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Chronic stress associated to contraceptives use on the progression of ligature-induced periodontitis in rats

Avaliação do estresse crônico associado ou não ao uso de contraceptivos na progressão de periodontite induzida por ligadura em ratos

Débora Prado MARTINS[a], Tereza Aparecida Delle Vedove SEMENOFF[a], Álvaro Henrique BORGES[a], Natalino Francisco da SILVA[a], Alex SEMENOFF-SEGUNDO[a*]

[a]UNIC – Universidade de Cuiabá, Cuiabá, MT, Brasil

Resumo

Introdução: Os contraceptivos são amplamente utilizados pelas mulheres em grande parte do mundo. O estresse associado a sua utilização pode conduzir respostas adversas no organismo. **Objetivo:** O objetivo deste trabalho foi avaliar os efeitos do estresse crônico associado ou não com o uso de contraceptivos na progressão de periodontite induzida por ligadura em ratas Lewis. **Material e método:** Para tanto, quarenta ratas foram divididas aleatoriamente em 5 grupos (n = 8): Grupo Contraceptivo e Ligadura (CG); Grupo Estresse e Ligadura (SG); Grupo Contraceptivo, Estresse e Ligadura (CSG); Grupo Ligadura (LG); e Grupo Controle (CtrlG). Um contraceptivo injetável foi administrado nos grupos CG e CSG no início do experimento. No dia seguinte, a periodontite foi induzida nos grupos CG, SG, LG e CSG, através da colocação de ligaduras no segundo molar superior direito destes animais. No terceiro dia, o SG e o CSG foram submetidos a um modelo de estresse crônico usando contenção física e exposição ao frio. Após 60 dias, os animais foram sacrificados e submetidos a medidas histométricas. Foram considerados dois parâmetros: perda de inserção histológico (CEJ-PL) e perda óssea (CEJ-BC). **Resultado:** Os resultados indicaram que o CtrlG (CEJ-PL 0,10 ± 0,00; CEJ-BC 0,34 ± 0,02) apresentaram os valores mais baixos ($p<0,05$). O grupo SG (CEJ-PL 0,90 ± 0,24; CEJ-BC 1,30 ± 0,28) apresentou valores mais elevados que foram significativamente diferentes ($p<0,05$) dos resultados do LG. Os outros grupos apresentaram resultados estatísticos semelhantes ($p>0,05$). **Conclusão:** Os resultados deste estudo não permitem uma avaliação da susceptibilidade dos animais à periodontite induzida por ligadura. Sendo necessários, portanto, outros estudos que elucidem melhor a questão.

Descritores: Periodontite; anticoncepcionais; ratos; estresse.

Abstract

Introduction: Contraceptives are widely used by women in much of the world. The stress associated with their use can lead adverse responses in the body. **Objective:** The aim of this paper was to evaluate the effects of chronic stress associated with contraceptive use on the progression of ligature-induced periodontitis in female Lewis rats. **Material and method:** Therefore, forty rats were randomly divided into 5 groups (n = 8): contraceptive and ligature group (CG); stress and ligature group (SG); contraceptive, stress and ligature group (CSG); ligature group (LG); and control group (CtrlG). An injectable contraceptive was administered to the CG and CSG groups at the beginning of the experiment. On the following day, periodontitis was induced in the CG, SG, LG and CSG groups by placing ligatures on the upper-right second molar. On the third day, the SG and the CSG were subjected to a chronic stress model using physical restraint and cold exposure. After 60 days, the animals were euthanized and submitted to histometric measurements. Two parameters were considered: histological attachment loss (CEJ-PL) and bone loss (CEJ-BC). Regarding CEJ-PL/CEJ-BC. **Result:** The results indicated that the CtrlG (CEJ-PL 0.10 ± 0.00; CEJ-BC 0.34 ± 0.02) showed the lowest values ($p<0.05$). The SG group (CEJ-PL 0.90 ± 0.24; CEJ-BC 1.30 ± 0.28) presented higher values that were significantly different ($p<0.05$) from the results of the LG. The other groups exhibited similar statistical results ($p>0.05$). **Conclusion:** The present results do not allow an assessment of the susceptibility of animals to ligature-induced periodontitis.

