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**EFEITO DA RESTRIÇÃO CALÓRICA NA PERDA ÓSSEA ALVEOLAR INDUZIDA POR
LIGADURA EM RATOS**

PONTA GROSSA

2020

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**EFEITO DA RESTRIÇÃO CALÓRICA NA PERDA ÓSSEA ALVEOLAR INDUZIDA POR
LIGADURA EM RATOS**

Tese apresentada para obtenção do título de Doutora na
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Orientador: Prof. Dr. Gilson Cesar Nobre Franco

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Efeito da restrição calórica na perda óssea alveolar induzida por ligadura em ratos.

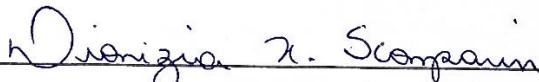
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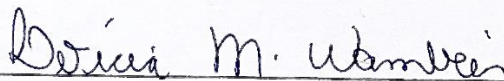
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Dedico esta tese de Doutorado aos meus pais,

Elias (*in memoriam*) e Marilda,

pelo amor incondicional para

que eu chegasse até aqui,

ao meu filho **Gustavo,**

o maior incentivo para

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e ao meu esposo **Eduardo,**

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RESUMO

Soares, E.L. **Efeito da restrição calórica na perda óssea alveolar induzida por ligadura em ratos.** [Tese] Doutorado em Clínica Integrada. Ponta Grossa: Universidade Estadual de Ponta Grossa; 2020.

A periodontite é uma condição infecciosa e inflamatória que inicia com a gengivite, no tecido de proteção do dente e progride para os tecidos de sustentação. Várias cascatas de reações inflamatórias estão envolvidas e resultam na perda óssea alveolar, que pode ser modificada por fatores locais, sistêmicos e comportamentais, como dietas. A restrição calórica é uma diminuição na ingestão calórica e está relacionada a mecanismos benéficos na redução de doenças crônicas, como a periodontite. Portanto, o objetivo deste estudo foi avaliar o efeito da restrição calórica na perda óssea alveolar induzida por ligadura em ratos. Este estudo utilizou 96 ratos Wistar, os quais foram divididos inicialmente em grupos com dieta normocalórica (Experimento 1) ou grupos com dieta de cafeteria (Experimento 2) e subdivididos em ambos estudos em grupos que foram submetidos à restrição calórica e grupos que receberam indução da periodontite experimental por ligadura. Dentre as análises, foram realizadas: macroscópica morfológica para análise da perda óssea alveolar, análise biométrica, níveis séricos de perfil glicêmico e lipídico, análise clínica periodontal e níveis dos biomarcadores de tecido ósseo. Os resultados mostram que a restrição calórica e a indução por ligadura tiveram interação significativa com a perda óssea alveolar apresentada pelos animais. Na comparação entre os animais que receberam indução por ligadura, os grupos que receberam indução de restrição calórica demonstraram redução significativa na perda óssea alveolar, com relação aos grupos que não receberam a restrição calórica, tanto para os grupos com dieta anteriormente normocalórica quanto para os grupos com dieta de cafeteria. Os dados encontrados comprovam o método de indução de restrição calórica, pois houve redução significativa do peso corporal, circunferência abdominal e da massa adiposa das gorduras, em ambos experimentos. Na análise do nível glicêmico, os animais submetidos a restrição calórica demonstraram redução significativa em comparação com os que não receberam restrição calórica. Quanto à análise clínica periodontal, entre os grupos que receberam a indução por ligadura, foram encontradas diferenças significativas nos parâmetros: sangramento, índice gengival e mobilidade, após restrição calórica, principalmente nos animais anteriormente obesos. Entretanto, com relação ao parâmetro biofilme não houve diferença significativa, sendo encontrado biofilme em todos os grupos com periodontite experimental. Os achados quanto aos níveis de biomarcadores do tecido ósseo, cálcio e fosfatase alcalina, comprovam que o método aplicado de indução de restrição calórica não mostrou diminuição nutricional. Levando em conta os resultados encontrados, a restrição calórica reduz significativamente tanto a perda óssea alveolar, quanto os parâmetros sérico e biométrico.

Palavras-chave: Periodontite, Restrição calórica, Inflamação

ABSTRACT

Soares, E.L. **Effect of caloric restriction on alveolar bone loss ligature-induced in rats.** [Tese] Doutorado em Clínica Integrada. Ponta Grossa: Universidade Estadual de Ponta Grossa; 2020.

Periodontitis is an infectious and inflammatory condition that starts with gingivitis in the teeth protective tissue and progresses to the supporting tissues. Several cascades of inflammatory reactions are involved and result in alveolar bone loss, which can be modified by local, systemic and behavioral factors, such as diets. Caloric restriction is a decrease in caloric intake and is related to beneficial mechanisms in reducing chronic diseases, such as periodontitis. Therefore, the aim of this study was to evaluate the effect of caloric restriction on ligature-induced alveolar bone loss in rats. This study used 96 Wistar rats, which were initially divided into groups with normocaloric diet (Experiment 1) or groups with cafeteria diet (Experiment 2) and subdivided in both studies in groups that were submitted to caloric restriction and groups that received induction of ligature-induced experimental periodontitis. Among the analyzes, were performed: macroscopic morphometric for analysis of alveolar bone loss, biometric analysis, serum levels of glycemic and lipid profile, clinical periodontal analysis and bone tissue biomarkers levels. The results show that caloric restriction and ligature induction had a significant interaction with the alveolar bone loss presented by the animals. In the comparison between animals that received induction by ligature, the groups that received caloric restriction induction showed a significant reduction in the alveolar bone loss in relation to the groups that did not receive caloric restriction, both for the groups with a previously normocaloric diet and for the groups with a cafeteria diet. The data found support the caloric restriction induction method, as there was a significant reduction in body weight, abdominal circumference and fat mass, in both experiments. In the analysis of the glycemic level, animals submitted to caloric restriction showed a significant reduction with those that did not receive caloric restriction. As for the clinical periodontal analysis, among the groups that received ligature induction, significant differences were found in the parameters: bleeding, gingival index and mobility, after caloric restriction, mainly in previously obese animals. However, regarding the biofilm parameter, there was no significant difference, biofilm being found in all groups with experimental periodontitis. The findings regarding the bone tissue biomarkers levels, calcium and alkaline phosphatase, prove that the applied method of induction of caloric restriction did not show nutritional decrease. Considering the results found, caloric restriction significantly reduces both alveolar bone loss and serum and biometric parameters.

Key-words: Periodontitis, Caloric restriction, Inflammation

LISTA DE FIGURAS

Figure 3.1.1	- Effect of caloric restriction on biometric parameters	27
Figure 3.1.2	- Blood concentration of biochemical parameters	28
Figure 3.1.3	- Comparison of clinical periodontal measures between groups with periodontitis ligature-induced	29
Figure 3.1.4	- Diagram of macroscopic morphometric analisys and images of alveolar bone loss representing the experimental groups	30
Figure 3.1.5	- Mean (EPM) of alveolar bone loss values (mm) of rats submitted to caloric restriction (CR) with and without periodontitis ligature-induced (LIG) in the left mandibular first molar	31
Figure 3.1.6	- Effect of caloric restriction on bone tissue biomarkers (A) Calcium, and (B) Alkaline phosphatase	31
Figure 3.2.1	- Effect of caloric restriction on biometric parameters	48
Figure 3.2.2	- Blood concentration of (A) Glucose (B) Triglycerides (C) HDL-cholesterol (D) LDL-cholesterol (E) Total cholesterol	49
Figure 3.2.3	- Comparison of clinical periodontal measures between groups that received ligature-induced experimental periodontitis, expressed as a percentage	50
Figure 3.2.4	- Diagram of macroscopic morphometric analisys and images of alveolar bone loss representing the experimental groups.....	51
Figure 3.2.5	- Mean (EPM) of alveolar bone loss values (mm) of rats submitted to caloric restriction (CR) with and without experimental periodontitis ligature-induced (LIG) in the mandibular first molar	52
Figure 3.2.6	- Effect of caloric restriction on bone tissue biomarkers (A) Calcium (B) Alkaline phosphatase	52

LISTA DE TABELAS

Table 3.1.1	- Biometric parameters in the experimentals groups submitted a caloric restriction and experimental periodontitis	26
Table 3.1.2	- Distribution of frequency of the periodontal clinical parameters based in scores	29
Table 3.2.1	- Biometric parameters in the experimentals groups submitted a caloric restriction and experimental periodontitis	47
Table 3.2.2	- Distribution of frequency of the periodontal clinical parameters based in scores	50

LISTA DE ABREVIATURAS E SIGLAS

ABL	Alveolar bone loss
ANOVA	Analysis of Variance
ARRIVE	Animal Research: Reporting of <i>In vivo</i> experiments
BOP	Bleeding on probing
CR	Caloric restriction
FoxO1	Forkhead box protein O1
HDL	High Density Lipoprotein
IgG	Immunoglobulin G
IL	Interleukin
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IP	Intraperitoneal
LDL	Low Density Lipoprotein
MMP-8	Matrix metalloproteinase-8
MMP-9	Matrix metalloproteinase-9
n	Número amostral
NF- κ B	Nuclear transcription factor kappa beta
NIH	National Institutes of Health
MMP-8	Matrix metalloproteinase-8
MMP-9	Matrix metalloproteinase-9
OPG	Osteoprotegerin
POA	Perda óssea alveolar
PPAR- γ	Peroxisome proliferator-activated receptor gama
RANK	Receptor activator of nuclear factor-kB
RANKL	Receptor activator of nuclear factor-kB ligand
RC	Restrição calórica
ROS	Reactive oxygen species
SEM	Standard error of mean
SIRT's	Sirtuins
SIRT1	Sirtuin 1

SIRT3	Sirtuin 3
SOD	Superoxide dismutase
SPSS	Statistical Package Social Sciences
TNF-alpha	Tumor necrosis factor - alpha

LISTA DE SÍMBOLOS

%	Porcentagem
g	Grama
mg	Miligrama
kg	Kilograma
°C	Grau Celsius
h	Hora
>	Maior que
<	Menor que
mm ²	Milímetros quadrados
cm	Centímetro
dL	Decilitro
x	Vezes
mL	Mililitro
mm	Milímetro
μL	Microlitro
±	Mais ou menos
β	Beta
γ	Gama

SUMÁRIO

1	INTRODUÇÃO	16
2	PROPOSIÇÃO	18
2.1	ARTIGO 1	18
2.1.1	Proposição geral	18
2.1.2	Proposição específica	18
2.2	ARTIGO 2	18
2.2.1	Proposição geral	18
2.2.2	Proposição específica	18
3	ARTIGOS	20
3.1	ARTIGO 1 - EFFECT OF CALORIC RESTRICTION ON ALVEOLAR BONE LOSS IN RATS	20
3.2	ARTIGO 2 - CALORIC RESTRICTION PREVENTS ALVEOLAR BONE LOSS IN THE EXPERIMENTAL PERIODONTITIS IN OBESE RATS.....	41
4	DISCUSSÃO	61
5	CONSIDERAÇÕES FINAIS	66
	REFERÊNCIAS	67
	ANEXO A - PARECER DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAL	72

1 INTRODUÇÃO

A periodontite é uma condição infecciosa e inflamatória dos tecidos de proteção e suporte do dente, que incluem: gengiva, cemento, ligamento periodontal e osso alveolar (HAJISHENGALLIS *et al.*, 2019; PEDROSO *et al.*, 2019). A periodontite é uma das doenças infecciosas mais comuns, no contexto mundial, e nos países desenvolvidos atinge aproximadamente metade da população adulta (TAKEUCHI ET AL, 2019). É o resultado de complexas inter-relações entre agentes infecciosos e fatores relacionados ao hospedeiro. Fatores ambientais, genéticos e comportamentais podem modificar a expressão da doença e, adicionalmente, alterar a severidade e progressão dessa condição (VELIDANDLA *et al.*, 2019). A reação imunoinflamatória da periodontite é marcada pelo aumento da produção de mediadores inflamatórios, como citocinas, espécies reativas de oxigênio e prostaglandinas (VARGAS-SANCHEZ *et al.*, 2017). O dano tecidual resultante da interação biofilme e hospedeiro, com a liberação dessas substâncias, está relacionado principalmente a consequente perda óssea alveolar (POA) e encontra-se bem estabelecido como resultado da resposta excessiva do hospedeiro aos microrganismos (TOKER *et al.*, 2019).

A resposta atuante ativamente na perda óssea alveolar pode ser influenciada por modificações comportamentais, dentre elas as modificações nutricionais, como a terapia dietética (PARK *et al.*, 2015). Nesse sentido, estratégias dietéticas de modulação das vias de resposta do hospedeiro na periodontite, como a restrição calórica, podem exercer um papel benéfico, além de terapêutico indicada no tratamento da obesidade e recomendada para aumento da longevidade, como também na prevenção e redução de doenças crônicas, podendo reduzir ou alterar o padrão de POA na periodontite (GONZALEZ *et al.*, 2012).

A restrição calórica (RC) é uma redução no consumo de energia entre 30-40%, sem má nutrição (COLMAN *et al.*, 2014; WALDMAN *et al.*, 2018). Mundialmente, a RC é aceita como método não genético que atua no processo de alteração fisiológica, principalmente relacionado ao avanço da idade ou disfunções metabólicas, como a obesidade, em que pode haver maior predisposição ao aumento nos níveis circulantes de citocinas pró-inflamatórias e agravamento na severidade e progressão de doenças inflamatórias, como a periodontite (RUGGENENTI *et al.*, 2017; REYNOLDS *et al.*, 2009).

