Effect of variable moderate chronic stress on ligature-induced periodontal disease in Wistar rats

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The aim of this investigation was to study the impact of stress on ligature-induced periodontal disease in rats by means of a variable moderate chronic stress model. Thirty male Wistar rats were randomly assigned to six groups. Control groups received only ligatures around the second maxillary molars, while experimental groups were exposed to stress in additional. Stress was imposed by means of flashing light, isolation, rat blood smelling, new environment exposure, immobilization in cold temperature and immobilization at room temperature. Stress was applied randomly, thereby diminishing adaptation of the animals to the model. The animals were killed after 29, 43, and 57 days. The distance between the cementum-enamel junction and the alveolar bone (CEJ-AB) was measured. Alveolar bone loss was statistically different between stressed and control animals, whereas differences were not observed between experimental periods. The mean CEJ-AB distance in animals exposed to stress was 154.50 μm smaller than the corresponding distance in the controls. It might be concluded that variable moderate chronic stress decreased alveolar bone loss in a ligature-induced periodontal disease model. □ *Alveolar bone loss; periodontal diseases; rats; stress*

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Periodontal diseases are inflammatory and affect the tooth supporting structures, and periodontal pathogens and their products are responsible for inducing an inflammatory response in the host. The host capacity to control a hyperactive response to pathogens is regarded as a key factor in the severity of tissue destruction. Environmental, behavioral, and biological factors can disrupt this balance leading to the establishment and progression of periodontal destruction. Diabetes, smoking, and certain bacteria have been identified as risk factors for periodontal diseases (1).

It is well established that stress is a risk factor for some infectious, autoimmune, and inflammatory diseases, as well as tumors. Decreased activity of leukocytes and other immune cells, down-regulation of the inflammatory mediators and impaired immunological response, among other host response modifications, are likely to occur after stress exposure (2–13).

Although the pathways by which stress influences the inflammatory response are not completely understood, activation of the hypothalamus-pituitary-adrenal (HPA) axis seems to be a key mechanism. This results in a cascade of events leading to the release of corticotropin-releasing hormone, glucocorticoids, corticotropin, endorphin, and arginine vasopressin that have different immune and inflammatory properties (4, 6, 8, 14, 15). Furthermore, stress up-regulates the HPA axis, leading to a shift towards an antibody mediated immune response, which is dominated by type 2 T helper cells (Th2). These cells secrete interleukin-4 (IL-4), IL-5, IL-10, and IL-13 that activate B-lymphocytes, eosinophils, and mast cells, possibly leading to increased susceptibility to infectious diseases. Conversely, a low respondent HPA axis may increase the resistance to infections; probably due to a cell mediate immune response dominated by type 1 T helper cells (Th1), which secrete interferon gamma (IFN-γ), IL-2 and tumor necrosis factor alpha and beta (TNFα and TNFβ) (13).

Stress has been linked to periodontal disease since the middle of the last century, and most reports comprise necrotizing forms of periodontal disease. In the past decade, more evidence has emerged from epidemiological studies relating periodontitis to stress, depression, and negative life events (16–18). Animal studies conducted in the 1960s have demonstrated a possible detrimental role of stress in periodontal tissues (19–22). Recently, Gaspersic et al. (23) found more fiber attachment and alveolar bone loss after exposing the experimental animals to restraint stress. In a series of studies on rats, Breivik et al. (13) demonstrated that periodontal disease susceptibility and progression could be explained, at least partly, by brain-neuroendocrine-immune regulatory mechanisms. Genetically determined HPA reactivity seems to play an important role and a possible feedback is likely to occur from periodontal disease (14).

Behavioral and endocrinological consequences of stress have been studied using different stress models—among them those trying to mimic stressing situations. Short and long stress exposure, predictable or variable regimens, mild, moderate, or severe stressors have all been used to

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verify the impact of stress (8, 24–27). Compared to other studies that have employed one single modification in the stress mechanism, these stress models are more likely to provide results that are applicable to the human situation. Also, the hypothesis that the presence of a ligature could be a stressor, and thus activate, per se, the HPA axis, should not be ruled out.

