means followed by different letters show statistically significant differences
Significant one-way ANOVA followed by Bonferroni

Figure 5 shows the plasmatic levels of FRAP at the end of the experimental period in the Control, Perio, VitC, and Perio+VitC groups. The groups that received vitamin C and/or induction of periodontitis exhibited lower values when compared to the Control group. This means that both vitamin C and periodontal disease were able to interfere with the serum levels of FRAP. However, vitamin C seemed to negatively affect the plasmatic levels of FRAP in rats with induced periodontal disease.

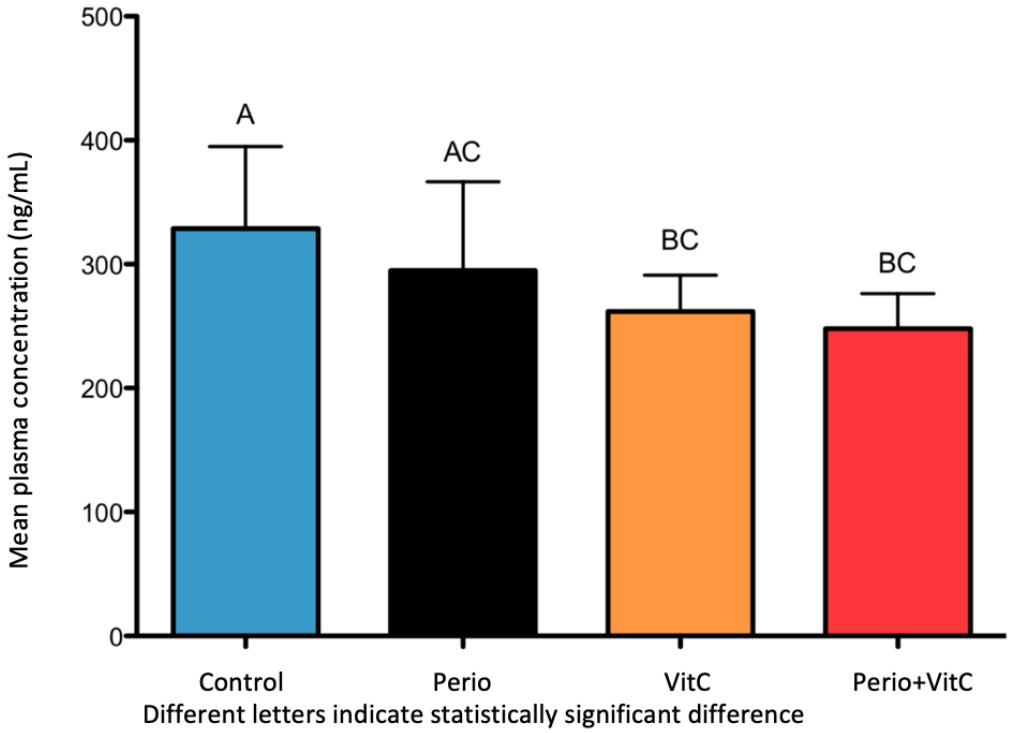

Figure 5. Mean plasmatic levels of Frap (±DP) at the end of the experimental period according to the experimental groups.

Figure 6 shows the plasmatic levels of sulfhydryl at the end of the experimental period in the experimental groups. The Perio and Perio+VitC groups exhibited higher values when compared to the Control and VitC groups. This demonstrates that the animals with periodontal disease presented a higher plasma concentration of sulfhydryl than the animals not exposed to experimental periodontitis. In this sense, the enzymatic antioxidant defense was altered, with higher concentrations in the animals with periodontal disease.