Modelos animais têm sido os mais utilizados mundialmente, em ambos métodos para indução, tanto da periodontite quanto da RC (VARGAS-SANCHEZ *et al.*, 2017; MATTISON

et al., 2017). O rato tem sido o animal predominantemente utilizado para analisar perda óssea alveolar na periodontite, em razão da sua similaridade com relação a destruição tecidual que ocorre em humanos. No estudo de Branch-Mays (2008) e colaboradores foram avaliados parâmetros relacionados a periodontite, em macacos não obesos, com resultados vantajosos e promissores relacionados à restrição calórica. Entretanto, na literatura ainda não existem relatos da avaliação da progressão e severidade da periodontite em animais obesos submetidos a restrição calórica, além da lacuna na literatura sobre o comportamento da dieta para aumento da longevidade também, na principal consequência da periodontite, a perda óssea alveolar.

Torna-se fundamental, portanto, entender o mecanismo da RC, tanto em animais obesos quanto não obesos, diretamente na POA, que é considerada o principal desfecho da periodontite e que ainda não se encontra elucidado na literatura. Com isso, o objetivo deste estudo foi verificar os efeitos na perda óssea alveolar, nos dados biométricos, clínicos e séricos, após indução de restrição calórica em ratos não obesos e obesos com periodontite experimental induzida por ligadura.

2 PROPOSIÇÃO

2.1 ARTIGO 1

2.1.1 Proposição geral

- Avaliar os efeitos de uma restrição calórica de 30% na periodontite experimental em ratos Wistar

2.1.2 Proposição específica

- Investigar os efeitos da restrição calórica na perda óssea alveolar em ratos Wistar com periodontite experimental, por meio de análise macroscópica morfométrica
- Avaliar os parâmetros clínicos periodontais após indução da restrição calórica e periodontite experimental
- Verificar o perfil biométrico dos animais após restrição calórica
- Analisar os perfis glicêmico e lipídico após restrição calórica e periodontite experimental
- Identificar os níveis dos biomarcadores do tecido ósseo, cálcio e fosfatase alcalina.

2.2 ARTIGO 2

2.2.1 Proposição geral

- Avaliar os efeitos de uma restrição calórica de 30% na periodontite experimental em ratos Wistar submetidos à dieta de cafeteria

2.2.2 Proposição específica

- Verificar os efeitos da restrição calórica na perda óssea alveolar analisada por macroscopia morfométrica em ratos obesos
- Investigar os parâmetros clínicos periodontais após indução da restrição calórica e periodontite experimental
- Verificar o perfil biométrico dos animais após restrição calórica e indução da obesidade pelo método de dieta de cafeteria
- Analisar os perfis glicêmico e lipídico após restrição calórica, obesidade e periodontite experimental

- Avaliar os níveis dos biomarcadores do tecido ósseo, cálcio e fosfatase alcalina, na presença de periodontite experimental, restrição calórica e obesidade.

3. ARTIGOS

3.1 EFFECT OF CALORIC RESTRICTION ON ALVEOLAR BONE LOSS IN RATS

STATUS: PROCESSO DE SUBMISSÃO

REVISTA: LIFE SCIENCES

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Abstract

Aims: Recent research suggests an association between longevity, caloric restriction (CR) and reduction of chronic inflammatory diseases, such as periodontitis. Currently, the mechanisms related to the effects of CR are not yet fully understood. In the present study, we aimed to evaluate the effects of caloric restriction on alveolar bone loss (ABL) in Wistar rats with experimental periodontitis.

Main methods: The impact of CR on biometric data, clinical periodontal parameters, serum biomarkers and biochemical parameters were also investigated. The experimental protocol lasted 24 weeks. The CR rats were fed for 12 weeks with 30% of the standard food compared to the animals with *ad libitum* diet. Experimental periodontitis was induced in Wistar rats during 15 days before the end of the experiment. The animals were divided into four experimental groups (n = 12 animals/group), according to the presence or absence of caloric restriction and ligature-induced periodontitis. Hemimandibles were used to assess ABL.

Key findings: Biometric data demonstrated a significant decrease in animals submitted to CR, when compared to animals *ad libitum*. Biochemical data showed a significant reduction in the glycemic profile after caloric restriction. Regarding the groups with experimental periodontitis induction, a significant decrease in alveolar bone loss was observed in animals submitted to caloric restriction, in addition to attenuating gingival inflammation, demonstrated by the significant difference in gingival index. Caloric restriction did not significantly alter bone tissue biomarkers.

Significance: According to the findings, caloric restriction reduced alveolar bone loss, glycemic profile and biometric parameters in rats with experimental periodontitis.

Keywords: Caloric restriction, periodontitis, rats.

Introduction

Periodontitis is a worldwide prevalent immunoinflammatory condition, affecting more than 700 million people worldwide [1]. Periodontitis is characterized by defense responses against bacterial colonization and involves the protective and supporting tissues of the tooth [2,3]. The extent and severity of periodontal destruction and alveolar bone loss (ABL) can be affected by the magnitude and characteristics of the host response and be modulated by environmental, systemic and genetic factors. The immunoinflammatory reaction of periodontitis is marked by

increased production of cytokines, such as tumor necrosis factor alpha (TNF-alpha), reactive oxygen species and prostaglandins [4]. Tissue damage, especially alveolar bone loss, is well established as result of the excessive response to subgingival pathogens [5]. In this sense, dietary strategies for modulating the host response pathways in periodontitis, such as caloric restriction, can play a beneficial role in the prevention and reduction of ABL.

Caloric restriction (CR) is defined as a reduction in energy consumption of approximately 30%, without malnutrition [6,7,8,9]. Worldwide, CR is accepted as the only non-genetic method in the process of physiological change for extend lifespan [10]. These changes are mainly related to advancing age, when there is a greater predisposition to increase circulating levels of pro-inflammatory cytokines and worsening of inflammatory diseases [11]. Among inflammatory diseases, periodontitis is the oral condition considered the major cause of tooth loss in adults and which can have systemic repercussions [12]. On the other hand, CR seems to reduce inflammatory processes related to aging and periodontitis. One of the reasons for this is the decrease in the levels of nuclear transcription factor kappa beta (NF- κ B) and oxidative stress mediators [11,13-15]. Additionally, CR attenuates the effects of the host's immune and inflammatory response, which in the periodontitis leads to consequent alveolar bone loss [16]. Therefore, CR is related, in addition, to increasing longevity, but also quality of life during advancing age [17].

Animal models have been the most used worldwide, in both periodontitis and CR induction methods [4,18]. The rat has been the animal predominantly used to analyze alveolar bone loss in periodontitis, due to they similarity with regard to tissue destruction that occurs in humans [19].

According to these findings, it is essential to understand the CR mechanism directly in ABL, which is considered the main outcome of periodontitis and which is still not well understood in the literature. The aim of this study was to evaluate macroscopic, biometric, biochemical and clinical outcomes, after induction of 30% caloric restriction for 12 weeks in rats with experimental periodontitis.

Material and Methods

Animals

This study was conducted after review and approval by the Animal Use Ethics Committee of the State University of Ponta Grossa (Protocol 12203/2017). Forty-eight male Wistar rats (*Rattus norvegicus, albinus*) were used, maintained in polypropylene cages, with controlled temperature, fed with free access to the normocaloric commercial diet and natural water (*ad libitum*), until the beginning of the experiment, following the ARRIVE guidelines (Animal Research: Reporting of *In vivo* experiments) and was carried out in accordance with the National Institutes of Health (NIH) guide for the care and use of Laboratory animals [20,21]. For the sample calculation, the G*power 3.1 program (G*Power, Universität Düsseldorf, Düsseldorf, North Rhine-Westphalia, Germany) was used and it was found that for a power of 85% the number used should be 10 animals per group. Considering possible losses during the experimental process and possible methodological error during the research, n=12 animals per group were used.

Animals were allocated to the following groups (n=12): AL (*ad libitum* diet); AL-LIG (*ad libitum* diet with induction of experimental periodontitis); CR (animals subjected to 30% caloric restriction); CR-LIG (induction of 30% caloric restriction and experimental periodontitis).

Induction of CR

At 30 days of age the animals started the experiment (Day 0). All groups remained on *ad libitum* diet and free access to water for the first 12 weeks. In the 13th week, up to the 24th week (with a total of 12 weeks), the animals were divided into 4 groups according to the induction of caloric restriction and experimental periodontitis. The AL and AL-LIG groups were maintained on an *ad libitum* diet for 24 weeks, and the CR and CR-LIG groups were subjected to caloric restriction during the final 12 weeks of the experiment. The CR and CR-LIG animals were fed with 70% of the feed consumed by the AL group, with the respective times adjusted according to the weeks of the experiment [9,10]. At the end of 24 weeks, all animals were euthanized.

Induction of experimental periodontitis

The protocol used to induce experimental periodontitis was the placement of periodontal ligature, according to the method established in previous studies [4,22]. The AL-LIG and CR-LIG groups were weighed and sedated with intraperitoneal (IP) anesthesia with 10% ketamine

solution (90 mg/kg) and 2% xylazine muscle relaxant (10 mg/kg). After anesthesia, the animals were placed on the operating table and with the aid of an exploratory probe, the cotton thread was inserted subgingivally around the mandibular first molars. Then, the correct wire positioning and retention were verified. The induction of periodontitis occurred during 15 days, prior to the animals euthanasia.

Biometric parameters

Body weight

In order to calculate and monitor the evolution of body weight, all animals were weighed twice a week to verify weight reduction after caloric restriction.

Lee index and naso-anal length

To determine the Lee Index of all animals, the naso-anal length (centimeters) and the final body weight (grams) were measured, according to the calculation used in Nascimento *et al* (2013) [23].

Abdominal circumference

The comparison between diets and proof of caloric restriction was further investigated with the aid of a tape measure, by measuring abdominal circumference (centimeters).

Adiposity index

The adiposity index was analyzed by sum of the weights of mesenteric, epididymal and retroperitoneal fats, divided by body mass x 100, expressed as percentage of adiposity, according to study of Teixeira *et al* (2018) [24].

Clinical periodontal measures

Prior to the animals euthanasia, clinical measures of the mandibular molars and also of the adjacent periodontium was performed based on four parameters: Biofilm (0-absent; 1-present); Bleeding on probing – BOP (0-absent; 1-present); Mobility: 0-absent; 1-slight mobility (vestibular-palatal); 2-moderate mobility (vestibular-palatal and mesial-distal); 3-severe mobility (vertical, movement inside and outside the tooth socket); Gingival index (0-normal gingiva; 1-mild inflammation, mild edema, slight color change and no bleeding; 2-moderate inflammation, edema, redness and bleeding; 3-severe inflammation, extreme redness, presence

of ulcer, edema and severe bleeding). To perform this analysis, the animals were initially weighed and sedated with intraperitoneal (IP) anesthesia with 10% ketamine solution (90 mg/kg) and 2% xylazine muscle relaxant (10mg/kg). Subsequently, the animals were positioned on the operating table and with the help of a spatula and probe, the clinical analysis of periodontal parameters was performed as previous described. [25-27].

Biochemical analysis

After euthanasia, blood was collected, centrifuged and stored at -80°C. Glucose levels, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were determined using commercial kits following the manufacturer's instructions using CT300i Wiener analyzer.

Macroscopic morphometric analysis

To assess the extent of alveolar bone loss, measurements were made on the animals left hemimandibles. After euthanasia, the mandible of each animal was removed and immersed in 70° alcohol. Subsequently, the samples were boiled during 30 minutes, immersed in hydrogen peroxide solution (3%) and stained with methylene blue (1%) for differentiation between the tooth and bone tissue, fixed on a support and photographed. The images were made for the macroscopic analysis of the mandibles with the aid of a Zeiss stereoscopic loupe with a 20x magnification. The digital images were analyzed for alveolar bone loss with the Image J Software. The measurements were made on the lingual surface. In this procedure, the area (mm²) corresponding to the exposed root of the molars was calculated. All acquired images were compared to an already known area (1.0x1.0mm²) [4,28,29].

Bone biomarkers

Bone tissue biomarkers, calcium and alkaline phosphatase were measured using commercial kits, following the manufacturer's instructions, and analyzed using the CT300i Wiener equipment.

Statistical analysis

Data regarding the clinical measures biofilm and bleeding on probing were analyzed using the Chi-square test and the mobility and gingival index data were analyzed using the Mann-Whitney test, evaluated by the Statistical Package for the Social Sciences - SPSS Program, version for Windows.

The remaining data were analyzed using analysis of variance (Two-way ANOVA) with Tukey's post-test, used for statistical comparisons (GraphPad Prisma software version 8.2.1, San Diego, CA, USA). The data were expressed as mean $\pm$ standar error of mean (SEM). The level of significance used was 5% ($p < 0.05$).

Results

Biometric parameters

Caloric restriction caused a reduction in the values of final body weight, weight gain, adiposity index and abdominal circumference ($p < 0.05$) compared to the groups with *ad libitum* diet. The CR did not change the naso-anal length parameter ($p > 0.05$) (Table 1). In the comparison between the AL and CR groups, caloric restriction was efficient in reducing 28% of body weight and 44.7% of the adiposity index.

Table 3.1.1. Biometrics parameters in the groups submitted to caloric restriction and experimental periodontitis.

	AL	AL-LIG	CR	CR-LIG
Initial body weight (g)	35 $\pm$ 1.79	37 $\pm$ 1.13	36 $\pm$ 2.43	35 $\pm$ 0.95
Final body weight (g)	433 $\pm$ 12.44 ^a	472 $\pm$ 11.62 ^a	311 $\pm$ 9.78 ^b	314 $\pm$ 7.52 ^b
Body weight gain (g)	398 $\pm$ 10.74 ^a	435 $\pm$ 12.21 ^a	275 $\pm$ 9.54 ^b	279 $\pm$ 7.68 ^b
Adiposity index (%)	3.8 $\pm$ 0.38 ^a	4.5 $\pm$ 0.28 ^a	1.8 $\pm$ 0.26 ^b	2.1 $\pm$ 0.33 ^b
Naso-anal lenght (cm)	26 $\pm$ 0.32	26 $\pm$ 0.47	25 $\pm$ 0.29	25 $\pm$ 0.35
Lee index (g/cm)	288 $\pm$ 2.59 ^a	295 $\pm$ 5.57 ^a	269 $\pm$ 3.95 ^b	272 $\pm$ 2.57 ^b
Abdominal circumference (cm)	19 $\pm$ 0.34 ^a	20 $\pm$ 0.47 ^a	17 $\pm$ 0.28 ^b	17 $\pm$ 0.43 ^b

Data represented as mean $\pm$ SEM. ANOVA-two way, Tuke's test. Different letters indicated $p < 0.05$.