The aim of the present study was to investigate the impact of stress in periodontal disease by means of a variable moderate chronic stress model on alveolar bone loss in ligature-induced periodontal disease in Wistar rats.

Materials and methods

Animals

Thirty male Wistar rats, 2 months old, were used in the present study. The animals were acclimatized to the housing conditions during the course of 4 weeks and a 12 h light and dark cycle was applied. Five rats were housed in each cage at a temperature of around 20°C. Standard rat chow pellets and water *ad libidum* were available.

Experimental groups

After weight stratification, the animals were randomly assigned into three control and three experimental groups of five rats each. Control groups received only ligatures, while experimental groups were exposed to stress in addition. Each group of experimental and control animals was killed on days 29, 43, and 57. All procedures that could generate pain were performed under anesthesia. The Ethics Committee of the Lutheran University of Brazil approved the study.

Experimental procedures

Cotton ligatures were placed around the 2nd maxillary molars on both sides on the first day. Knots were made palatally to prevent loss. The ligatures were examined once a week and replaced when necessary.

A variable moderate chronic stress model was utilized for the experimental animals. Stress was performed at different time-points in the period from 8 a.m. to 8 p.m. The sequence was randomly changed every week and each stressful stimulus was used only once a week.

The stress model (20–23) was performed as follows: (a) *Flashing light*: the experimental animals were exposed to 1 min of darkness followed by 1 h of flashing light (40 watts/45 flashes per minute). (b) *Isolation*: this procedure consisted of individual housing for a period of 20 to 28 h. (c) *Immobilization*: restraint was carried out by placing each animal within a 15 × 7 cm plastic tube. Tube size was adjusted by the operator from the outside until the animal was unable to move. The device allowed normal breathing. This procedure was held for 1 h. (d) *Immobilization in cold temperature*: the immobilization protocol described above was performed with the animals placed in a cold environment with the temperature ranging between 6°C and 12°C. (e) *Rat blood smelling*: 2 ml of fresh blood was placed in a plastic device containing heparin. Donor rats were submitted to individual housing for 24 h. This procedure was performed for 1 h. (f) *New environment exposure*: rats were placed, for 30 min, on platforms of 14 × 14 cm located in an aquarium containing 8 cm of water (24–28°C). Animals that fell from the platform were gently placed on it again. (g) *No stress*: one day per week was used to assess ligature presence, with no other stressful stimulus.

Histological procedures

Following sacrifice, the left and right segments of the maxillae were dissected out and fixed in 10% neutral buffered formalin. The specimens were then decalcified in 5% nitric acid, dehydrated in graded alcohol concentrations, embedded in paraffin and sectioned along the long axis of the teeth. Histological analysis was performed to verify whether the sections were obtained from the central part of the specimen. As a prerequisite, the coronal pulp had to be clearly identified in at least 2 teeth and the root pulp in 1. When these prerequisites were fulfilled, 6 serial sections, representative of each specimen (taken with step of 10), were stained with hematoxylin and eosin.

Measurements

Body weight of the animals was registered using a standardized scale three times on the first and last weeks of the experimental period. Bone level at the mesial aspect of the mesial root of the 2nd maxillary molar, on both sides, was estimated. Histometric analysis of the distance between the cemento-enamel junction and alveolar bone (CEJ-AB) was performed using a digital image processing system (Imagelab 2.3, Brazil). All measurements were carried out in micrometers (μm), in triplicate for each section, by a blind examiner.

Reproducibility

Before the histometric analysis was performed, the examiner twice measured 60 sections, with an interval of 1 week between measurement. The difference between the first and the second measurement was 20.33 μm ± 18.82. In order to evaluate reproducibility during the measurement procedure, one section in every 15 was randomly selected and analyzed. The difference between the measurements observed was 17.40 μm ± 16.86. The intraclass correlation coefficient was 0.99 for both occasions and no systematic error was observed (paired t test, $P = 0.71$ and 0.99, respectively).