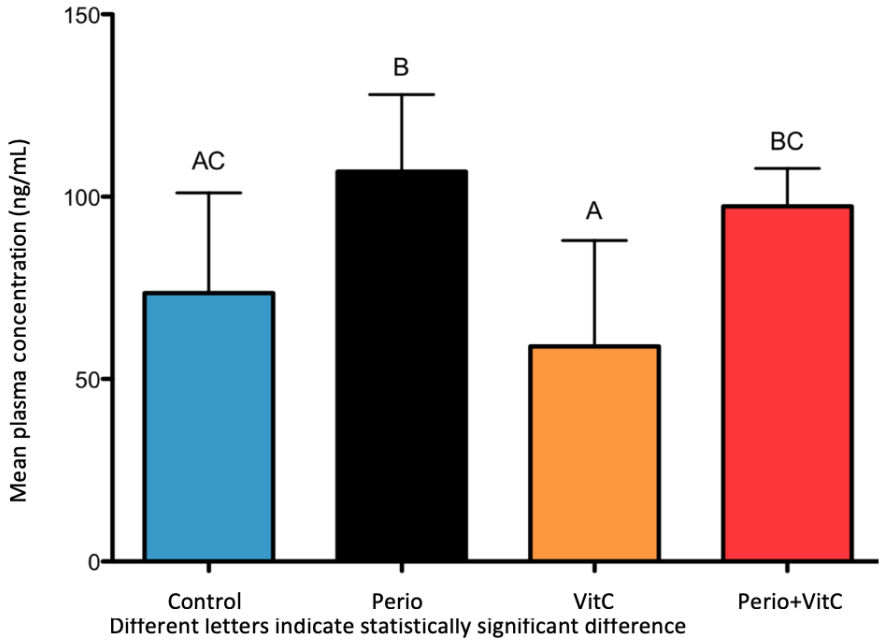

Figure 6. Mean plasmatic levels of sulfhydryl (±SD) at the end of the experimental period according to the experimental groups.

Figure 7 shows the median (25% and 75% percentile) of the plasma carbonyl concentration at the end of the experimental period by group. Substantial variation existed in the plasma carbonyl concentration within the same group, but there was no statistical difference in the median carbonyl concentration between the Control, VitC, Perio, and Perio+VitC groups.

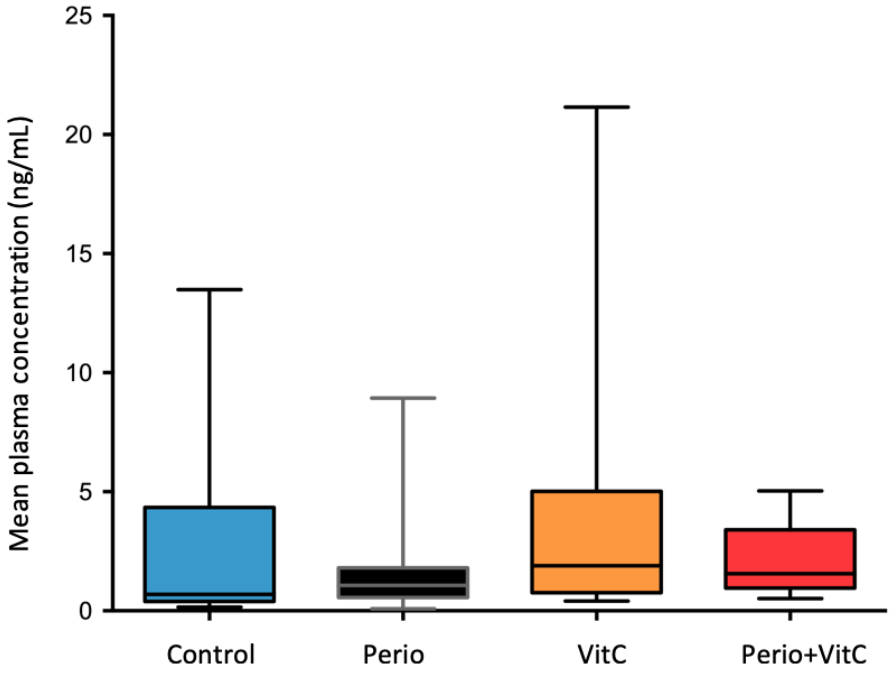

Figure 7 Median (25% and 75% percentile) of the plasma carbonyl concentration at the end of the experimental period according to the groups.

DISCUSSION

The present study aimed to evaluate the effect of exposure to vitamin C on periodontal destruction and oxidative stress parameters in Wistar rats. Vitamin C presented the potential for reduction of ABL, as well as modulation the parameters of oxidative stress.

An important aspect to be reported is that the number of animals analyzed in the present study varied from 11 to 17, due to the loss of ligature in some animals. Studies with these models have used periodic verification of the presence of ligatures and eventual replacement when they are lost (TOMOFUJI, 2009). However, this could generate stress in animals, which could negatively affect the results. Moreover, as the period for induction of periodontal disease was 14 days, the loss of ligature for a short period could generate a bias. Therefore, animals that lost the ligatures were excluded from all analyses (both periodontal destruction and oxidative stress markers) due to the exact time of the loss being unknown. The results presented thus relate always to the same group of animals. It is important to emphasize that studies on the etiopathogenesis of periodontal diseases normally have used the number of similar or even inferior animals in the present study (DUNDAR, 2016; LEVINE, 1986; JUNIOR, 2017; ÖZDEN, 2017; TOMOFUJI, 2009; VARGAS-SANCHES, 2016).