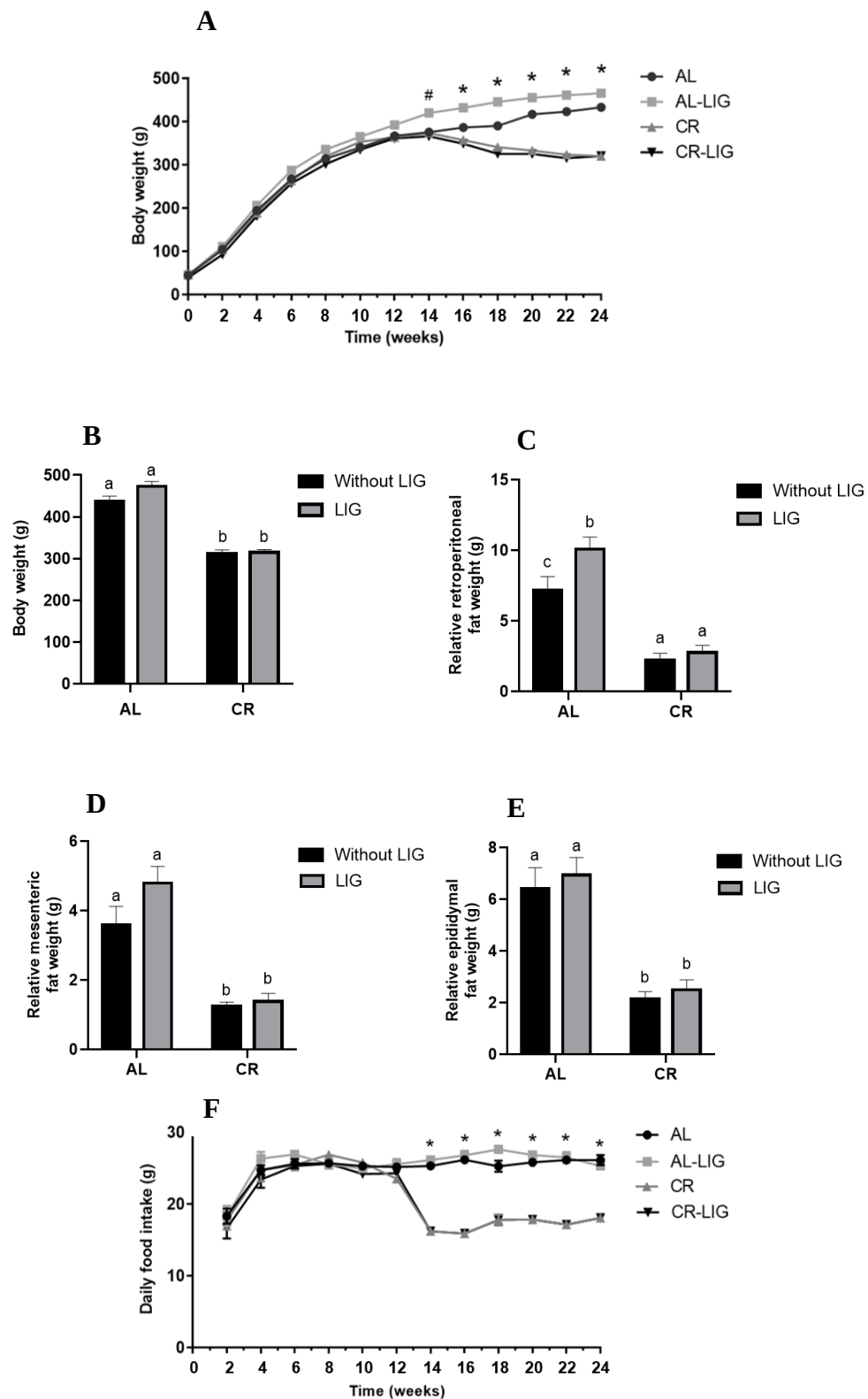


Figure 3.1.1. Effect of caloric restriction on biometric parameters. (A) Body weight curve; (B) Final body weight; (C), (D) and (E) Relative retroperitoneal, mesenteric and epididymal fat weight, respectively; and (F) Daily food intake. AL= *ad libitum* diet, AL-LIG = *ad libitum* diet with ligature-induced, CR = caloric restriction, CR-LIG = caloric restriction and ligature-induced. Different letters mean a significant difference ($p < 0.05$). Two-way ANOVA followed by Tukey's post-test. Data expresses mean $\pm$ SEM. Significant difference ($p < 0.05$) (#) between AL-LIG and CR groups and (*) between groups that received CR with AL groups.

Biochemical parameters

The glucose levels of the groups submitted to caloric restriction were shown to be influenced by the CR, with a reduction in plasma levels in comparison with *ad libitum* diet groups ($p < 0.05$). On the other hand, the concentrations of triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol were not altered by the diet ($p > 0.05$) (Figure 2).

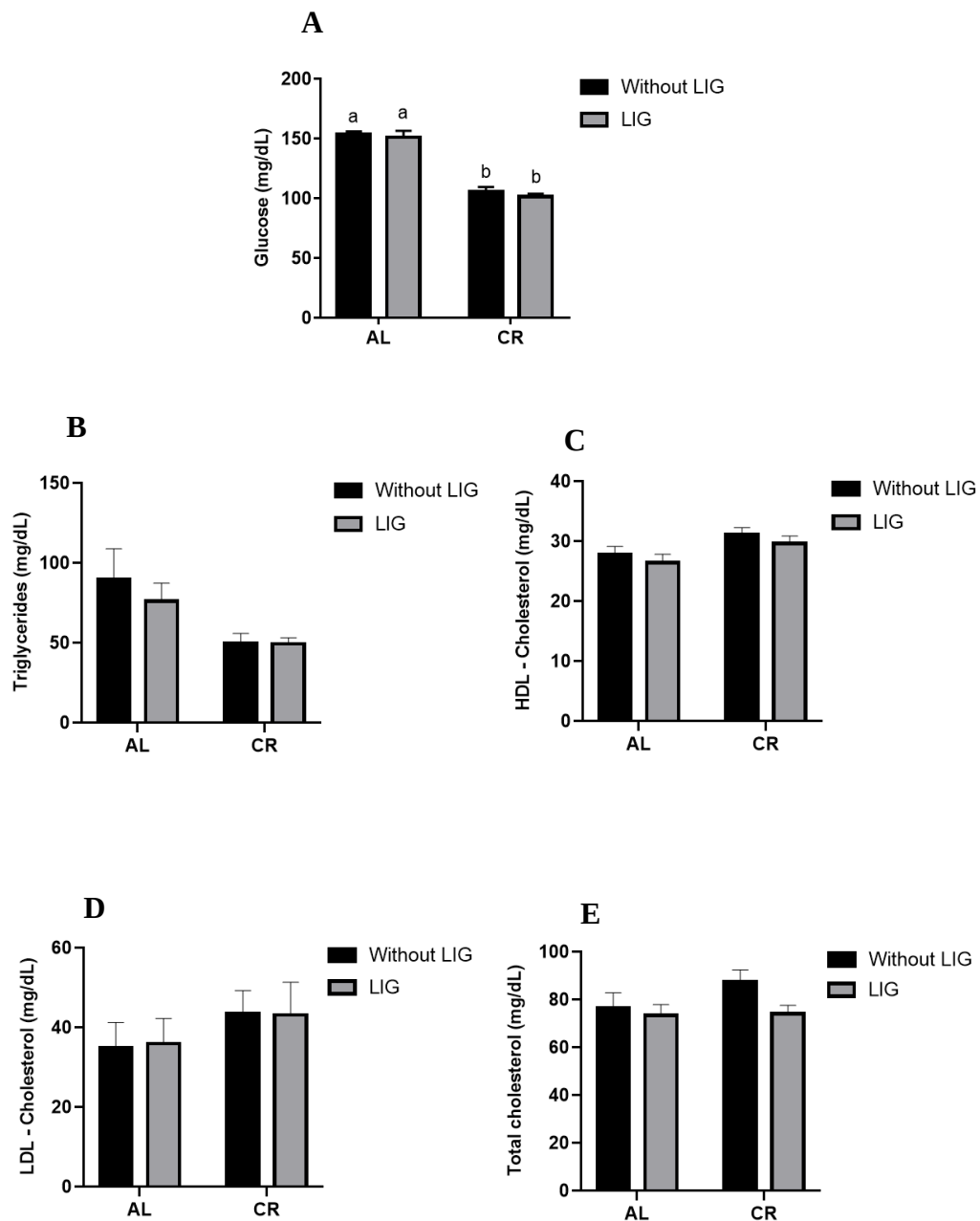


Figure 3.1.2. Blood concentration of biochemical parameters. (A) Glucose (B) Triglycerides (C) HDL-cholesterol (D) LDL-cholesterol (E) Total cholesterol. Different letters mean a significant difference ($p < 0.05$). Two-way ANOVA followed by Tukey's post-test. Data expresses mean $\pm$ SEM.

Clinical periodontal measures

As expected, AL-LIG and CR-LIG groups that had experimental periodontitis induction showed bleeding on probing (100% and 75%, respectively) and biofilm (100% and 80%, respectively), with no significant difference between groups ($p > 0.05$) (Figure 3). The highest scores of the gingival index parameter were predominant in the AL-LIG group compared to the CR-LIG rats ($p < 0.05$). However, there was no impact of the diet on the different mobility scores, when the two groups were compared ($p > 0.05$) (Table 2).

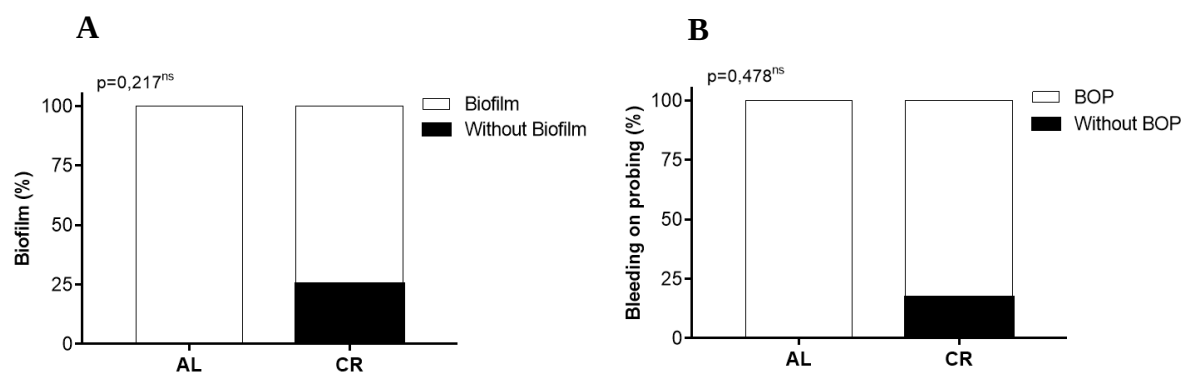


Figure 3.1.3. Comparison of clinical periodontal measures between groups with periodontitis ligature-induced. Data expressed as percentage. Chi-square test. (A) Biofilm ($p = 0.217$) (B) Bleeding on probing ($p = 0.478$). ns * indicates no significant difference.

Table 3.1.2. Distribution of frequency of the periodontal clinical parameters based in scores.

Parameter	Gingival index* ($p = 0.004$)				Mobility ($p = 0.091$)			
Score	0	1	2	3	0	1	2	3
AL-LIG	0	2	6	4	0	5	6	1
CR-LIG	1	8	3	0	4	5	2	1

*Significant differences between the groups. Mann-Whitney Test. AL-LIG: experimental group without caloric restriction and with ligature-induced. CR-LIG: experimental group with caloric restriction and with ligature-induced.

Alveolar bone loss

Caloric restriction significantly decreased alveolar bone loss, in the comparison between groups with experimental periodontitis ligature-induced ($p < 0.01$). In contrast, in the groups without experimental periodontitis, the alveolar bone loss did not show any significant change ($p > 0.05$). Both factors, caloric restriction and experimental periodontitis, had significant isolated

effects and, in addition, the interaction between the factors was significant ($p < 0.01$) (Figure 5).