Descriptors: Periodontitis; contraceptive agents; rats; stress.

INTRODUCTION

During different life stages, sexual hormones – especially female hormones – have a significant influence on periodontal tissues. Changes in periodontal conditions are associated with variations in the levels of these substances[1]. In this respect, the use of oral contraceptives plays an important role[2], as these drugs modify the estrous cycle to prevent pregnancy. Nevertheless, it seems that estrogen and progesterone receptors located in the gingival tissue allow a greater accumulation of immunoinflammatory products, altering defense responses to microbial biofilm challenges[3].

In periodontal disease, stress is considered an environmental factor that can modify the response to microorganisms[4]. It is believed that the mechanism that leads to the progression of periodontitis is associated with stress-induced changes and stimuli of hypothalamic-pituitary-adrenal (HPA) axis, which affects and modifies the body's defense system. As a result, greater amounts of cortisol and adrenaline are generated, altering the pattern of the immune response in the periodontium and therefore contributing to the progression of periodontal disease[5,6].

Current research indicates a link between male and female hormones and reduced disease progression or some protective effect, especially in the cardiorespiratory system[7]. In particular, estradiol, which was used in this study, is a hormone that is related to a reduction in dendritic cells, which are responsible for memory in mammals. It is known that lesions in this region can affect the progression of ligature-induced periodontitis in rats[8,9]. Stress variables seem to invoke greater destruction of the periodontal tissues in animals[5,] and in humans[10]. There is no clear description of the effect of hormones, such as estradiol, on the periodontium[11], although evidence indicates that clinical parameters of periodontal health, including relevant changes in the macrobiotic of the biofilm[12], worsen with the use of such hormones[13,14]. Given the above information, it is important to understand how stress, ligature-induced periodontitis and contraceptive use are associated as well as the relationship between contraception and ligature-induced periodontitis.

Understanding the effect of chronic stress[15] associated with contraceptive use seems to be another important hypothesis that needs to be studied. Thus, the aim of this study was to evaluate the effect of chronic stress associated or not with contraceptive use on the progression of ligature-induced periodontitis in Lewis female rats.

MATERIAL AND METHOD

For the present experiment, forty adult female inbred Lewis *Rattus norvegicus* originating from the vivarium of the School of Dentistry, University of Cuiaba (University of Cuiaba, UNIC, State of Mato Grosso, Brazil) were selected. The animals had an initial mean weight of 196.622 ± 19.98 g. The animals were kept in housing boxes (16 × 40 × 30 polyethylene) in groups of four, with standardized rations (Presence – Animal Nutrition, Paulínia, SP, Brazil) and water ad libitum, under a light/dark cycle of 12 hours, controlled temperature at 23 °C and ± 40% humidity. They were given a four-week adaptation period after they were placed in their new environment. The present research was previously approved by the Research Ethics Committee under protocol number 0307-319, UNIC/2009.

Initially, the animals were randomly divided into five groups (Figure 1), as follows: the contraceptive and ligature group (CG, n = 8); the stress and ligature group (SG, n = 8); the contraceptive, stress and ligature group (CSG, n = 8); the ligature group (LG, n = 8); and the control group (CtrlG, n = 8).

On the first day, the animals from the CG and CSG groups received intramuscular injections of 0.1 ml Perlutan (Boehringer Ingelheim, Itapecerica da Serra, SP, Brazil). This procedure was repeated on the thirty-first day of the study.

The day after the contraceptive drug was administered[14]; the animals from the CG, SG, LG and CSG groups underwent the