Monitoring body weight of animals has been used to infer whether experimental procedures influence systemic health (WAGNER, 2016). In the present study, the animals of all groups gained weight as a result of their development, with no statistically significant differences observed. Thus, it can be suggested that the experimental procedures (both placement of ligatures and exposure to vitamin C) did not impair the systemic health of the animals. The results obtained can therefore be credited to the experimental procedures *per se* and not to systemic health damage (CHAPPLE, 1997). It is also important to emphasize that the weight range found in the animals participating in this study resembles other published studies (AKMAN, 2013).

Solid and liquid intake can also be affected by experimental procedures, either due to the eventual presence of pain because of manipulations or difficulties adapting to the taste of the solutions (WAGNER, 2016). For solid ingestion, adaptation was not necessary since the rat feed used in the study was the same that the animals received from birth. The results of the analyses of solid and liquid ingestion also indicated similar feeding patterns in the different experimental groups, which facilitated isolation of the variables of exposure to ligature and/or vitamin C in the findings.

Vitamin C, the main independent variable, was administered in water at a concentration of 1 g/L. The use of the vitamin orally has advantages and disadvantages. Notably, the topical effect of vitamin C can also be obtained. In terms of disadvantages, it was only possible to evaluate the average consumption per housing box, which may not accurately reflect individual consumption. However, this is a current laboratory practice in similar studies and does not seem to mask or negatively interfere in the results (CAVAGNI, 2013).

One important outcome of the present study was the ABL found at the end of the experimental period. This is one of the few opportunities in health research to quantify the effect of the inflammatory process. Results obtained by comparing the sides with and without ligatures clearly demonstrated that the presence of ligatures was able to generate inflammation that resulted in destruction of the periodontal tissues, which supports the effectiveness of the method, similar to other studies (DUNDAR, 2016; KOSE, 2015).

Regarding the comparison between the groups themselves, no statistically significant differences were observed in the evaluation of the non-ligature sides. This implies that the experimental protocol did not interfere with spontaneous ABL. Studies have analyzed both spontaneous and induced bone loss, with conflicting results (LIBERMAN, 2011; VERZELETTI, 2012).

The comparison between groups where ligature was installed and the VitC and Control groups showed that the presence of ligatures generated additional ABL. The presence of vitamin C generated lower degrees of bone loss in the group with periodontitis. On the other hand, the simple presence of vitamin C did not differ from the Control group. This suggests that vitamin C, in the presence of a consistent microbial challenge such as ligature, minimizes periodontal damage.

The vast majority of studies published so far on this topic show positive results in relation to the complementary use of vitamin C with conventional periodontal treatment, and in relation to ABL in studies using animal models. However, the mechanisms underlying the positive effect of vitamin C in the periodontal etiopathogenic process are still the subject of research. One possibility lies in the effects that vitamin C has on oxidative stress. Epidemiological studies indicate a negative association between the plasmatic level of vitamin C and the severity of periodontitis (TIMMERMAM, 2007). The increase in the plasma level of vitamin C has a beneficial effect on periodontitis. However, it is unclear how vitamin C ingestion

affects gingival oxidative stress, gene expression that encodes inflammation, and cellular behavior in periodontal lesions, although some studies suggest that it increases the number of bundles of collagen, regenerating periodontal tissue and detoxifying histamine in gingival inflammation (NAKOMOTO, 1984; VAN DER VELDEN, 2011; ALAGL, 2015).

The analysis of the FRAP, which shows the ability of plasma to reduce iron, used in this study to detect non-enzymatic antioxidant capacity, was not in line with the first hypotheses raised. Plasmatic levels of FRAP were reduced with exposure to vitamin C, demonstrating a decrease in antioxidant capacity in these groups. The literature indicates that increased levels of this marker are expected given the challenges caused by supplementation with antioxidant agents. A possible explanation for this is the complexity of the relationships between the antioxidant agents in the inflammatory process in periodontal disease, as well as the possibility of vitamin C being present as pro-oxidative when in contact with iron, being able to produce radical species at the systemic level.

