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Impacto do estresse precoce de vida sobre a progressão da periodontite apical e da doença periodontal: estudo comportamental e histológico em ratos

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Orientador: Prof. Dr. Daniel Galera Bernabé
Coorientadores: Prof. Dr. Luciano Tavares Angelo Cintra
Profa. Dra. Sandra Helena Penha Oliveira

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“A realidade não consiste apenas nas coisas que já conhecemos. Ela inclui o que existe, mas ainda ignoramos — e que só viremos a conhecer no futuro, talvez quando tivermos construído instrumentos melhores para auxiliar nossos cinco sentidos. Sempre existiram átomos, mas só recentemente tivemos certeza disso, e é provável que nossos descendentes saibam muitas outras coisas que hoje desconhecemos. É o fascínio e o prazer da Ciência: ela revela coisas continuamente.”

Richard Dawkins

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LISTA DE ABREVIATURAS

ABL	Alveolar bone loss
AP	Apical Periodontitis
EDTA	Ethylenediamine tetraacetic acid
ELS	Early life stress
EZM	Elevated Zero Maze Test
H&E	Hematoxylin and Eosin
HPA	Hypothalamic-pituitary-adrenal axis
MS	Maternal Separation
PBD	Post-birth
PD	Periodontal Disease
SNS	Sympathetic Nervous System
WHO	World Health Organization

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RESUMO GERAL

Estímulos estressores durante a infância afetam negativamente as funções fisiológicas, alterando as respostas inflamatórias, imunes, neuro-hormonais e comportamentais. Há evidências de que o estresse precoce de vida (EPV) pode ser um fator de risco direto ou indireto para o desenvolvimento de algumas doenças metabólicas e inflamatórias na idade adulta. No entanto, apesar de existirem evidências de que o estresse influência na progressão da periodontite apical (PA) e da doença periodontal (DP), o impacto do EPV sobre estas doenças é pouco estudado. Portanto, este estudo teve como objetivo avaliar a influência do EPV sobre a PA e DP induzidas experimentalmente em ratos. No presente estudo foram utilizados sessenta ratos machos Wistar, divididos em 6 grupos experimentais (n=10): Grupo controle: ratos não submetidos ao EPV ou a indução das doenças; Grupo EPV: ratos submetidos ao EPV; Grupo PA: ratos com PA; Grupo EPV+PA: ratos submetidos à indução da PA e do EPV; Grupo DP: ratos com DP e Grupo EPV+DP: ratos submetidos à indução da DP e do EPV. O EPV foi induzido pelo método de separação materna (SM) por um período de 3 horas durante 21 dias consecutivos. Ao completarem 90 dias, os animais dos grupos PA e EPV+PA foram submetidos à indução da PA através da exposição pulpar do primeiro e segundo molares superiores do lado direito. A indução da DP foi realizada nos grupos DP e EPV+DP através da inserção e manutenção de uma ligadura no segundo molar superior esquerdo. O protocolo de exposição pulpar para a PA e manutenção da ligadura para os animais com DP, foi de cinco semanas. O teste labirinto elevado em zero (LEZ) foi realizado em todos os animais com 37 dias após a indução das doenças para avaliar o comportamento de ansiedade. Após 3 dias do teste LEZ, os animais foram eutanasiados e as maxilas foram coletadas. Posteriormente, foi realizada análise histológica e histométrica para avaliação do infiltrado inflamatório e extensão das lesões induzidas experimentalmente. A análise histológica revelou maior intensidade do infiltrado inflamatório para os animais com PA e DP expostos ao EPV ($p<0.05$). A

