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ORIGINAL ARTICLE

Effect of neonatal clomipramine in the pathogenesis of ligature-induced periodontitis in Lewis rats

ANA CRISTINA SOLETTI[1], EDUARDO JOSÉ GAIO[2] & CASSIANO KUCHENBECKER RÖSING[1,2]

[1]*Department of Periodontology, Faculty of Dentistry, Lutheran University of Brazil, Canoas, Brazil and* [2]*Department of Periodontology, Faculty of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil*

Abstract

Objective. The aim of this study was to verify the association between an endogenous depression model and the development of ligature-induced periodontitis in rats. ***Material and methods.*** Nine male Lewis rats received 30 mg/kg clomipramine from neonatal day 8 to day 21 (depressed group), while 13 control Lewis rats were left untouched (control group). On day 150, ligatures were placed around the 2nd upper molars in both groups, i.e. the contralateral molar of the intra-group control. On day 190, the rats were killed and the maxillae were defleshed. The distance between the cemento-enamel junction and the alveolar bone crest was measured by a blinded examiner using standardized digital photographs. ***Results.*** The depressed rats showed hyperactivity in open field on day 142 and greater attempts to escape on day 143 compared to controls. The other behavioral data did not show statistically significant differences between the groups (Mann-Whitney, $p > 0.05$). In teeth with ligature, mean alveolar bone loss varied from 0.51 to 0.60 and from 0.63 to 0.64 mm for tests and controls, respectively (t-test, $p > 0.05$). In teeth without ligature, these values varied from 0.38 to 0.43 and 0.42 to 0.45 mm in the test and control groups, respectively (t-test, $p > 0.05$). ***Conclusion.*** Induced depression did not alter ligature-induced bone loss in Lewis rats.

Key Words: *Behavioral test, clomipramine, neonatal antidepressant drugs, periodontitis, rats*

Introduction

Studies have established that there is a bi-directional communication between the central nervous system and the immune response [1,2]. This relationship is mediated by the activity of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system [2–4]. Behavioral factors (stress, depression, and anxiety) can therefore alter the immune response to microbial challenges and stimulate release of inflammatory mediators such as interleukin and endotoxin [5–7].

Periodontitis is an infectious inflammatory disease related to the imbalance between subgingival dental biofilm and the immune-inflammatory response of the host. Genetic, environmental, and behavioral factors are involved in the modulation of this process [8,9]. In this respect, the role of alterations in the HPA axis in the establishment and development of periodontitis has been investigated in several studies. In addition, clinical and epidemiological findings have associated different degrees of stress, depression, and negative life events with periodontitis [10–12].

An endogenous depression model in rats using neonatal clomipramine is well established in the literature [13,14]. This treatment frequently results in later adult abnormalities, such as decreased sexual activity and REM sleep abnormalities. However, the possible influence of this negative mood state over the periodontal tissues is poorly understood. The present study was carried out to further investigate whether depression in rats modifies susceptibility to periodontal destruction, and to explore whether behavioral tests (number of sectors explored, sexual test) are impaired by the use of neonatal clomipramine. The purpose of the present study was thus to test the hypothesis that endogenous depression accelerates alveolar bone loss in Lewis rats.

Correspondence: Cassiano Kuchenbecker Rösing, Rua Dr. Valle, 433/701-90560-010, Porto Alegre, RS, Brazil. Fax: +55 51 3346 8789. E-mail: odonto@ufrgs.br

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Material and methods

Experimental animals and groups

Twenty-five male Lewis rats were used in the present study from birth. On neonatal day 8, the animals were randomly assigned to a test group and a control group. The test group received 30 mg/kg bodyweight per day of clomipramine subcutaneously from day 8 to day 21 [15]. Rats from the control group were only weighed. During the experimental period, 3 rats from the test group died (1 on day 15 during medication and 2 on day 40 from pneumonia; histologically confirmed). The final composition of the study population was thus 9 rats in the test group and 13 in the control group. Water and standard food were available for both groups *ad libitum*. The animals were weighed at this point on neonatal days 8, 11, 14, 17, and 21.

Sample size calculations

Sample size estimates were calculated based on the data of a study by Susin & Rösing [16]. Considering an alpha error of 0.05 and a beta error of 0.10, a minimum number of 9 animals per group was considered necessary.

Experimental procedures

The present study comprised a previously described initial phase (from day 8 to day 21) in order to induce depression, a second phase of behavioral evaluation (from day 120 to day 150), and a third phase of ligature-induced periodontitis (from day 150 to day 190), after which the animals were killed. Figure 1 demonstrates the experimental design.

Behavioral evaluation

The rats were submitted to open field under video surveillance and randomly analyzed in triplicate by two trained, blinded examiners. The total number of sectors, number of peripheral sectors, number and timing of standings, and number of groomings were assessed [17].