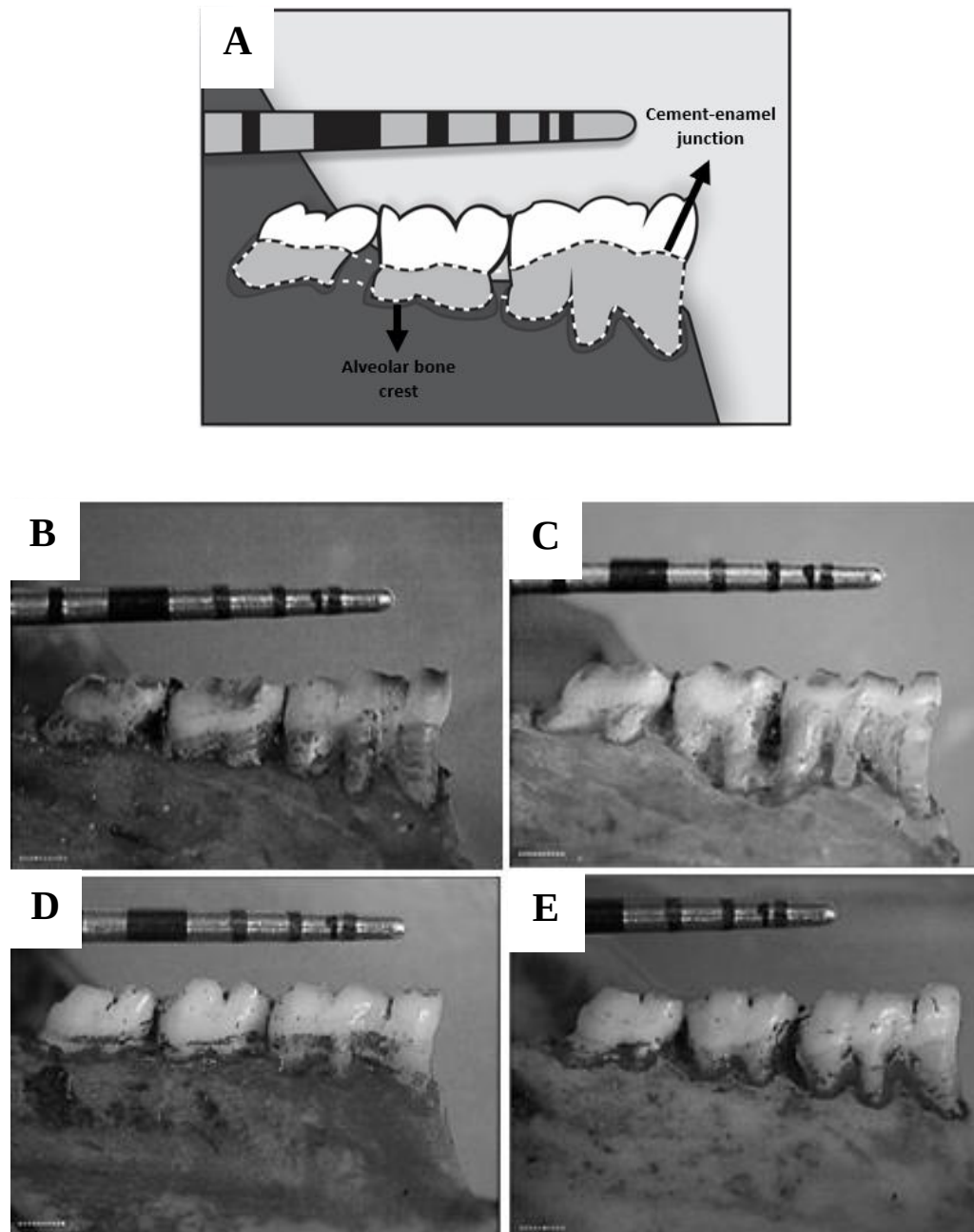


Figure 3.1.4. Diagram of macroscopic morphometric analysis and images of alveolar bone loss representing the experimental groups. (A) Parameters used for measurement of the total area of alveolar bone loss. (B) AL, without caloric restriction and without experimental periodontitis. (C) AL-LIG, without caloric restriction and with experimental periodontitis. (D) CR, with CR induction and without experimental periodontitis. (E) CR-LIG, with CR and with periodontitis ligature-induced.

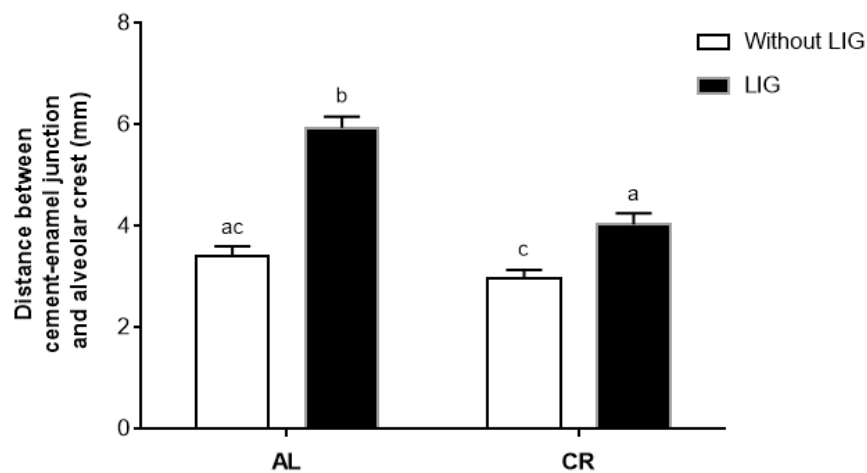


Figure 3.1.5. Mean (EPM) of alveolar bone loss values (mm) of rats submitted to caloric restriction (CR) with and without periodontitis ligature-induced (LIG) in the left mandibular first molar. Calorie restriction factor, $p < 0.0001$ (significant difference); Ligature-induced factor, $p < 0.0001$ (significant difference); Interaction of factors (calorie restriction with ligature), $p = 0.0008$ (significant difference). Different letters means significant differences ($p < 0.01$) between groups (two-way ANOVA with Tukey's post-test)

Levels of bone biomarkers

Blood concentrations of calcium and alkaline phosphatase were determined, as bone tissue biomarkers, to investigate whether induction of caloric restriction could affect the levels of these mediators. For both, no significant differences were found between groups ($p > 0.05$) (Figure 6).

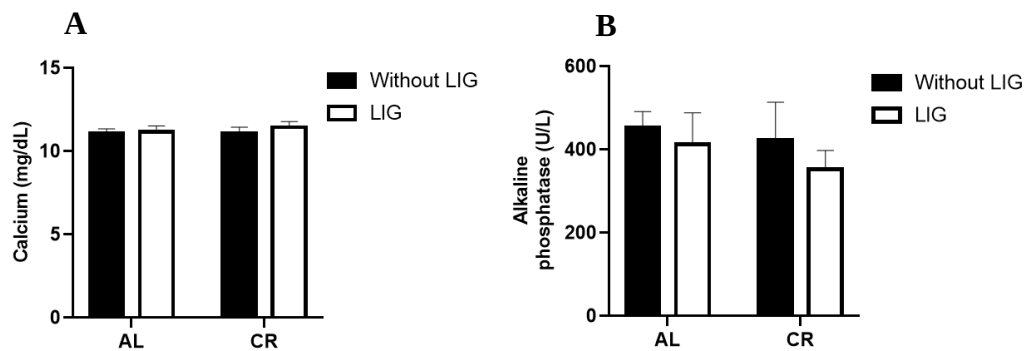


Figure 3.1.6. Effect of caloric restriction on bone tissue biomarkers (A) Calcium, and (B) Alkaline phosphatase. Two-way ANOVA, followed by Tukey's post-test. Data expressed as mean $\pm$ SEM. No significant difference between the experimental groups.

Discussion

In this study, we demonstrated that the values of alveolar bone loss were reduced in the presence of 30% caloric restriction, with experimental periodontitis ligature-induced in rats. This pioneer finding suggests a robust influence of caloric restriction on local bone metabolism under periodontitis, which reflects the main outcome of the disease, bone resorption, being considered the cause of tooth loss in adults [1].

Among the evidences about the benefits of caloric restriction, mainly related to the increase in lifespan and the prevention of inflammatory conditions, there are few reports about the interaction with oral diseases, such as periodontitis. Although still scarce, studies on this topic have shown a strong impact of the presence of caloric restriction on the reduction of the inflammatory condition, as a consequence of its major change in cellular and molecular aspects, such as less transcription and expression of inflammatory cytokines (e.g. TNF-alpha and interleukins) [30,31]. The hypothesis of our study fills the gap on the influence of 30% caloric restriction on alveolar bone loss, after experimental periodontitis ligature-induced in rats.

Caloric restriction of 30% has been described as related to the reduction in mortality rates in *Rhesus* monkeys, increased longevity, improved health, such as reduction of risk factors for atherosclerosis [6,7]. In addition, the literature reports improvement in periodontal clinical measures after 30% caloric restriction [16,32]. Considering this findings, the 30% calorie restriction was chosen as the method in our study.

The experimental periodontitis ligature-induced method is well established and considered a worldwide proposed model to promote the accumulation of biofilm and the consequent inflammatory response, by placing a ligature thread around the cervix region of the molars [4,33]. The investigation of experimental periodontitis was considered the focus of the study due to the increasing relevance of this oral disease, in a worldwide context, due to the high reported prevalence rates [12]. In addition to complex nature, periodontitis is associated with an immunoinflammatory response, which can have systemic repercussions [34,35]. Additionally, it can be strongly modulated by behavioral, genetic or nutritional factors, such as dietary therapies [17,36]. Caloric restriction had a great influence on experimental periodontitis, as our findings demonstrated a significant decrease in alveolar bone loss, by analyzing the distance between the cemento-enamel junction and the bone crest of the molars, a established method in the literature [4,37]. These findings are in agreement with others studies, which found promising results after caloric restriction in male monkeys, in which diet therapy played a key

role in inflammation, with a decrease in the release of antibodies (IgG) and levels of interleukin 8 (IL- 8). Additionally, lower values were found after caloric restriction, for the clinical periodontal measures of probing depth and clinical attachment loss [16].

Caloric restriction has been widely researched and is characterized as one of the major interventions for increasing longevity and improving the immunoinflammatory profile in various diseases, such as cancer and diabetes [7,18,38-40] . Aging is accompanied by an increase in oxidative damage, an inflammatory process and consequent dysfunction and loss of cellular homeostasis [13]. Cellular damage due to increased oxidative stress is caused mainly by the accumulation of reactive oxygen species (ROS) and free radicals. This production and the increase in ROS has been one of the main brands related to the aging process [41]. This mechanism causes damage to important cellular components, such as genetic material, macromolecules and lipids.

The theory to explain the mechanism involved between caloric restriction and increased longevity with consequent disease prevention, is based on the reduction of oxidative stress through enzymatic activity and intracellular signaling pathways [13]. ROS are synthesized as bioproducts of cellular respiration, approximately from about 90% of the mitochondria. The inactivation of ROS is regulated by antioxidant enzymes, such as superoxide dismutases (SOD) and then, the intracellular microenvironment remains in balance. The imbalance between this ROS production and cell antioxidant capacity leads to oxidative stress. This dynamic in balance occurs constantly in the organism, however with biological aging, the amount of ROS becomes in excess in the cell [41]. The mechanism to contain this oxidative increase is called the antioxidant system, composed of enzymes such as SOD and sirtuins (SIRT's), which are deacetylase proteins, widely researched in the aging process [9,42]. SIRT's, especially SIRT1 and SIRT3, have been heavily studied for their important role in caloric restriction [9,41]. They are responsible for activating the expression of antioxidant enzyme genes and inactivating signaling pathways, such as NF- κ B, with a consequent reduction in the inflammatory response [43,44]. In addition, acts on the maintenance and repair proteins of genetic material. In caloric restriction, due to energy deprivation, there is an increase in the levels of NAD + in the intracellular environment, thus increasing the expression of the genes encoding sirtuins [14, 45]. SIRT1 is located primarily between the nucleus and the cytosol and is related to the inhibition of signaling pathways that lead to the inflammatory response [43]. While SIRT3, present in the mitochondria, is associated with the process of increasing the antioxidant capacity by acting together with superoxide dismutase (SOD) and is associated with the regulation of

glutathione, the main antioxidant molecule [9,41,46]. Besides, SIRT-3 plays a role in maintaining bone homeostasis, by inhibiting osteoclast differentiation and consequent interference with its cell transcription factor [47].

This hypothesis of mechanism allows to explain the effects of reduction of the alveolar bone loss in the context of experimental periodontitis, because the periodontitis presents the main activation of the NF- κ B signaling pathway, which results in the synthesis and secretion of pro-inflammatory cytokines, such as TNF-alpha and IL-1 β , leading to the process of bone resorption through osteoclast activation and consequent bone loss [44,48-50]. Therefore, the effect of caloric restriction on alveolar bone loss in rats with experimental periodontitis is more evident and through our findings and mechanisms proposed in the literature. Additionally, the data found for clinical periodontal measures converge with the literature, in which a significant decrease was observed in the modified gingival index parameter, in animals submitted to caloric restriction, compared to the *ad libitum* group [32].

Regarding the biometric data used, such evaluations proves the efficiency of caloric restriction, through the decrease in the parameters of body weight, weight gain, adiposity index, Lee index and abdominal circumference. These findings corroborate with the literature, where research indicates decreased body weight and adipose tissue after caloric restriction [7,45,51]. The accumulation of abdominal fat deposits has been strongly associated with insulin resistance, systemic inflammation, cancer and lower adiponectin values [51]. Interestingly, adiponectin is also involved in calorie restriction, being reported to have overexpression in CR related to longevity [45]. Additionally, adipose tissue is one of the main sources of production of pro-inflammatory mediators, called adipokines, such as TNF-alpha and IL-1 [16]. In the present study, the decrease in adipose tissue, demonstrated by the reduction in the adiposity index, suggests a decrease in the production and release of adipokines and, therefore, a reduction in inflammatory mediators, which may have an influence on inflammatory diseases, such as periodontitis [16]. Caloric restriction also results in metabolic and transcriptional reprogramming of adipose tissue, which reduces adiposity by altering gene expression, and it is globally accepted that the peroxisome proliferator-activated receptor γ (PPAR γ) is primarily responsible for this regulation [52].

Interestingly, the literature shows that CR has a powerful protective effect against inflammatory conditions, such as insulin resistance, which generally increases with advancing age [6,52]. In this sense, the significant decrease in the averages of the glycemic profile of animals submitted

to caloric restriction, suggests an influence after caloric restriction in the insulin signaling pathways. The immunological basis for this mechanism also consists of a reduction in oxidative stress, a reduction in NF- κ B and especially in the overexpression of SIRT-3, related in the literature to a decrease in glucose levels [53]. Additionally, SIRT-1 acts as a protective and regulating molecule against inflammation and metabolic syndrome. The literature demonstrates that inhibition of SIRT-1 results in metabolic imbalance [9,54]. In contrast, aging has been considered one of the main risk factors for the development of diabetes, through intracellular damage and consequent pathological vascular remodeling, mediated by endothelial dysfunction [55]. The other biochemical parameters, such as lipid profile, showed no significant difference, corroborating with study of Ding and collaborators (2017) [56]. The levels of calcium and alkaline phosphatase, assessed to check for possible changes in bone or nutritional tissue biomarkers, did not show significant differences between the groups, which probably suggests that there was no aspect of malnutrition after caloric restriction.