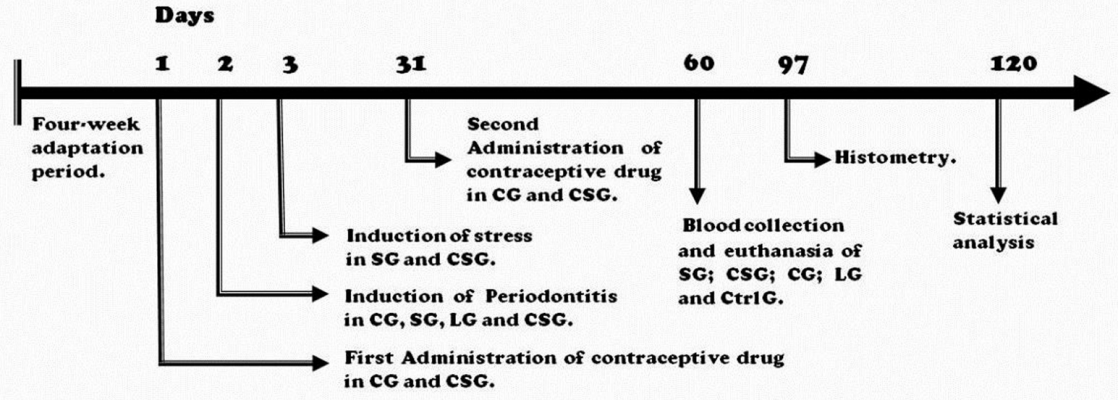

Figure 1. Demonstrative diagram of whole experimental procedure. SG - Stress and ligature Group; CSG - Contraceptive, Stress and Ligature Group; CG - Contraceptive and Ligature Group; LG - Ligature Group; CtrlG - Control Group.

induction of experimental periodontal disease. Control group animals were maintained in their housing boxes throughout the experiment without any procedure.

All of the animals except those from the control group (CtrlG) were anesthetized with an intramuscular injection of 0.1 ml ketamine (Dopalen; Vetbrands, Jacarei, SP, Brazil) combined with 0.05 ml xylazine chloride (Rompum; Bayer Animal Health, São Paulo, SP, Brazil) per 100 grams of body weight.

After anesthesia, a sterile suture (4-0 silk; Ethicon, Johnson and Johnson, São Paulo, Brazil) was placed around the upper-right second molar. The homologous left side was used as a control without the induction of disease[9].

For the chronic stress model[5], the animals in SG and CSG were subjected to physical restraint and cold exposure. The animals were placed in polyvinyl tubes (PVC; Amanco, Mexichem Brazil, São Paulo, SP, Brazil) compatible with their size. Later, the entrances of the tubes were blocked with metal wire to allow the animals to breathe while remaining immobilized at temperatures as low as 7 °C for four hours daily, six days a week for 60 days.

After 60 days, the animals were anesthetized with an intramuscular injection of 0.1 ml ketamine hydrochloride (Dopalen, Agribrands Animal Health, Paulínia, SP, Brazil) combined with 0.05 ml of ketamine hydrochloride (Rompun, Bayer Animal Health, São Paulo, Brazil) per 100 g of body weight. Next, the skin on the abdominal wall was split diagonally at the base of the abdomen to make a "V"-shaped opening.

When access to the abdominal cavity was obtained, the internal organs were displaced to allow a view of the posterior vena cava. Blood was collected into a 5-mL tube via a vena cava puncture with a 25 × 7 needle (Vacutainer - Becton Dickinson, Plymouth, UK). Samples of all groups involved in this study were sent to a specialized laboratory to analyze the cortisol and progesterone level, using colorimetry method by means of spectrophotometer (Femto 700S, São Paulo, SP, Brazil).

After blood collection, the animals were euthanized with an excess of anesthetics. The right and left hemi-maxillae were removed and fixed in 10% formaldehyde for 48 hours. The specimens were then decalcified in ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich Corp., St. Louis, MO, USA) for a period of approximately five weeks (with six EDTA changes). Immediately thereafter, the specimens were washed, dehydrated, cleared and embedded in paraffin. The specimens were then cut into 6-µm sections, stained with Harris hematoxylin and alcoholic eosin and mounted.

Ten serial slides were selected for analysis. The slides included the 1st and 2nd upper (right and left) molars and the following structures[9]: a) the coronal pulp, b) the radicular pulp, c) the cementum enamel junction on the mesial face of the second molar, d) the interproximal bone crest and e) the conjunctive attachment. Histometry was performed by capturing (Leica DMLB Microscope, Wetzlar GmbH, Germany) and digitizing images and measuring them in mm using ImageLab 2000 software (Bio Diracon Informatics Ltda., Vargem Grande do Sul, SP, Brazil). The examiner was trained to calibrate the study variables and blinded to the groups for all parameters measured; the examiner was considered calibrated when a standard error of measurement (SEM) greater than 0.8 was achieved[9].