Statistical analysis

Body weight analysis was performed using one-way

Table 1. Final number of rats per group (considering the three discarded specimens)

Period	Experimental group (stress) (*n*)	Control group (*n*)
29 days	5	5
47 days	5	4
59 days	4	4
Total	14	13

ANOVA at day zero. At the end of each experimental period, the control and experimental groups were compared by independent *t* test.

Multiple linear regression analysis was carried out using CEJ-AB distance as the dependent variable, while experimental groups and time periods were used as independent variables. Regression diagnosis was performed in order to verify whether or not the assumptions for regression analysis were valid (28). The 95% confidence intervals (95% CI) were estimated for the regression coefficients. Power analysis and sample size estimation were performed for the 29-day period.

Considering the animals as the statistical unit, the mean value of the CEJ-AB distance for each rat was calculated. Statistical package STATA 7 (STATA Corporation, College Station, TX, USA) and SPSS 11.0 (SPSS Inc., Chicago, IL, USA) were used to perform the statistical analysis. The level of significance was set at 5%.

Results

During the study, 2 rats from the control and 2 from the experimental groups died and were replaced. One animal died during the first and 3 during the second week of the experiment. The final study sample is given in Table 1. Body weight analysis at day zero and at the end of each experimental period did not show statistically significant differences in any comparison. In both the control and the experimental group the animals gained weight during the study.

Residual analysis searching for outliers demonstrated that four observations should be investigated. The sections were histologically analyzed by an external blinded examiner with experience in this field. In two sections, a great amount of hair impaction was observed and abscesses were diagnosed. In order to provide better model fitting, these sections were not included in the statistical analysis and a final R^2 of 0.45 was obtained. Moreover, sections from one animal were lost due to laboratorial problems.

Fig. 1 shows the mean CEJ-AB distance in control and experimental groups in different experimental periods. Since experimental periods did not have any significant impact on the results ($P = 0.19$), a combined CEJ-AB distance measurement was estimated. Using the present fitted regression model, estimated mean was 928.21 μm (95% CI 878.06–978.36) for control groups and 773.71 μm (95% CI 717.64–829.78) for experimental groups. Thus the CEJ-AB distance for animals exposed to

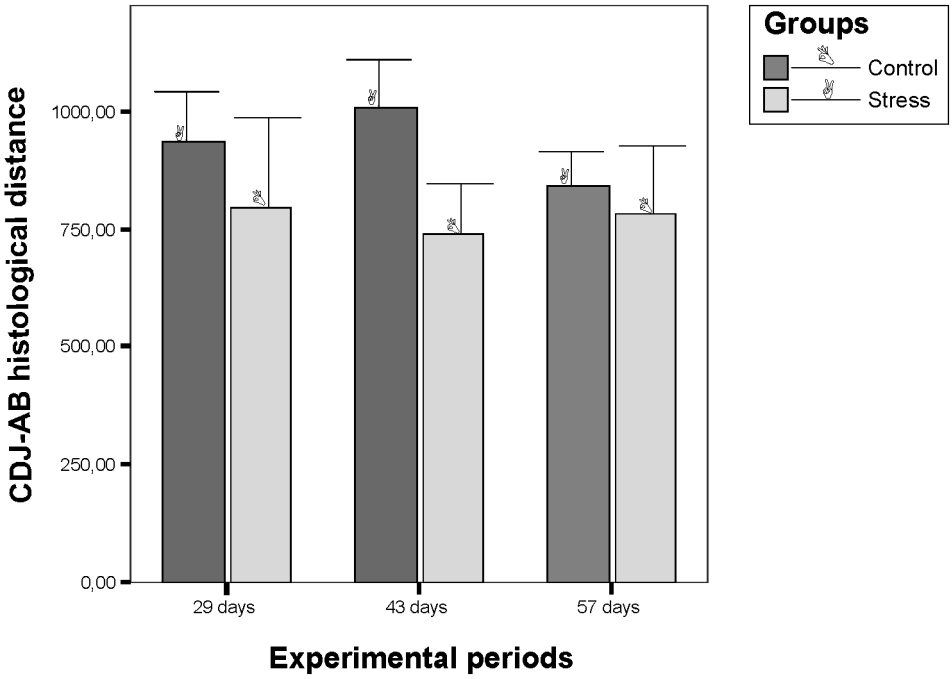

Fig. 1. CEJ-AB distance (μm) in control and experimental groups at different experimental periods. Bars show mean and error bars show 95% confidence interval of mean.

stress was 154.50 μm (95% CI 79.27–229.73) smaller than the corresponding distance in controls ($P = 0.001$).