Conclusion

In summary, we demonstrated that caloric restriction reduces alveolar bone loss in ligature-induced periodontitis. Our results may be relevant to broaden the understanding of the role of caloric restriction in periodontitis, especially its effect on bone tissue, which had not yet been found in the literature. This relationship between periodontitis and systemic changes can contribute to the development of future strategies to modulate the main outcome of periodontitis, the alveolar bone loss.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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3.2 CALORIC RESTRICTION PREVENTS ALVEOLAR BONE LOSS IN EXPERIMENTAL PERIODONTITIS IN OBESE RATS

STATUS: PROCESSO DE SUBMISSÃO

REVISTA: LIFE SCIENCES

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Abstract

Aims: It has been shown that periodontitis, can be modified by systemic changes, including behavioral factors, such as diet. Caloric restriction is one of the dietary therapeutic strategies indicated for obesity. It is associated with several benefits, among them, modulation of the inflammatory response. The aim of this study was to verify whether caloric restriction in obese rats changes the progression of experimental ligature-induced periodontitis.

Main methods: Forty-eight Wistar rats were used for 24 weeks and initially fed with cafeteria diet during 12 weeks. The animals were divided into four groups according to the caloric restriction and experimental periodontitis. The cotton thread was placed around the mandibular first molars, for 15 days, before the end of the experiment. The rats submitted to caloric restriction received, from the 13th week of the experimental protocol, 70% of the food intake compared to the *ad libitum* animals of other study of the our research group. Alveolar bone loss was assessed using macroscopic morphometric analysis. Analyzes of clinical periodontal measures, biometrics, serum biomarkers and biochemical parameters were performed.

Key findings: Caloric restriction decreased the alveolar bone loss in the periodontitis group when compared to the group that received a cafeteria diet with periodontitis. Besides that, the results demonstrate the improvement in the glycemic profile, without prejudice to bone tissue biomarkers.

Significance: Based on the results obtained, caloric restriction reduces the progression of alveolar bone loss in rats with experimental periodontitis, in addition to presenting benefits in biometric data, decreasing both glycemic profile and clinical periodontal measures.

Key words: Obesity, Caloric restriction, Periodontitis

Introduction

The deleterial effects of obesity on the progression and severity of periodontitis have been reported in epidemiological and experimental studies [1-3]. Among the therapeutic methods indicated for reducing obesity, there are diets and exercise [4].

Caloric restriction (CR) is one of the most recommended strategies for reducing body weight, currently considered extremely efficient [5]. It is mainly related to the host responses to inflammatory processes, such as obesity and periodontitis. In addition, CR can act on the mechanisms for maintaining hormonal balance and systemic physiological functions, regulates

gene expression, reduces damage caused by oxidative stress and reduces the expression of inflammatory cytokines, such as tumor necrosis-alpha (TNF-alpha) and interleukin 6 (IL-6). These mechanisms have been suggested as the basis of the relationship between CR and periodontitis [6].

Actually, caloric restriction has been suggested to attenuate periodontal parameters and inflammatory molecules in human studies and animals submitted to experimental periodontitis ligature-induced [6,7]. However, the effect on alveolar bone tissue and the condition of the diet used as therapy for obesity, still needs to be elucidated. Experimental animal models have been used to evaluate the inflammatory, immunological and clinical processes of progression and severity of periodontitis, for the reason that studies in humans of progression of periodontal disease are considered extremely difficult to be evaluated due to the unpredictability of progression [4].

Periodontitis is characterized by immune and inflammatory responses in defense against bacterial colonization and involves the protective and supporting tissues of the tooth [8,9]. The inflammatory process in periodontitis can result in degradation of connective fibers and resorption of alveolar bone in a susceptible individual. The susceptibility of each individual can be explained due to differences in the immune response in the context of imbalance in periodontal tissues. This imbalance is marked by the consequent clinical implication of alveolar bone loss, mainly through the release of inflammatory cytokines. Based on the increase in the circulating level of inflammatory molecules, the association of periodontitis with systemic and behavioral factors, such as CR, has been reported [7].

Therefore, the aim of this study was to evaluate whether the effect of caloric restriction can be beneficial in alveolar bone loss in experimental periodontitis after obesity induction with the cafeteria diet method in rats.

Material and methods

Experimental design and animals treatment

Male Wistar Rats (*Rattus norvegicus albinus*) were obtained (State University of Ponta Grossa), the protocol was approved by the Ethics Committee on the Use of Animals (Protocol 12203/2017) and was in accordance with the ARRIVE (Animal Research: Reporting of *In vivo* experiments) guidelines and following the National Institutes of (NIH) guide for the care and

use of Laboratory animals [10,11]. The rats were housed in polypropylene cages, maintained at a controlled temperature ($\pm 23^{\circ}\text{C}$) and on a 12/12h light-dark cycle; fed a cafeteria diet without caloric restriction (CAF) or fed a cafeteria diet and 30% caloric restriction (CR), divided also according to the placement of periodontal ligature (CAF-LIG and CR-LIG). All animals had free access to water. For the sample calculation, the G* power 3.1 program (G * Power, Universität Düsseldorf, Düsseldorf, North Rhine-Westphalia, Germany) was used and it was found that for a power of 85% the number used should be 10 animals per group. Considering possible losses during the experimental process and possible methodological error during the research, n of 12 animals per group were used, divided into four groups.

Diet protocol and experimental periodontitis ligature-induced

In order to evaluate the effect of caloric restriction as a treatment, obesity was induced in animals by the cafeteria diet method. All animals, at the beginning, received the cafeteria diet, available 3 times/week. The components of this diet consisted of 3 combinations with different menus containing high-caloric foods, distributed over the 3 days of the week [12-14]. After 12 weeks, half of the animals were submitted to caloric restriction (CR) and the other half remained on the cafeteria diet (CAF), until the 24th week. From the 13th week, in the groups submitted to CR, the animals were fed with 70% of the amount ingested by the group that received an *ad libitum* diet from another study that followed in parallel, in our research group, following the diet protocol established in the literature [4,15]. The animals remained fed the caloric restriction or cafeteria diet until the end of the 24 weeks. On the 22nd week, the rats in the CAF-LIG and CR-LIG groups were anesthetized with intramuscular injection of 10% ketamine (90mg/kg) and 2% xylazine (10mg/kg) and received the cotton thread placement in the mandibular first molars, to induce experimental periodontitis, according to the protocol recommended in the literature [16,17]. The animals that did not receive the experimental periodontitis, remained only submitted to diets until the 24th week. Euthanasia and blood collection, hemimandibles and analysis of clinical periodontal measures were performed at the end of the 24-week experiment. Biometric data and adipose tissue were collected for analysis and proof of the effectiveness of the diets.

Biometric data

Body weight was measured twice a week and the graph of evolution of body weight was made to verify the increase or decrease in weight according to the diet provided. The naso-anal length was determined to calculate the Lee Index, together with the measurement of abdominal

circumference, according to Merigo et al (2013) [3]. The adipose tissue from the epididymal, mesenteric, retroperitoneal regions were removed and weighed to determine the adiposity index, as proposed in previous studies [18,19].

Clinical periodontal measures

Previously to the euthanasia, clinical periodontal analysis of the molars that received the ligatures and also of the adjacent periodontal tissue was performed based on four parameters: Biofilm (0-absent; 1-present); Bleeding on probing (BOP) (0-absent; 1-present); Mobility: 0-absent; 1-slight mobility (vestibular-palatal); 2-moderate mobility (vestibular-palatal and mesial-distal); 3-severe mobility (vertical, movement inside and outside the tooth socket); Gingival index (0-normal gingiva; 1-mild inflammation, mild edema, slight color change and no bleeding; 2-moderate inflammation, edema, redness and bleeding; 3-severe inflammation, extreme redness, presence of ulcer, edema and severe bleeding). To perform this analysis, the animals were initially weighed and sedated with intraperitoneal (IP) anesthesia with 10% ketamine solution (90 mg/kg) and 2% xylazine muscle relaxant (10 mg/kg). Subsequently, the animals were positioned on the operating table and with the help of a spatula and probe, the clinical analysis of periodontal measures was performed [20-22].

Biochemical parameters

After experiment, the blood was collected, centrifuged and stored at -80°C. Glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride levels were determined using commercial kits following the manufacturer's instructions, using the Wiener CT300i analyzer equipment.

Macroscopic morphometric analysis

To assess the extent of alveolar bone loss, measurements were made on the left hemimandibles. After euthanasia, the mandible of each animal was removed, boiled during 30 minutes, immersed in hydrogen peroxide solution (3%) for 12 hours and stained with methylene blue (1%) to differentiate between the tooth and bone tissue, fixed on a support and photographed. The images were made for the macroscopic analysis of the mandibles with a Zeiss stereoscopic loupe with a 20x magnification. The digital images were analyzed for alveolar bone loss with the Image J Software. The measurements were made on the lingual surface. In this procedure, the area (mm²) corresponding to the exposed root of the molars was calculated. All acquired images were compared to an already known area (1.0x1.0mm²) [23].

Bone biomarkers

The measurement of biomarkers bone tissue, calcium and alkaline phosphatase, was performed using commercial kits, following the manufacturer's instructions, and analyzed using the CT300i Wiener equipment.

Statistical analysis

Data regarding the biofilm and bleeding on probing, clinical periodontal measures, were analyzed using the Chi-square test and the mobility and gingival index data were analyzed using the Mann-Whitney test, evaluated by the Statistical Package for the Social Sciences - SPSS Program, version for Windows. The remaining data were analyzed using analysis of variance (two-way ANOVA) with Tukey's post-test, used for statistical comparisons (GraphPad Prisma software version 8.2.1, San Diego, CA, USA). The data were expressed as mean $\pm$ SEM. The level of significance used was 5% ($p < 0.05$).

Results

Biometric parameters

The effectiveness of caloric restriction was evidenced by the significant reduction in body weight of the CR groups compared to the obese animals ($p < 0.05$). The rats submitted to CR had a 42% decrease in body weight compared to the animals that received a cafeteria diet. The values of Lee index and abdominal circumference were also significantly reduced compared to groups with a high calorie diet ($p < 0.05$). Caloric restriction reduced the adiposity index 77% in CR animals compared to obese groups. (Table 1).

Table 3.2.1. Biometric parameters in the experimental groups submitted a caloric restriction and experimental periodontitis.

	CAF	CAF-LIG	CR	CR-LIG
Initial body weight (g)	36 ± 1.93	36 ± 1.20	34 ± 1.37	34 ± 2.15
Final body weight (g)	571 ± 14.14 ^a	555 ± 18.81 ^a	334 ± 11.69 ^b	321 ± 9.77 ^b
Body weight gain (g)	535 ± 13.93 ^a	519 ± 18.27 ^a	300 ± 11.19 ^b	287 ± 9.59 ^b
Adiposity index (%)	8.5 ± 0.73 ^a	9.0 ± 0.63 ^a	2.1 ± 0.24 ^b	2.0 ± 0.20 ^b
Naso-anal lenght (cm)	27 ± 0.40	26 ± 0.34	25 ± 0.39	25 ± 0.26
Lee index (g/cm)	313 ± 3.75 ^a	315 ± 4.14 ^a	276 ± 5.07 ^b	277 ± 2.99 ^b
Abdominal circumference (cm)	23 ± 0.47 ^a	23 ± 0.45 ^a	17 ± 0.20 ^b	17 ± 0.33 ^b

Data represented as mean ± SEM. ANOVA-two way, Tukey's post-test. Different letters indicated $p < 0.05$.