Additionally, a sexual behavior test was performed, i.e. 7 days prior to testing, 11 female Lewis rats were ovariectomized; 48 and 24 h prior to testing, subcutaneous estrogen (5 μg estradiol benzoate – Benzoginoestril®; Aventis Pharma, São Paulo, Brazil) was injected. Hence, 4–6 h prior to sexual testing, 500 μg medroxiprogesterone acetate (Depo-Provera®; Pfizer, São Paulo, Brazil) was given. Thirty minutes video surveillance was carried out after 10 min of male–female presentation. Number of mounts, number of intromissions, and timing to the first mount were assessed [17].

Induction of periodontitis

Cotton ligatures (4.0) (Ethicon®; Johnson & Johnson, São Paulo, Brazil) placed around the cervix of the 2nd maxillary molars with a knot tied bucally [18,19] on the right side on day 150 were checked weekly. The animals were killed 40 days after placement of the ligatures and all ligatures were positioned perfectly. Bodyweight was measured three times a week in the first and last weeks of ligature-induced periodontitis in order to verify the systemic conditions of the animals. The Ethics Committee of the Lutheran University of Brazil approved the study protocol.

Laboratory procedures

Following sacrifice, the left and right segments of the maxillae were defleshed after immersion in sodium hypochlorite with 9% active chloride (Q,boa®; Osasco, São Paulo, Brazil) for 5 h [18–21]. The soft tissues were then removed mechanically. Methylene blue 1% was used for 1 min to stain the cemento-enamel junction, followed by rinsing in water.

Measurements

Standardized pictures of each specimen were taken with a digital camera and medical lenses (D100®; Nikon, Ayuthaya, Thailand) [18,20,21]. A minimal focal distance was used and the specimen was placed with the occlusal surfaces parallel to the floor. A tripod was used to minimize errors. Additionally, a calibration ruler was photographed together in order to transform the data with accuracy. Pictures taken from the buccal and palatal aspects of the specimens were computer-measured by means of Image Tool 3.0 (Image Tool 3.0; UTHSCSA, San Antonio, Texas, USA). Bone level at the mesial aspect of the mesial root of the 2nd maxillary molar, buccally and palatally, on both sides (with or without ligatures) was measured. Bone level was measured between the cemento-enamel junction and the bone crest in the picture. In the present study, this measure was considered as the alveolar bone loss.

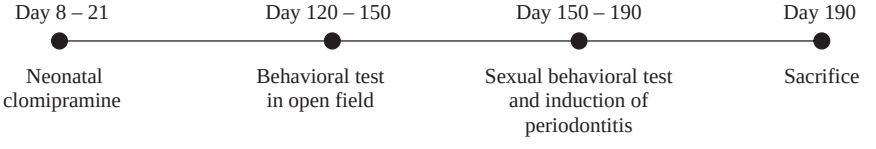

Figure 1. Experimental design.

Reproducibility

Reproducibility of measurements was tested before and during the analysis. Double measurements at 1-week intervals were performed. Paired *t*-test statistics were run and no differences were observed in the mean values for comparison ($p > 0.05$). Additionally, the Pearson correlation coefficient obtained between the repeated measurements revealed very high correlations (0.95–1.0; $p < 0.001$).

Statistical analysis

The rat was considered the unit of analysis in this study. A normal distribution was not obtained for open field and sexual behavior. Thus, comparisons between test and control groups were performed by Mann-Whitney. Mean bodyweight and alveolar bone loss values (normally distributed) were compared by independent and paired sample *t*-test as appropriate. The level of significance was set at 5%.

Results

The bodyweights of the animals throughout the study are given in Table I. On neonatal day 8, the test and control groups presented the same average weight. During the first experimental period, when test animals were submitted to clomipramine injections, weight gain occurred similarly up to day 14. On days 17 and 21, the test and control groups presented statistically significant differences in bodyweight.

During the ligature-induced periodontitis period, neither the test nor the control animals exhibited statistically significant differences in bodyweight, except for day 152. No differences were observed on days 186 and 188. At sacrifice, a statistically significant difference was observed between test and control animals.

The results obtained in open field revealed statistically significant differences in the mean (SD) total of explored sectors (24.88 (7.74) vs. 15.75 (6.09); $p < 0.03$), total of peripheral sectors (18.88 (5.33) vs. 12.58 (5.04; $p < 0.03$), and standings (10.63 (2.45) vs. 6.92 (3.15); $p < 0.01$) for tests and controls, respectively. No differences were observed in the number and time of standings nor in groomings. Sexual behavior testing did not reveal statistically significant differences between groups.

The main outcome of this research (alveolar bone loss) is indicated in Table II. No statistically significant differences were observed between the test and control groups in any of the evaluations.

Table I. Mean and standard deviation (SD) of bodyweight of the animals.