The parameter "histometric analysis" was defined as the distance in millimeters between the cementum enamel junction on the mesial face of the second molar and the coronal portion of the junctional epithelium. This parameter was established as histological attachment loss (CEJ-PL). Additionally, the distance in millimeters between the cementum enamel junction and the alveolar bone crest was analyzed as an indicator of histological bone loss (CEJ-BC) – Figure 2.

The parameter "body weight" was compared using the final mean weights of the groups.

The data were statistically analyzed using one-way (body weight and cortisone) and fixed-factor two-way (histological parameters) analysis of variance (ANOVA), followed by Tukey's post-test with a significance level (alpha error) of 5% (IBM SPSS Statistics version 20, Armonk NY, United States).

RESULT

Some blood samples suffered hemolysis, and they were discarded (Tables 1 and 2). For the final weight analysis of the animals and for the histological measurements of attachment loss (CEJ-PL) and bone loss (CEJ-BC), all groups comprised eight animals.

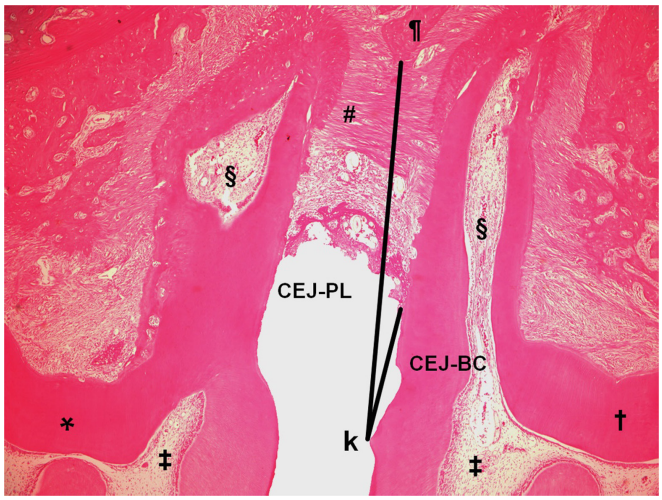

Figure 2. Demonstrates 1st (*) and 2nd (Ĥ) upper molars and: coronal pulp (‡); radicular pulp (§); cementum enamel junction (k); interproximal bone crest (¶); conjunctive attachment (#). CEJ-PL: means histological attachment loss and CEJ-BC: means histological bone loss.

Table 1. Comparisons of the final weight of the animals (in grams) in the studied groups

Groups	N	Mean	SD
CSG	8	259.62 * †	74.27
SG	8	257.01 *	93.06
CG	8	251.37 *	78.74
LG	8	278.25 † ‡	75.80
CtrlG	8	289.12 ‡	87.83

N: Number of animals; SD: Standard Deviation; CSG: Contraceptive+Stress and Ligature Group; SG: Stress and Ligature Group; CG: Contraceptive and Ligature Group; LG: Ligature Group; CtrlG: Control Group. Symbols *, †, ‡ in the lines indicate significant differences between groups (ANOVA and Tukey's tests, $p<0.05$).

The animals' weight indicated that CtrlG had the highest body mass gain (289.12 ± 87.83) of all groups (p<0.05). There are no statistics differences between them (p>0.05). CSG (259.62 ± 74.27), SG (257.01 ± 93.06) and CG (251.37 ± 78.74) did not differ significantly from one another (p>0.05). Moreover CSG was statistically similar with LG (278.25 ± 75.80) respectively (p>0.05) (Table 1).

Table 2. Progesterone collected from the blood (ng/mL) of the animals in the analyzed groups

Groups	N	Mean	SD
CSG	5	27.02 *	18.56
SG	6	13.86 † *	06.63
CG	6	25.55 † *	17.53
LG	5	06.49 †	02.91
CtrlG	7	05.42 †	03.76

N: Number of animals; SD: Standard Deviation; CSG: Contraceptive+Stress and Ligature Group; SG: Stress and Ligature Group; CG: Contraceptive and Ligature Group; LG: Ligature Group; CtrlG: Control Group. Symbols *, † in the lines indicate significant differences between groups (ANOVA and Tukey's tests - p<0.05).