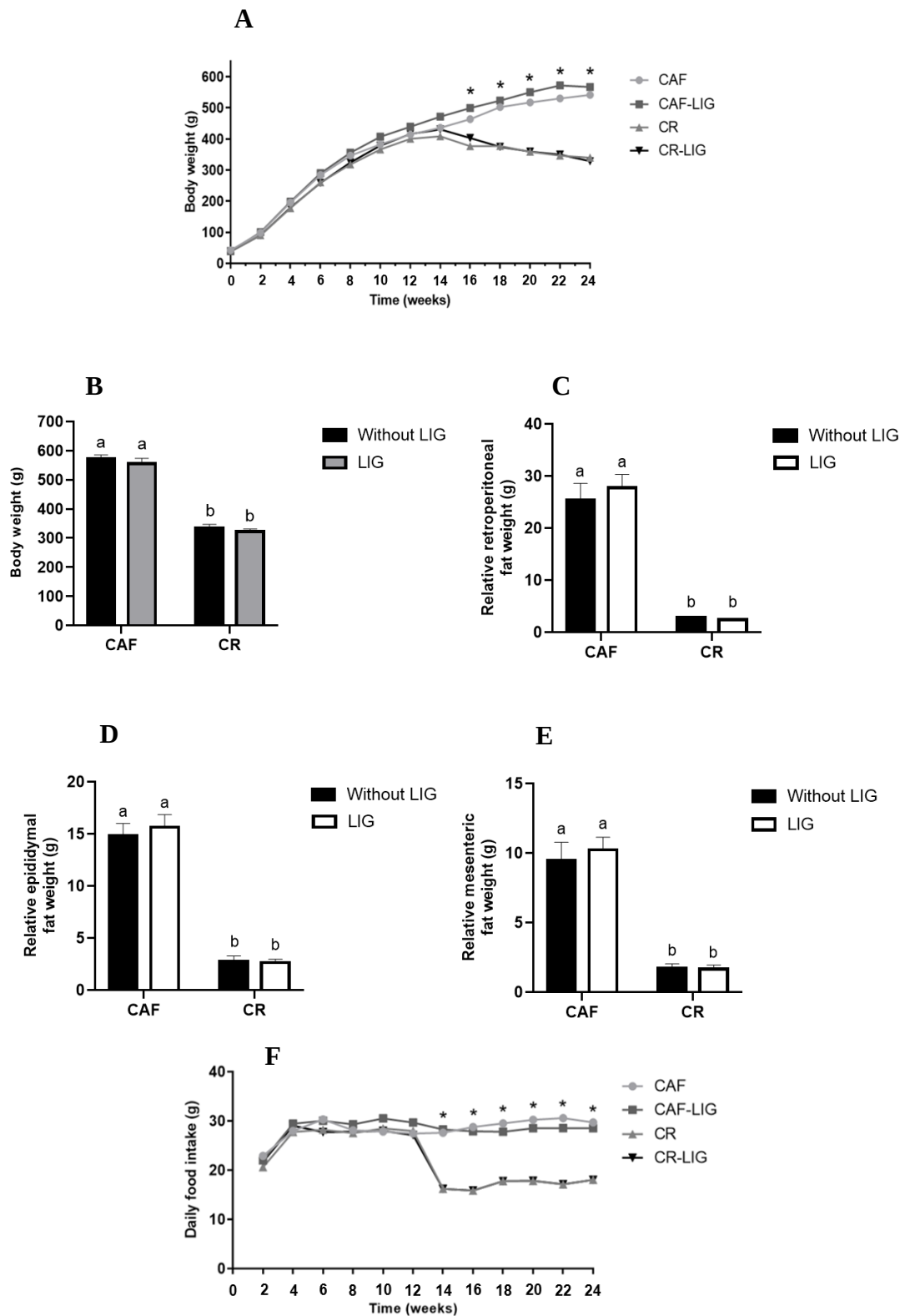


Figure 3.2.1. Effect of caloric restriction on biometric parameters. (A) Body weight curve; (B) Final body weight; (C), (D) and (E) Relative retroperitoneal, mesenteric and epididymal fat weight, respectively; and (F) Daily food intake. CAF= cafeteria diet, CAF-LIG = cafeteria diet with experimental periodontitis ligature-induced, CR= caloric restriction, CR-LIG = caloric restriction and periodontitis ligature-induced. Different letters means a significant difference ($p < 0.05$). Two-way ANOVA followed by Tukey's post-test. Data expresses mean $\pm$ SEM. *Significant difference ($p < 0.05$) between groups that received CR with CAF groups.

Biochemical parameters

Caloric restriction reduced glucose concentrations, with a significant decrease compared to obese rats. The other biochemical parameters of triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol showed similar values, with no significant differences between groups ($p > 0.05$).

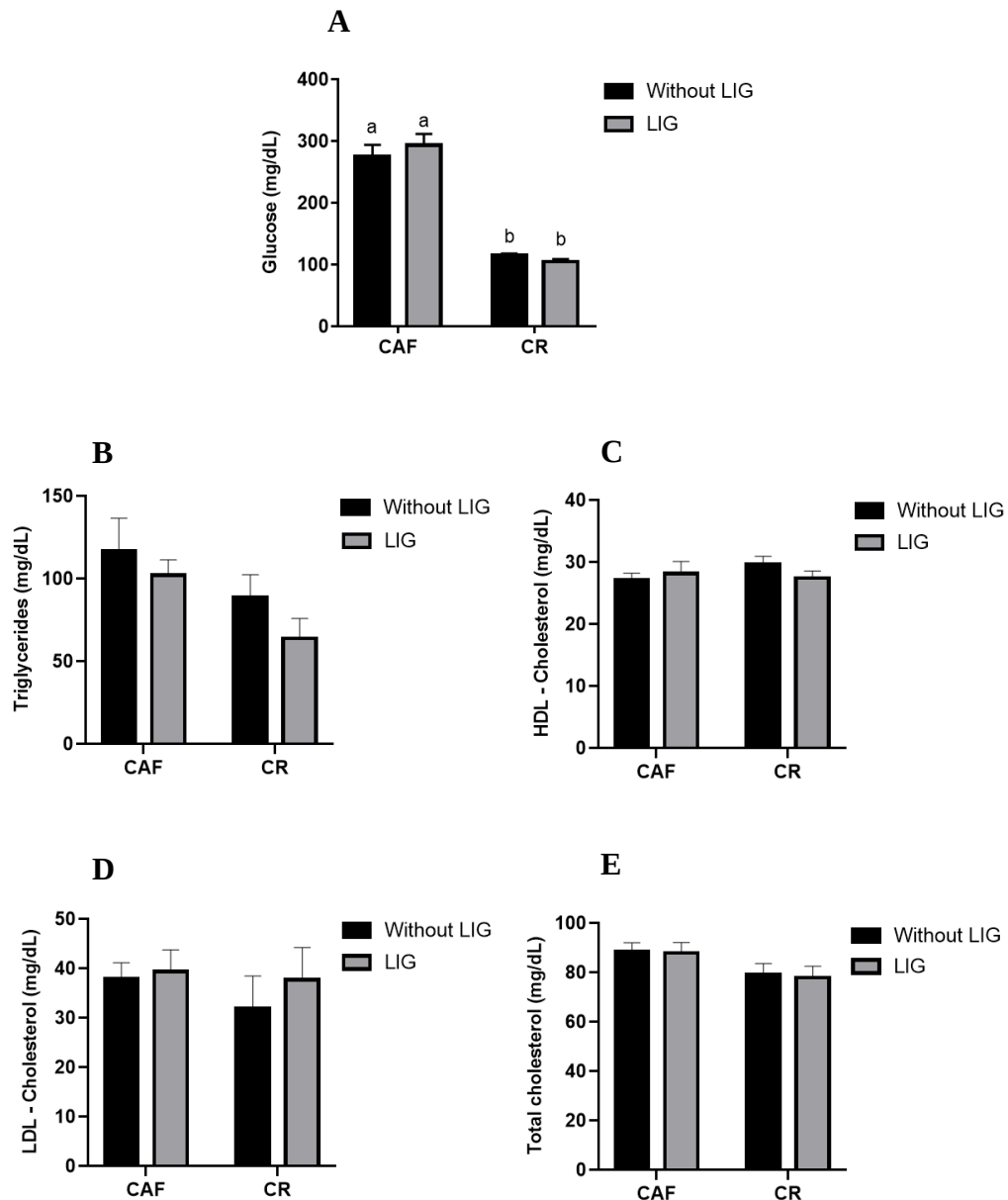


Figure 3.2.2. Blood concentration of (A) Glucose (B) Triglycerides (C) HDL-cholesterol (D) LDL-cholesterol (E) Total cholesterol. Different letters means a significant difference ($p < 0.05$). Two-way ANOVA followed by Tukey's post-test. Data expresses mean $\pm$ SEM.

Clinical periodontal measures

Bleeding on probing was shown to be significantly increased in groups without caloric restriction, in which 100% of the rats were detected with bleeding, while approximately 70% of the animals with CR showed no BOP. However, for the biofilm parameter, no significant difference was found, and all animals in both groups were detected with the presence of biofilm. Beneficially, CR had a significant effect on the parameters of gingival index ($p < 0.001$) and mobility ($p = 0.001$), when comparing groups with and without induction of CR (Figure 3 and Table 2).

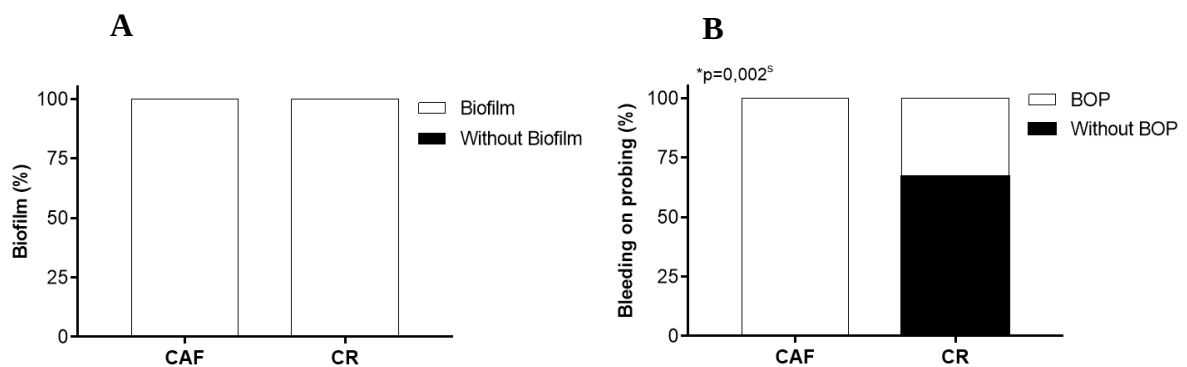


Figure 3.2.3. Comparison of clinical periodontal measures between groups that received ligature-induced experimental periodontitis, expressed as a percentage. Chi-square test. (A) Biofilm (B) Bleeding on probing ($p = 0.002$).

Table 3.2.2. Distribution of frequency of the periodontal clinical parameters based in scores.

Parameter	Gingival index* ($p < 0.001$)				Mobility* ($p = 0.001$)			
Score	0	1	2	3	0	1	2	3
CAF-LIG	0	2	8	2	0	5	4	3
CR-LIG	1	10	1	0	7	5	0	0

*Significant differences between the groups. Mann-Whitney Test. CAF-LIG: cafeteria diet experimental group without caloric restriction and with ligature-induced. CR-LIG: cafeteria diet experimental group with 30% caloric restriction and with ligature-induced.

Macroscopic morphometric – alveolar bone loss

The effect of CR was significant in reducing the values of alveolar bone loss, when comparing the groups that received ligature to induce experimental periodontitis ($p < 0.01$). Rats that were not induced to experimental periodontitis had no significant changes in alveolar bone loss after induction of CR ($p > 0.05$). Both in the analysis of the isolated factors ($p < 0.001$) and in the interaction between the factors ($p < 0.008$), the effect of the three conditions evaluated was significant (Figure 5).

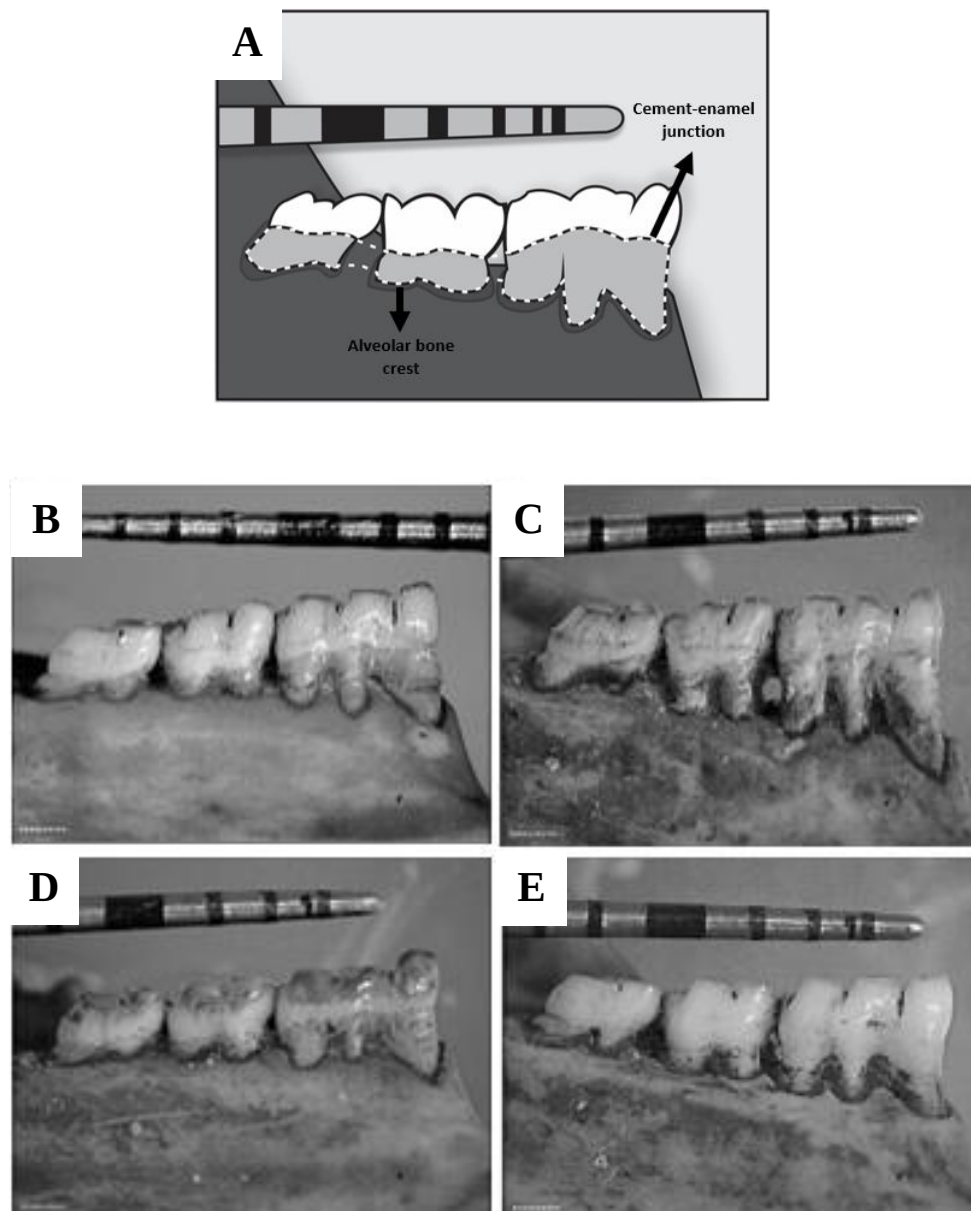


Figure 3.2.4. Diagram of macroscopic morphometric analysis and images of alveolar bone loss representing the experimental groups. (A) Parameters used for measurement of the total area of alveolar bone loss. (B) CAF, cafeteria diet. (C) CAF-LIG, without caloric restriction and with experimental periodontitis. (D) CR, with CR induction and without ligature. (E) CR-LIG, with CR and with experimental periodontitis ligature-induced.