Power analysis between control and experimental groups at day 29 demonstrated that the power of the comparison was 0.50. Considering the present material and power of 0.80 and 0.90 a sample size of 9 and 12 pairs, respectively, would be necessary to achieve an existing statistical significance at day 29.

Discussion

In the present study we investigated the impact of stress in ligature-induced periodontal disease in Wistar rats. The less alveolar bone loss observed in rats exposed to variable moderate chronic stress compared to the control animals is not consistent with previous investigations (19–23, 29).

In seeking an explanation for the results, the high values of the CEJ-AB distance observed in the 43-day control group were evaluated because they could have influenced the analysis towards significance. Thus, an additional regression model dropping this group was fitted and the combined control groups mean CEJ-AB distance was 114.73 μm (95% CI 38.49–190.97) greater than the experimental groups ($P = 0.005$). It may be assumed that the values observed in the 43-day control group did not influence the results. An eventual activation of the HPA axis by the ligature should not be discarded.

The hypothesis that a shift in the Th1/Th2 dominance towards a cell-mediated response may lead to a decreased susceptibility to infection has been demonstrated previously (13, 14). The possibility that the present stress model might have led to a Th1 dominance, thus increasing the resistance to infection, is still unknown. Also, Wistar rats are genetically diverse and respond unpredictably to the HPA axis. Although this could be a drawback in the experiment, also human beings have this diversity in terms of HPA axis response (14).

Nevertheless, previous studies have shown that, in some circumstances, stress may lead to decreased susceptibility to infection, attenuated immunosuppression (9, 10, 13) and less periodontal breakdown (14). These findings might be an explanation for the results in this study. However, the present findings are not consistent with previous studies, which observed more periodontal breakdown (20–22), reduced reparative response (22), detrimental changes in the connective tissue (19, 20, 22) less macrophages recruitment and less TNFα levels as well as more nitric oxide secretion (30–31).

Differences in the experimental design may partly explain the results observed in other studies. The great number of animals dying and gaining less weight might be due to general health problems, which could have influenced the results in previous investigations (21, 22). Although 4 animals died during the present study, 2 were not exposed to any stressing agents. Moreover, no significant difference was observed in body weight between control and experimental groups, reinforcing the idea that the stress model employed was well tolerated by the animals. Additionally, the use of a predictable chronic stress, in contrast to the present model, increases the possibility of adaptation that was likely to occur in some studies (23, 29). Furthermore, the lack of parameters assessed and statistical analysis in previous studies makes comparison of the results difficult (19, 20). Whether the different experimental design and characteristics of the animals explain the different findings in the present investigation is unknown.

The present stress model has been tested by different authors previously and has been associated with different glucocorticoid levels in experimental animals in comparison to controls (20–23).

The information current in the literature about the time needed for periodontitis to establish is still controversial. Three experimental periods were therefore selected. Nevertheless, in the present study, the factor time did not have any significant impact on alveolar bone level, as demonstrated by regression analysis. In future investigations a sample size of 9 or 12 pairs of rats could be used for 29 days to shorten the experimental period.

The present investigation contributes to the unknown role of stress in periodontal destruction. Further research is necessary until better elucidation of the biological processes, related to stress and periodontal diseases, is achieved. Generally, the results of the present study suggest that the proposed stress model is suitable for studying the relationship between stress and periodontal destruction. Moreover, 4 weeks seems adequate for differences to emerge, so long as sample sizes similar to those estimated in the present study are adopted. The assumption that every stress stimulus increases periodontal tissue destruction should not be overlooked, because, as demonstrated in the present investigation, stress may lead to less alveolar bone loss.

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