The progesterone levels in the blood were significantly different for CtrlG (5.42 ± 3.76) and LG (6.49 ± 2.91) compared with CSG (27.02 ± 18.56) (p<0.05), whereas there were no differences when CtrlG and LG were compared with SG (13.86 ± 6.63) CG (25.55 ± 17.53) (p>0.05) (Table 2).

The study results indicate that when histological attachment loss (CEJ-PL) (Figure 3) and histological bone loss (CEJ-BC) (Figure 4) were compared using the fixed-factor two-way ANOVA, the animals that were subjected to stress -CEJ-PL 0.90 ± 0.24; CEJ-BC 1.30 ± 0.28- presented significantly different results from those subjected to periodontitis induction alone -CEJ-PL 0.58 ± 0.33; CEJ-BC1.13 ± 0.41- (p<0.05). The animals subjected to a combination of chronic stress and contraceptive use -CEJ-PL 0.82 ± 0.25; CEJ-BC1.25 ± 0.21- and those subjected to contraceptive use only -CEJ-PL 0.82 ± 0.24; CEJ-BC1.32 ± 0.23- did not demonstrate significant differences from those of animals with ligature-induced

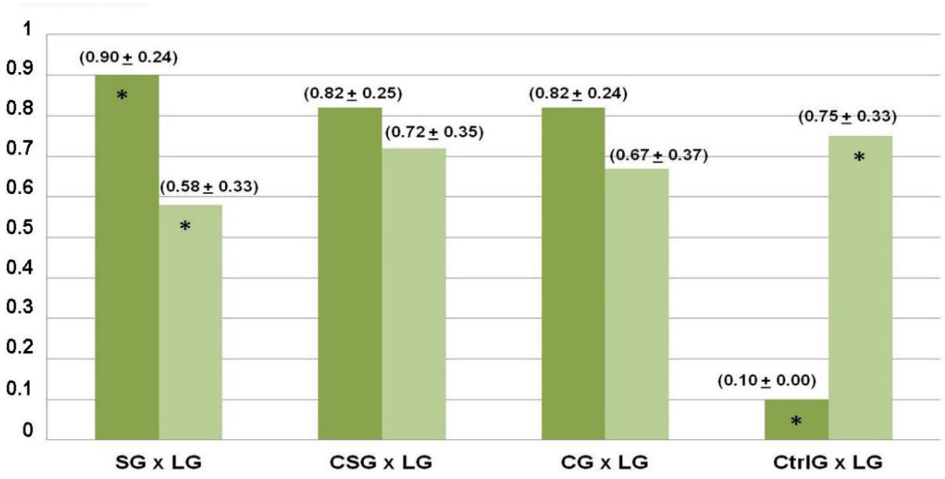

Figure 3. Histological measurements in millimeters (mm) corresponding to histological attachment loss (CEJ-PL) and the respective standard deviations. SG: Stress Group; LG: Ligature Group; CSG: Contraceptive + Stress Group; CG: Contraceptive Group; CtrlG: Control Group. * Indicates statistical difference between groups. (Fixed-factor two-way ANOVA - p<0.05).