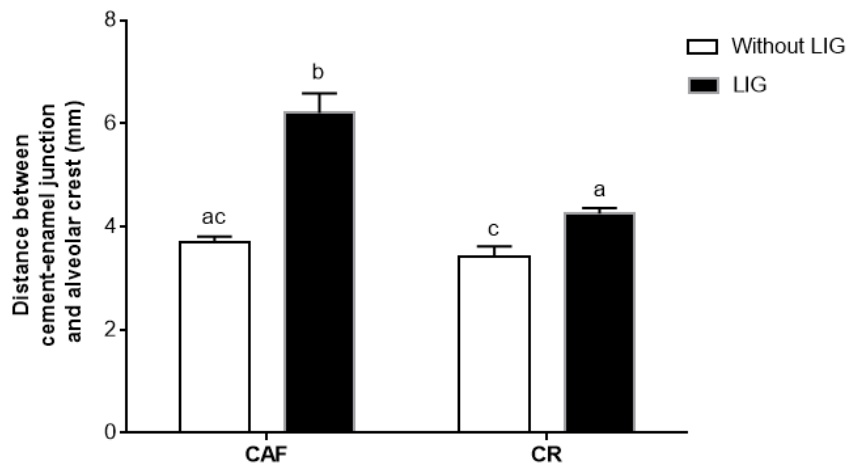


Figure 3.2.5. Mean (EPM) of alveolar bone loss values (mm) of rats submitted to caloric restriction (CR) with and without experimental periodontitis ligature-induced (LIG) in the mandibular first molar. Caloric restriction factor, $p < 0.0001$ (significant difference); Ligature factor, $p < 0.0001$ (significant difference); Interaction of factors (caloric restriction with ligature), $p = 0.0008$ (significant difference). Different letters means significant differences ($p < 0.01$) between groups (two-way ANOVA with Tukey's post-test)

Bone biomarkers

The blood collected showed no significant changes in calcium and alkaline phosphatase levels when comparing the experimental groups (Figure 6).

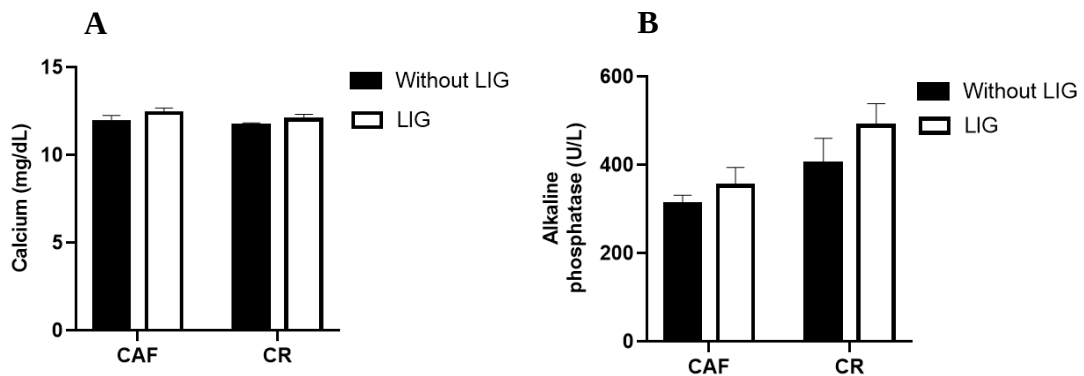


Figure 3.2.6. Effect of caloric restriction on bone tissue biomarkers (A) Calcium (B) Alkaline phosphatase. Two-way ANOVA, followed by Tukey's post-test. Data represents mean $\pm$ SEM. No significant difference between the experimental groups.

Discussion

Caloric restriction is worldwide used as a therapy for obesity and it is established in the literature that the reduction of caloric intake in the percentage between 20-40%, without malnutrition, results in several benefits, widely researched, such as the reduction of body weight and improvement in the health condition [24-29]. However, no study has tested the role of caloric restriction on bone tissue in oral conditions, such as periodontitis. In this study, Wistar rats were submitted to a cafeteria diet and subsequently to a caloric restriction of 30%, to analyze alveolar bone loss in the context of experimental periodontitis. Our results reveal a significant reduction in alveolar bone loss after caloric restriction for obesity therapy in rats with experimental periodontitis. These data demonstrate a pioneering and important finding in the impact and influence of caloric restriction on bone tissue, under the condition of ligature-induced periodontitis.

Periodontitis is an inflammatory condition that affects the protective and supporting tissues of the tooth, including the alveolar bone and that, if untreated, may lead to tooth loss [30]. The method of ligature-induced periodontitis is currently well understood and widely used [16,22,31]. The fact that the presence of experimental periodontitis has been significantly influenced by caloric restriction, by decreasing the values of alveolar bone loss, interestingly reinforces the key role of caloric restriction on the body's inflammatory and immune response. The literature shows that obese patients, submitted to caloric restriction, showed a significant decrease in gingival fluid biomarkers, such as interleukin IL-1 β , matrix metalloproteinases (MMP-8 and MMP-9) [7]. The cafeteria diet method for obesity induction is considered an experimental diet model that highly reflects the prevalent consumption of unhealthy foods in actual society and is a method proposed and established in the literature, considered efficient to result in obese rats [32-35]. Obesity has been associated with a low degree of chronic inflammation, which leads to the release, by adipocytes, of inflammatory mediators called adipokines, such as TNF-alpha and IL-6 [36-38]. Consequently, the increase in inflammation is accompanied by oxidative imbalance, with a greater possibility of damage to cellular structures, genetic material, lipids and proteins [33,39-41]. This imbalance in cellular processes and increased synthesis of pro-inflammatory cytokines trigger the overexpression of molecules related to the NF- κ B signaling pathway, such as the nucleus kappa beta activator receptor ligand (RANKL), by osteoblasts that act in differentiation and activation of the osteoclasts, resulting in alveolar bone resorption. The RANK / RANKL / OPG system is decisive in alveolar bone resorption, in this process bone cells activated by pro-inflammatory cytokines secrete a greater

amount of RANKL. While the RANK membrane receptor is found in osteoclast precursor cells, responsible for bone resorption. In this mechanism, an osteoclastogenesis regulating molecule, osteoprotegerin (OPG) has an affinity for RANKL and, in large quantities in the cell, it helps in bone balance and maintenance [42]. In periodontitis, this imbalance and accumulation of RANKL, with less OPG production and secretion, makes the disease progression mainly resulting in alveolar bone loss [43].

In contrast, caloric restriction alters intracellular signaling pathways, such as NF- κ B and thereby decreases the release of pro-inflammatory cytokines, triggering the overexpression of regulatory molecules, such as SIRT-1 (sirtuin-1) [44-46]. SIRT-1 belongs to enzymes called deacetylases, of the sirtuin family and among the main beneficial functions are: modulation of genetic stability, regulation of cell survival, endocrine signaling and gene transcription [45,47]. Sirtuins, more specifically SIRT-1 and SIRT-3, are involved in the processes of caloric restriction, more widely described and associated with health benefits [48-50]. With the regulation of the oxidative and inflammatory mechanism in cells, are greater the chances of preventing and decreasing inflammatory diseases, such as obesity and periodontitis [37]. In periodontitis, this mechanism suggests a local modification of the local immunoinflammatory response, with reduction of cellular damage, improvement in the oxidative and inflammatory profile, regulation of the NF- κ B pathway and consequent decrease in the release of inflammatory mediators, such as TNF- α and IL-1 β , characteristic of periodontitis [51]. In this sense, through the lower activation of bone resorption, it is suggested a decrease in the progression and severity of periodontitis, represented in our study by the significantly reduced values of alveolar bone loss after caloric restriction.

Regarding periodontal clinical parameters, mobility, bleeding and gingival index showed significant differences between obese groups and those who received caloric restriction. The findings of attenuated periodontal clinical parameters after caloric restriction suggest that the mechanism associated with the process of alveolar bone loss may be associated with the clinical manifestations of periodontitis. In addition, all animals tested presented biofilm, which indicates that the ligature-induced periodontitis method resulted in the expected microbiological accumulation. There are still no studies in the literature that evaluated periodontal clinical parameters in obese animals, submitted to both caloric restriction and experimental periodontitis. Therefore, our data suggest an important scientific basis for future research on this topic.

The caloric restriction markedly reduced the values of body weight and other biometric parameters, such as adiposity index, abdominal circumference, Lee index and body weight gain, in comparison with the groups that were not submitted to caloric restriction. Our data converge with the literature, in which both obese patients and animals showed a reduction in body weight after caloric restriction [7,47]. Interestingly, patients from a randomized clinical study also showed a decrease in body weight and glucose level, after caloric restriction in obesity. Converging with our findings, the clinical study also found no significant difference in the parameters of triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol [26]. Additionally, the literature is established regarding the reduction of body weight and blood glucose levels, after caloric restriction [52]. SIRT-1 regulates several biological processes via deacetylation of the main metabolic molecules including NF- κ B, Forkhead box protein O1 (FoxO1) and peroxisome proliferator activated receptor (PPAR γ). The deacetylation of these molecules results in an increase in the concentration of adiponectin, decreasing inflammation and increased sensitivity to insulin [53]. In contrast to our finding, Ding et al (2017) observed a significant difference in the parameters of triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol after caloric restriction [54].

Other data from our study, such as calcium and alkaline phosphatase levels, did not show significant difference after induction of caloric restriction. This fact suggests that the 30% caloric restriction did not cause malnutrition. Alkaline phosphatase has been postulated as a prognostic predictor and adjunct in routine methods to determine periodontitis activity. Alkaline phosphatase is a calcium and phosphate binding glycoprotein, present in various organs and tissues, mainly in bone tissue. It is produced by several cells, such as polymorphonuclear leukocytes, macrophages, osteoblasts, present in the periodontium [55,56]. Therefore, it is an important indicator of osteoblastic activity and therefore, it was also the target of investigation in our study.

Conclusion

The caloric restriction effectively reduced the alveolar bone loss and attenuated the clinical periodontal measures in the CR-LIG group, after cafeteria diet. Moreover, this therapy also improved the biometric parameters and glycemic profile. Considering our findings, we demonstrate the important role of caloric restriction in inflammatory conditions, mainly the periodontitis and potential regulation of alveolar bone metabolism.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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4 DISCUSSÃO

A restrição calórica tem sido mundialmente utilizada e estabelecida na literatura como a redução da ingestão calórica na porcentagem entre 20-40%, sem má-nutrição, resulta em vários benefícios, amplamente pesquisados, como a redução do peso corporal e melhora no quadro de saúde (DUTRA *et al.*, 2012; JENSEN *et al.*, 2014; RUGGENENTI *et al.*, 2017; OTT *et al.*, 2017; FRANCOIS *et al.*, 2018; WANG *et al.*, 2018). Entretanto, nenhum estudo testou o papel da restrição calórica sobre o tecido ósseo, mais especificamente na perda óssea alveolar, na periodontite. Neste trabalho demonstramos que os valores de perda óssea alveolar se apresentaram diminuídos na presença de 30% de restrição calórica, com indução de periodontite experimental em ratos. Esse achado pioneiro sugere uma forte influência da restrição calórica no metabolismo ósseo local sob o quadro de periodontite experimental, o que reflete no principal desfecho da doença, a reabsorção óssea, sendo considerada uma das causas de perda dentária em adultos (KASSEBAUM *et al.*, 2014).

A periodontite é uma condição inflamatória que afeta os tecidos de proteção e suporte do dente, incluindo o osso alveolar e que, se não tratada, leva a perda dentária (IZUORA *et al.*, 2016). O método de indução da periodontite experimental por ligadura é atualmente bem elucidado e amplamente utilizado (VARGAS-SANCHEZ *et al.*, 2017; ANDRADE *et al.*, 2019; TOKER *et al.*, 2019). O fato da presença da periodontite experimental ter sido significativamente influenciada pela restrição calórica, por meio da diminuição dos valores de perda óssea alveolar, interessantemente reforça o papel-chave da restrição calórica sobre a resposta inflamatória e imunológica do organismo, além do seu efeito na perda óssea frente ao quadro de obesidade tratada com terapia dietética. A periodontite pode ser fortemente modulada por fatores comportamentais, genéticos ou nutricionais, como as terapias dietéticas (GONZALEZ *et al.*, 2012; BASU, MASEK E EBERSOLE, 2018). A grande influência da restrição calórica em nossos achados, revelou uma diminuição significativa na perda óssea alveolar, por meio da análise da distância entre a junção cimento-esmalte e a crista óssea dos molares, método de análise estabelecido na literatura (VARGAS-SANCHEZ *et al.*, 2017; SOUZA *et al.*, 2018). Esses achados estão de acordo com estudos, que encontraram resultados promissores após restrição calórica em macacos machos, no qual a terapia dietética exerceu papel-chave na inflamação, com a diminuição da liberação de anticorpos (IgG) e dos níveis de interleucina 8 (IL-8). Adicionalmente, foram encontrados menores valores após restrição calórica, nos parâmetros clínicos periodontais de profundidade de sondagem e perda clínica de inserção (REYNOLDS *et al.*, 2009). A literatura demonstra que pacientes obesos, submetidos

a restrição calórica, apresentaram diminuição significativa nos biomarcadores do fluido gengival, como IL-1 beta, metaloproteinases de matriz (MMP-8 e MMP-9) (PARK *et al.*, 2015). O método de dieta de cafeteria para indução da obesidade é considerado um modelo de dieta experimental que altamente reflete o consumo prevalente de alimentos não saudáveis e é um método proposto e estabelecido na literatura, considerado eficiente para resultar em ratos obesos (SAMPEY *et al.*, 2011; OLIVA *et al.*, 2017; BORTOLIN 2018; BUYUKDERE, GULEC, AKYOL, 2019).