The plasma concentration of sulfhydryl reports the dosage of this marker on plasma proteins, which participates in the enzymatic antioxidant defense. The group with induced periodontal disease presented higher plasmatic levels of sulfhydryl, demonstrating a greater antioxidant capacity than the other groups. This finding can be explained by the increased antioxidant response caused by oxidative stress triggered by ligature placement. The group that was exposed to vitamin C and received the induction of periodontitis, expressed itself in a similar way to the control group. In this case, vitamin C seems to have acted attenuating the oxidation process caused by the experimental periodontitis.

The plasma concentration of carbonyl reported the dosage of this marker on plasma proteins, which participates in the enzymatic antioxidant defense. In the present study, no significant differences were observed between experimental groups. This is possibly related to vitamin C acting on another marker or only on local tissue.

One possible explanation for the findings observed for oxidative stress markers is related to the local evaluation. It is possible that, at a plasma level, the markers are reacting with other molecules of the animal metabolism besides vitamin C that are eventually free in the bloodstream. In this sense, the benefits of vitamin C observed may be related to the fact that it has only a local tissue effect.

The present study presents strengths and limitations. The advantages are its

use of basic principles of contemporary research, with blinding, calibration, randomization, and use of control groups both intra- and intergroup, allowing the results to isolate the effects of ligature and vitamin C. It is also noteworthy that the concentration of vitamin C ingested has a relationship with recommendations for supplementation in humans (LEVINE, 1986). The main limitation is the impossibility of direct extrapolation of the results to humans. The loss of ligatures of some animals can also be considered a limitation. However, the observation of statistically significant differences between the groups suggests that this loss of animals did not interfere in the findings.

The interpretation of this study is relevant to the knowledge of periodontal disease pathogenesis, suggesting that exposure to vitamin C has the potential to modulate both periodontal destruction and oxidative stress parameters, which should be the object of continuous investigation.

CONCLUSION

Exposure to vitamin C potentially reduces ABL, modulating oxidative stress parameters in rats.

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3 CONSIDERAÇÕES FINAIS

O presente estudo teve como objetivo verificar o efeito da utilização de vitamina C sobre a periodontite experimental e parâmetros de estresse oxidativo em ratos Wistar. Como principal resultado encontrado no estudo foi que os animais que receberam vitamina C e indução de doença periodontal tiveram menores graus de perda óssea alveolar quando comparados aos animais que receberam apenas indução de periodontite. Com esse resultado podemos concluir que houve uma ação antioxidante da vitamina C frente a um estresse oxidativo induzido pela doença periodontal.

Os marcadores de estresse oxidativo plasmáticos analisados foram FRAP, sulfridil e carbonila. Em relação aos níveis plasmáticos de FRAP os grupos com vitamina C apresentaram diferença em relação ao grupo controle. Já em relação ao sulfidril, os grupos com doença periodontal apresentaram menor nível comparado com o grupo controle. Os níveis de carbonil também foram analisados e não apresentaram diferença estatísticas entre nenhum dos grupos.

Estudos utilizando ratos como plataforma são parte fundamental da construção do conhecimento dos processos etiopatogênicos das doenças periodontais. Ainda que importantes diferenças biológicas existam entre as estruturas periodontais de seres humanos e ratos, esse modelo mostra-se bastante atraente a medida que, do ponto de vista ético, seria impossível conduzir um estudo paralelo a este. Ainda, do ponto de vista metodológico, utilizou-se um estrito controle de qualidade que seguiu as diretrizes do ARRIVE Guidelines. Isso inclui um controle total das características do ambiente em que os animais permaneceram durante o estudo, princípios básicos de pesquisa como randomização, cegamento, reprodutibilidade dos examinadores entre outros. Tal fato aumenta a validade interna e amplia o potencial de translação dos achados.

Os resultados deste trabalho de conclusão de curso apontam para um efeito protetor da vitamina C sobre o desenvolvimento da doença periodontal de modo que os achados podem ser explicados por uma modulação do estresse oxidativo. De maneira nenhuma é objetivo extrapolar os achados da presente investigação diretamente para seres humanos. Entretanto, os resultados observados são bastante encorajadores e abrem uma miríade de possibilidades de investigação científica incluindo avaliação de citotoxicidade e realização de ensaios clínicos randomizados

com seu emprego como coadjuvante à terapia periodontal.

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