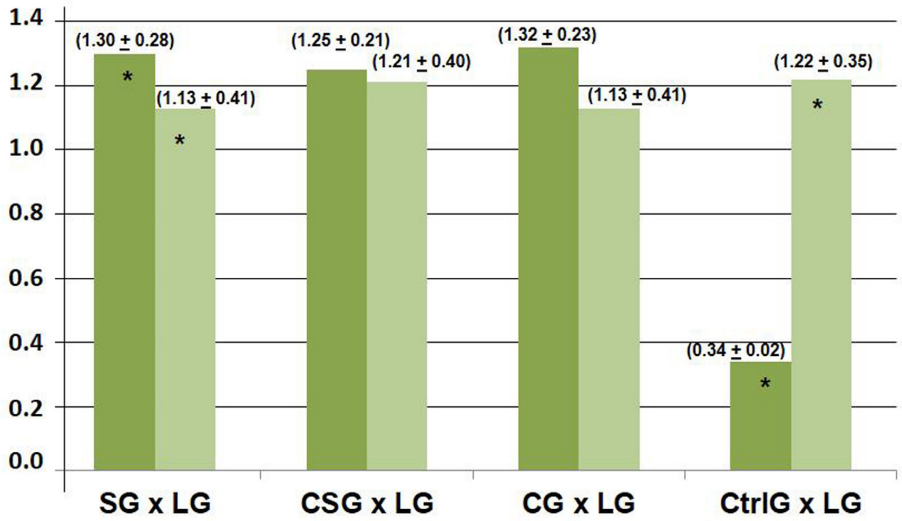

Figure 4. Histological measurements in millimeters (mm) corresponding to histological bone loss (CEJ-BC) and the respective standard deviations. SG: Stress Group; LG: Ligature Group; CSG: Contraceptive + Stress Group; CG: Contraceptive Group; CtrlG: Control Group. * Indicates statistical difference between groups. (Fixed-factor two-way ANOVA - p<0.05).

periodontitis alone -CEJ-PL 0.67 ± 0.37; CEJ-BC1.13 ± 0.41- (p>0.05). The results for the control group differed from those for all other groups -CEJ-PL 0.10 ± 0.00; CEJ-BC0.34 ± 0.02- (p<0.05).

With regard to plasma cortisol levels, SG showed the highest values and differed significantly from the other groups (p<0.05). CSG showed the second highest amount of cortisol, without significant differences compared with CG, but with significant differences compared with the other groups. LG and CG had the lowest amount of cortisol in the blood; there was no significant difference between LG and CG, but they differed from CSG, CG and SG (p<0.05) (Table 3).

DISCUSSION

This study aimed to histometrically evaluate the progression of ligature-induced periodontitis in female Lewis rats receiving contraceptives. The findings suggest that the combination of exposure to stress and contraceptives, or exposure to contraceptives only, did not modify the pattern of destruction of periodontal tissues in rats, but that chronic stress did alter the progression of periodontitis.

Periodontitis is an inflammatory disease of infectious origin and is directly connected to environmental and internal risk factors, such as hormones, which gives plausibility to this study[4,16].

Associations between stress, i.e., cortisol hormone from the posterior portion of the pituitary gland, and estradiol-based contraceptive use, i.e., natural sex hormones from gonadotropins (luteinizing and follicle-stimulating hormone) originating from the anterior portion of the same gland, with the progression of periodontal disease were determined in this study[16,17].

The findings of this study indicate that the combination of contraceptive use and stress do not have a cumulative effect on periodontal destruction in either periodontal soft tissue or in bone. However, stress severely alters the physiology of the endocrine system and the inflammatory process[18]. In this sense, our results are consistent with findings from Kudielka, Kirschbaum[17], who showed that hormones are able to conduce changes in the pathophysiology of cell ultrastructure definition, in many cases, in the production of pro-inflammatory cytokines such as IL-6, IL-1 and TNF in periodontal tissues[16].

Table 3. Cortisol collected from the blood (µg/dl) of the animals according to the analyzed groups

Groups	N	Mean	SD
CSG	7	3.05 *	1.61
SG	6	4.11 †	1.57
CG	6	2.61 *	0.70
LG	6	1.83 ‡	1.25
CtrlG	5	1.00 ‡	0.54

N: Number of animals; SD: Standard Deviation; CSG: Contraceptive+Stress and Ligature Group; SG: Stress and Ligature Group; CG: Contraceptive and Ligature Group; LG: Ligature Group; CtrlG: Control Group. Symbols *, †, ‡ in lines indicate significant differences between groups (ANOVA and Tukey's tests, p<0.05).

Several studies have shown that pathways involving the hypothalamus-pituitary- adrenal axis are related to periodontitis[6,15,19]. In this study, we attempted to combine the effects of the hypothalamic-pituitary-gonadal axis, which is strongly related to reproductive functions, with other organic response[17]. The study findings are based on the ability of the hormone estradiol to interfere with the initial formation of myeloid and lymphoid progenitor cells, which once stimulated, can differentiate into all defense cells[20].

In addition, increased levels of estradiol in an animal's body may interfere with osteoimmunology by changing bone metabolism and therefore avoiding ligature-induced periodontitis[16].