	Test ($n = 9$)		Control ($n = 13$)		
Day	Mean (g)	SD	Mean (g)	SD	$p^\star$
8	15.0	1.3	15.0	1.2	0.98
11	18.3	1.7	19.2	1.9	0.23
14	21.6	1.4	22.9	1.5	0.06
17	24.7	1.8	27.0	1.6	0.01
21	29.2	2.6	32.0	3.0	0.03
150	376.6	16.1	387.1	17.8	0.17
152	372.6	13.4	388.9	16.4	0.02
154	377.4	17.5	392.0	18.5	0.08
186	404.5	10.9	401.3	39.0	0.82
188	398.3	16.7	413.6	19.5	0.07
190	395.8	17.8	413.0	14.0	0.02

*Independent sample *t*-test, $\alpha = 0.05$.

Discussion

The possible interference of depression in the pathogenesis of ligature-induced alveolar bone loss in Lewis rats was evaluated in the present study. Experimental models of depression and periodontal bone loss have been utilized in the literature [3,14,15,17]. Animal studies are necessary in such research settings because of ethical requirements. However, the extrapolation of data from such studies should be performed with caution.

Ligature-induced periodontitis has been studied extensively in periodontal research, and Wistar rats are the most widely used species [16,18,19]. In the present study, isogenic Lewis rats were used, particularly because their response profile to stressors is not as variable as it can be with other species [7,22]. Lewis rats reflect a low response to stress and are considered one of the most suitable species for studies in which neonatal approaches are used, since heterogenic rats, like Wistar, for example, do not present a predictable reaction pattern. High responders to stressors, like Fischer 344 rats, have been studied and do not show differences in behavioral tasks from controls [23]. Hence, isogenic rats present similarities in antigen-presenting type II cells, a situation which warrants a uniform immune reaction, facilitating interpretation of the findings.

The depression model used in this study was crucial for the outcomes. An endogenous model enhances corticosteroid levels and possible immunologic alterations. This model has been shown to alter levels of corticosteroids, a link element between psychic and immune alterations [24]. It also alters REM sleep, which is a common finding in depression [15,25].

Control animals in the present study were left untouched, since it has been demonstrated that neonatal handling *per se* can lead to behavioral alterations in the rat [26]. A placebo group has not been indicated in neonatal-induced depression. In order to check for depressive states and general health, we performed behavioral tests and also monitored bodyweight.

The depressed animals gained less weight during medication intake, similarly to the results of Vogel

Table II. Mean alveolar bone loss in sites with and without ligature for the test and control groups.

	Alveolar bone loss in millimeters, mean (SD)			
	Site	Test group	Control group	$p^{\star}$
Without ligature	Buccal	0.38 (0.01)	0.42 (0.03)	0.06
	Palatal	0.43 (0.08)	0.45 (0.03)	0.06
With ligature	Buccal	0.60 (0.24)	0.63 (0.12)	0.13
	Palatal	0.51 (0.22)	0.64 (0.13)	0.33

*Independent sample *t*-test.

et al. [17]. However, at the moment when ligatures were placed, no differences were observed in body-weight. The statistically significant differences observed during the induction period were of low magnitude and probably did not influence the results negatively.

The results observed in the open field demonstrated significant differences between the test and control rats. These differences were evident in the total number of sectors explored (test animals were hyperactive, which is common in depressive states), as well as in the total number of peripheral sectors (inhibition of the exploratory capacity, which reflects a search for protection) on the second day of evaluation. A higher number of standings observed in the test animals is an indicator of attempts to escape, characteristic in this depression model [25,27].

The combination of results from open field and weight is indicative of a depressive state.

No differences were observed in sexual behavior. However, this test is frequently used in studies with more than 20 animals per group. The sample size for the present study was calculated based on the primary outcome – alveolar bone loss and thus the study groups were suffice for the aim. However, sexual behavior might not have been fully elucidated. The loss in the test group was also observed in the study by Vogel et al. [17]. However, the test group still comprised the necessary number of rats at the end.

The ligatures remained in place for 40 days. A study by our group has shown that no statistically significant differences are observed after 30 days of ligature-induced periodontitis in Wistar rats [16]. However, since the present study used Lewis rats, which tend to lose less bone after ligature placement [28], we decided that 40 days would allow more distinct outcomes.

The primary outcome of the present study was alveolar bone loss. The dry bone measurements for alveolar bone loss have been utilized in several studies [18,19,21,29], which correlates with the histometric measurements and is not subject to cut-positioning bias [21]. The results of the present study demonstrate no statistically significant differences between rats from the test and control groups. This was the case both in teeth with and without ligatures. However, when teeth with and without ligatures are compared, a statistically significant difference is observed, confirming that the induction of alveolar bone loss occurred satisfactorily.

It was evident that the depression model used in this study could not significantly alter the outcome. One might assume that depression is not linked to periodontitis. However, findings from different studies demonstrate that different forms of activation of the HPA axis, similarly to what occurs during depression, can modulate immune features. The role of depressive states in the pathogenesis of periodontitis cannot be ignored.

It may be concluded that induced depression by clomipramine cannot alter ligature-induced bone loss in Lewis rats.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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