A restrição calórica tem sido amplamente pesquisada, pelo fato de ser caracterizada como uma das principais intervenções para aumento da longevidade e melhora do perfil imunoinflamatório em várias doenças, como câncer e diabetes (COLMAN *et al.*, 2014; MATTISON *et al.*, 2017; DUSZKA *et al.*, 2018; RHOADS *et al.*, 2018). O envelhecimento é acompanhado pelo aumento de danos oxidativos, processo inflamatório e consequente disfunção e perda da homeostase celular (QIU *et al.*, 2010). Os danos celulares devido ao aumento do estresse oxidativo, são causados principalmente pelo acúmulo de espécies reativas de oxigênio (ERO's) e radicais livres. Essa produção e o aumento das ERO's têm sido uma das principais marcas relacionadas ao processo de envelhecimento (KINCAID E BOSSY-WETZEL 2013). Esse desequilíbrio oxidativo também é encontrado na obesidade, associado a um baixo grau de inflamação crônica, que leva a liberação, pelos adipócitos, de mediadores inflamatórios denominados adipocinas, como fator de transcrição nuclear alfa (TNF-alfa) e IL-6 (DEBOER, 2012; LUO *et al.*, 2018; PEREZ *et al.*, 2019). Consequentemente, o aumento da inflamação, acompanhado do desequilíbrio oxidativo, aumentam a possibilidade de danos as estruturas celulares, DNA, lipídios e proteínas (GALASSETTI, 2012; PONUGOTI, DONG E GRAVES, 2012; KARAM *et al.*, 2017; OLIVA *et al.*, 2017). A teoria para explicar o mecanismo envolvido entre a restrição calórica e consequente prevenção de doenças, é baseada na diminuição do estresse oxidativo por meio da atividade enzimática e vias de sinalização intracelulares (QIU *et al.*, 2010). As ERO's são sintetizadas como bioprodutos da respiração celular, advindas cerca de 90% das mitocôndrias. A inativação das ERO's é feita por enzimas antioxidantes, como as superóxidos dismutases (SOD's) e então, o microambiente intracelular permanece em equilíbrio. O desequilíbrio entre essa produção de ERO's e a capacidade antioxidante da célula, leva ao estresse oxidativo. Essa dinâmica em equilíbrio ocorre constantemente no organismo, entretanto com algumas condições como o envelhecimento biológico e a obesidade, a quantidade de ERO's torna-se em excesso na célula (KINCAID E BOSSY-WETZEL 2013). O mecanismo para conter esse aumento oxidativo é chamado

sistema antioxidante, composto por enzimas como as SOD's e as sirtuinas (SIRT's), que são proteínas desacetilases, amplamente pesquisadas no processo de envelhecimento (BORDONE *et al.*, 2007; WALDMAN *et al.*, 2018). As SIRT's, principalmente a SIRT1 e a SIRT3 têm sido fortemente estudadas pelo seu importante papel na restrição calórica (KINCAID E BOSSY-WETZEL 2013; WALDMAN *et al.*, 2018). São responsáveis por ativar a expressão de genes de enzimas antioxidantes e inativar vias de sinalização, como o fator nuclear de transcrição kappa beta (NF- κ B), com consequente redução da resposta inflamatória (HUANG *et al.*, 2012; QU *et al.*, 2017). Além disso, as SIRT's podem atuar nas proteínas de manutenção e reparo do material genético. Na restrição calórica, em razão da privação de energia ocorre aumento nos níveis de NAD⁺ no meio intracelular, aumentando assim a expressão dos genes que codificam as sirtuinas (BALASUBRAMANIAN, HOWELL, ANDERSON, 2017; HOSHINO, KOBAYASHI E HIGAMI, 2018). A SIRT1 está localizada primordialmente entre o núcleo e o citosol e está relacionada com a inibição de vias de sinalização que levam a resposta inflamatória (HUANG *et al.*, 2012). Enquanto a SIRT3, presente na mitocôndria, encontra-se associada ao aumento da capacidade antioxidante por atuar juntamente com a superóxido dismutase (SOD) e está relacionada a regulação da glutathione, a principal molécula antioxidante (SEBASTIAN E MOSTOSLAVSKY, 2010; KINCAID E BOSSY-WETZEL, 2013; WALDMAN *et al.*, 2018). Adicionalmente, a SIRT-3, desempenha um papel na manutenção da homeostase óssea, por inibir a diferenciação dos osteoclastos, pela consequente interferência com fatores de transcrição celular relacionados a inflamação (HUH *et al.*, 2016).

Essa hipótese de mecanismo permite explicar o efeito de redução da perda óssea alveolar no quadro de periodontite experimental, pelo fato da periodontite apresentar a ativação principal da via de sinalização do NF- κ B, que resulta na síntese e secreção de citocinas pró-inflamatórias, como TNF-alfa e IL-1beta, levando ao processo de reabsorção óssea por meio da ativação dos osteoclastos e consequente perda óssea (HIENZ, PALIWAL, IVANOVSKI, 2015; QU *et al.*, 2017; ZHAO, 2017; HIYARI *et al.*, 2018). Esse desequilíbrio nos processos celulares e aumento da síntese de citocinas pró-inflamatórias desencadeiam a superexpressão de moléculas como o ligante do receptor ativador do fator nuclear kappa beta (RANKL) pelos osteoblastos, que agem na diferenciação e ativação dos osteoclastos, que resulta na reabsorção óssea alveolar. O sistema RANK/RANKL/OPG é determinante na reabsorção óssea alveolar, em que células ósseas ativadas por citocinas pró-inflamatórias secretam maior quantidade de RANKL. O receptor de membrana RANK é encontrado nas células precursoras de osteoclastos, responsáveis pela reabsorção óssea. Nesse mecanismo, uma molécula reguladora da

osteoclastogênese, a osteoprotegerina (OPG) possui afinidade pelo RANKL e, se estiver em grande quantidade na célula, ajuda no equilíbrio e manutenção óssea (HIENZ, PALIWAL, IVANOVSKI, 2015). Na periodontite, esse desequilíbrio e acúmulo de RANKL, com menor produção e secreção de OPG, faz com que a progressão tenha como principal consequência a perda óssea alveolar (PAPATHANASIOU *et al.*, 2016).

Com a regulação do mecanismo oxidativo e inflamatório nas células, por meio do equilíbrio das biomoléculas secretadas, aumentam as chances de prevenção e diminuição de doenças inflamatórias, como a obesidade e a periodontite (LUO *et al.*, 2018). Na periodontite, esse mecanismo sugere uma modificação local da resposta imunoinflamatória, com redução dos danos celulares, melhora no perfil oxidativo e inflamatório, regulação do NF- κ B e consequente diminuição na liberação de mediadores inflamatórios, como TNF-alfa e IL-1beta, característicos da periodontite (HIYARI *et al.*, 2018). Nesse sentido, com a regulação e diminuição da resposta inflamatória exacerbada, que leva a ativação do processo de reabsorção óssea, sugere-se a redução na progressão e severidade da periodontite, representada em nosso estudo pelos valores significativamente reduzidos de perda óssea alveolar após restrição calórica. Por meio dos nossos achados e mecanismos propostos na literatura, o efeito da restrição calórica na perda óssea alveolar em ratos com periodontite experimental torna-se extremamente relevante.

Os dados encontrados dos parâmetros clínicos periodontais convergem com a literatura, em que foi observada diminuição significativa no parâmetro índice gengival modificado, em animais submetidos a restrição calórica, comparados ao grupo *ad libitum* (BRANCH-MAYS *et al.*, 2008). A mobilidade, o sangramento e o índice gengival apresentaram diferenças significativas entre os grupos obesos e os que receberam restrição calórica. Com relação ao parâmetro Biofilme, todos os animais testados apresentaram presença de biofilme, o que indica que o método de indução por ligadura resultou no acúmulo microbiológico esperado. Na literatura ainda não existem estudos que avaliaram os parâmetros clínicos periodontais em animais obesos, submetidos tanto a restrição calórica quanto a periodontite experimental.

A restrição calórica marcadamente reduziu os valores de peso corporal e demais parâmetros biométricos, como índice de adiposidade, circunferência abdominal, índice de Lee e ganho de peso corporal, em comparação com os grupos que não foram submetidos a restrição calórica. Nossos dados convergem com a literatura, estudos tanto pacientes quanto animais

obesos apresentaram redução no peso corporal após restrição calórica (PARK *et al.*, 2015; WALDMAN *et al.*, 2018). Pacientes de um estudo clínico randomizado, interessante, também apresentaram diminuição no peso corporal e no nível de glicose, após restrição calórica para o quadro de obesidade. Convergindo com os nossos achados, o estudo clínico também não encontrou diferença significativa nos parâmetros de triglicerídeos, colesterol total, HDL-colesterol e LDL-colesterol (RUGGENENTI *et al.*, 2017). Adicionalmente, a literatura encontra-se estabelecida quanto a redução do peso corporal e dos níveis de glicose no sangue, após restrição calórica (YANG *et al.*, 2017). A SIRT-1 é responsável por regular vários processos biológicos via desacetilação das principais moléculas metabólicas incluindo NF- κ B, Forkhead box protein O1 (FoxO1) e receptor ativado por proliferador de peroxissomo γ (PPAR γ). A desacetilação dessas moléculas resulta no aumento da concentração de adiponectina, diminuição da inflamação e aumento da sensibilidade a insulina (HEO E CHOUNG, 2018). Em contraste com nosso achado, Ding *et al.* (2017) observaram diferença significativa nos parâmetros de triglicerídeos, colesterol total, HDL-colesterol e LDL-colesterol após restrição calórica.

Outros dados do nosso estudo, como níveis de cálcio e fosfatase alcalina, não apresentaram diferença significativa após indução da restrição calórica. Esse fato sugere que a restrição calórica em 30% não ocasionou um quadro de má nutrição. A fosfatase alcalina tem sido postulada como preditor prognóstico e auxiliar na rotina de métodos para determinar a atividade da periodontite. A fosfatase alcalina é uma glicoproteína ligante de cálcio e fosfato, presente em vários órgãos e tecidos, principalmente no tecido ósseo. É produzida por várias células, como leucócitos polimorfonucleares, macrófagos, osteoblastos, presentes no periodonto, além de ser um importante indicador da atividade osteoblástica (CUTANDO *et al.*, 2013; SRIDHARAN *et al.*, 2017).

5 CONSIDERAÇÕES FINAIS

A restrição calórica de 30% reduziu significativamente os valores de perda óssea alveolar induzida por ligaduras para modelo de periodontite experimental em ratos, tanto não obesos quanto obesos. A atuação da restrição calórica está relacionada ainda a redução da glicose e dos parâmetros biométricos, comprovando a indução e as vantagens do método na melhora da saúde dos ratos. Os parâmetros periodontais atenuados sob indução da restrição calórica demonstram uma potencial modificação positiva no processo inflamatório, quando comparados aos animais sem a dieta. Enquanto que os níveis de biomarcadores do tecido ósseo não foram alterados na presença da restrição calórica, sugerindo que o método utilizado para restrição calórica não desencadeou diminuição nutricional.

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**ANEXO A – PARECER DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE
ANIMAL**



UNIVERSIDADE ESTADUAL DE PONTA GROSSA

**PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA DO USO DE ANIMAL****CARTA DE APROVAÇÃO****Processo CEUA – 039/2017****Protocolo UEPG – 12203/2017****Título –** .”Projeto de pesquisa “Efeito da restrição calórica na doença periodontal”**Interessado:** Leomar Emanuel Almeida Mecca (leoo.mecca@gmail.com)**Data de Entrada – 12/07/2017****Resultado:** Aprovado**Data/Prazo – 22/09/2017 a 22/09/2019****Considerações**

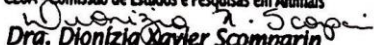
Prezado Leomar (PPG Odontologia):

Em relação a utilização de animais no protocolo de pesquisa sob sua responsabilidade, a CEUA deliberou pela aprovação da autorizada a utilização de 72 ratos heterogênicos de 350 gramas.

Ponta Grossa, 22 de setembro de 2017

UNIVERSIDADE ESTADUAL DE PONTA GROSSA
Pró-Reitoria de Pesquisa e Pós-Graduação

CEUA - Comissão de Estudos e Pesquisas em Animais


Dra. Dionizia Xavier Scomparin
Coordenadora



UNIVERSIDADE ESTADUAL DE PONTA GROSSA

**PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA DO USO DE ANIMAL****CARTA DE APROVAÇÃO****Processo CEUA – 049/2017****Protocolo UEPG – 12203/2017****Título – “Projeto de pesquisa “Efeito da restrição calórica na doença periodontal”****Interessado:** Leomar Emanuel Almeida Mecca (leoo.mecca@gmail.com) e Gilson Cesar Nobre Franco (gcnf@hotmail.com)**Data de Entrada – 12/07/2017****Resultado:** Aprovado**Data/Prazo – 18/12/2017 a 18/12/2019****Considerações**

Prezado Mestrando Leomar Emanuel Almeida Mecca e Professor Gilson (PPG C. Farmacêuticas):

Em relação a utilização de animais no protocolo de pesquisa sob sua responsabilidade, a CEUA deliberou pela aprovação da autorizada a utilização de 24 ratos de vinte e um dias.

Ponta Grossa, 18 de dezembro de 2017

UNIVERSIDADE ESTADUAL DE PONTA GROSSA
Pró-Reitoria de Pesquisa e Pós-Graduação
CEUA - Comissão de Estudos e Pesquisas em Animais
Dra. Dionizila Xavier Scamparini
Coordenadora