Another appropriate item to be discussed is the ability of estradiol (gonadotropins) to activate osteoprotegerin, which in turn can decrease the differentiation and function of osteoclasts and increase their apoptosis. Moreover, the increased concentration of estradiol reduces the release of interleukin and tumor necrosis factor, preventing bone resorption[21]. Furthermore, estrogen may decrease the chemotaxis ability of neutrophils. The degranulation process is directly connected to the progression of periodontitis and would still be able to produce a new inflammatory cascade and inhibit collagenase activity[21,22]. Estradiol also reduces the action of monocytes, including their release of interleukin-6, which is connected to the loss of periodontal structures[23].

With regard to the histometric analysis, we found that chronic physical stress was associated with greater periodontal destruction compared with induced periodontitis. These same results were also found by other studies in rats with radiographic[5] and histological analyses, even for a shorter period of time[15]. It is believed that the association between stress and ligature-induced periodontitis occurs due to the absence of oxygen in periodontal tissues[24]. The process of periodontal destruction appears to be worsened in humans, given the difficulties of patient hygiene when combined with emotional problems[25].

In this respect, the association between induced periodontitis, stress and contraceptive use was expected to induce greater soft and hard tissue attachment loss. The role of stress in periodontal disease has a plausible pathophysiological basis. Evidence suggests that stress is associated with an increased severity of periodontal disease[25]. In fact, prolonged chronic stress produces worse tissue indicators throughout the animal body[6].

It is known that genetic issues are linked to a greater or lesser progression of periodontitis[26]. Specific lineages of animals were chosen to test the study hypothesis because it seems that inbred rats are only minimally susceptible to periodontitis[27] and do not experience substantial destruction of periodontal structures.

In this study, a higher concentration of cortisol was observed in the groups submitted to stress, including further destruction of periodontal structures. Therefore, this study confirms the effect of stress on animals in terms of cortisol levels and corroborates other authors' results[6]. The long experimental period may have influenced the results[2]. This period was chosen based on the assumption that periodontitis is a chronic disease[4] and on the fact that hormonal changes combined with stress and contraceptive therapy could have synergistic effects over a shorter period of time[5]. After 15 days, the effects of disease progression are likely to be identified. However,

after 30 days, the responses begin to differentiate according to stress modulation[15,27]; furthermore, this length of time is a reasonable period within which to detect hormonal changes[28].

Body weight is an important indicator of health, especially in studies involving the relationship between stress and ligature-induced periodontitis in rats[15]. In addition to body-weight decrease, others indications of systemic change, such as changes in spleen, thymus and adrenal gland weight and alterations in lymphoid organs[15], can occur. In this study, the animals exhibiting lower body mass gain were CG, SG and SCG, which was in agreement with other animal tests involving stress[29] and estradiol use[14].

This could explain why only the SG presented significant differences from the LG, i.e., positive control group. The groups that received a contraceptive associated with ligature (CG) as well as the group that received a combination of stress and contraceptive (CSG) did not show more destruction periodontal structure compared to the LG. Therefore, it seems that the inflammatory processes in the periodontium, despite demonstrating systemic changes as shown by hormone tests, only confirm that greater periodontal destruction occurs in the SG, as previously described by several authors[10].

Estradiol has the ability to interfere with histological insertion loss and bone loss progression on periodontal tissue, and many variables, such as age, sex, genetics, diet[30] and stress, may be involved in the neuro-endocrine-immune process. In this sense, animals subjected to chronic stress may have less-effective Helper cells 1 and 2[8], which could compromise other immune system responses, making them even more susceptible to ligature-induced periodontitis[10]. Therefore, it was expected that the group of animals subjected to chronic stress would present a longer progression of ligature- induced-periodontitis.

CONCLUSION

The present results do not allow an assessment of the susceptibility of animals to ligature-induced periodontitis.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

*CORRESPONDING AUTHOR

Alex Semenoff-Segundo, Departamento de Pós-graduação em Ciências Odontológicas Integradas, UNIC – Universidade de Cuiabá, Avenida Manoel José de Arruda, 3100, Jardim Europa, 78065-900 Cuiabá - MT, Brasil, e-mail: semenoff@uol.com.br

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