análise histométrica mostrou maior extensão das lesões periapicais nos animais com PA expostos ao EPV quando comparado aos animais com PA não estressados ($p<0.05$). Já para os animais com DP, não houve diferença na extensão da perda óssea alveolar entre os animais com DP submetidos ou não ao EPV ($p>0.05$). A análise comportamental revelou um aumento do comportamento ansioso no grupo PA em relação ao grupo controle ($p<0.05$). No entanto, a exposição ao EPV anulou este efeito da PA sobre o comportamento, já que os animais do grupo EPV+PA frequentaram por mais tempo os braços abertos do aparelho ($p<0.05$). Já para os ratos submetidos à indução de DP, foi observado aumento do tempo nos braços abertos dos grupo DP em relação ao grupo controle ($p<0.05$). O mesmo aconteceu com o grupo DP em relação ao grupo DP-estressado ($p<0.05$). Os resultados do presente estudo indicam que o EPV afeta a progressão da periodontite apical, exacerbando o processo inflamatório e a reabsorção óssea periapical. Contudo, apesar de agravar a intensidade da inflamação na doença periodontal, o EPV não afeta a reabsorção óssea alveolar.

Palavras-chave: Estresse psicológico. Periodontite apical. Doença periodontal.

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GENERAL ABSTRACT

Stress stimuli during childhood negatively affect physiological functions, altering inflammatory, immune, neuroendocrine responses and behavior. There is evidence that early life stress (ELS) can be a direct or indirect risk factor for the development of some metabolic and inflammatory diseases in adulthood. However, although there is evidence that stress influences the progression of apical periodontitis (AP) and periodontal disease (PD), the impact of ELS on these diseases is little studied. Therefore, this study aimed to evaluate the influence of ELS on experimentally induced AP and PD in rats. Sixty Wistar male rats were used in this study, divided into 6 experimental groups (n=10): Control Group: rats not subjected to ELS or to induction of diseases; ELS Group: rats subjected to ELS; PA Group: rats with PA; ELS+AP Group: rats subjected to induction of AP and ELS; PD Group: rats with PD and ELS+PD Group: rats subjected to induction of PD and ELS. ELS was induced by the method of maternal separation (MS) for a period of 3 hours for 21 consecutive days. On completing 90 days, the animals of the AP and ELS+AP groups were subjected to induction of AP through pulpal exposure of the right upper and lower first and second molars. Induction of PD was performed in the PD and ELS+PD groups through the insertion and maintenance of a ligature in the upper second left molar. The pulp exposure protocol for AP and ligation maintenance for animals with PD was five weeks. The elevated zero maze test (EZM) was performed on all animals at 37 days after diseases induction to assess anxiety-like behavior. After 3 days of the EZM test, the rats were euthanized and the jaws were collected. Subsequently, histological and histometric analysis was performed to evaluate the inflammatory infiltrate and extent of the experimentally induced lesions. Histological analysis revealed greater intensity of inflammatory infiltrate for the animals with AP and PD exposed to ELS ($p<0.05$). Histometric analysis showed greater extension of periapical lesions in animals with AP exposed to ELS when compared to non-stressed animals with AP ($p<0.05$). As for the animals with PD, there was no difference in the extent of alveolar bone loss between

the animals with PD submitted or not to ELS ($p>0.05$). Behavioral analysis revealed an increase in anxious behavior in the AP group compared to the control group ($p<0.05$). However, exposure to ELS decreased this effect of AP on behavior, since the animals in the ELS+AP group frequented the open arms of the apparatus for longer. For the PD rats, an increase in time in the open arms was observed compared to the control rats ($p<0.05$). The same was true for the PD rats compared to the PD-stressed rats ($p<0.05$). The results of this study indicate that early life stress affects the progression of apical periodontitis, exacerbating the inflammatory process and periapical bone resorption. However, although it aggravates the intensity of inflammation in periodontal disease, early life stress does not affect alveolar bone resorption.

Keywords: Psychological stress. Apical periodontitis. Periodontal disease.

SUMÁRIO

Capítulo 1

Early life stress predicts more agressive experimental apical periodontitis in adult rats

Early life stress predicts more aggressive experimental apical periodontitis in adult rats *

Running Title: Early life stress and apical periodontitis

Maria Clara Bertolini Botelho[1], Luciano Tavares Angelo Cintra[2], Cristiane Cantiga da Silva[2], Giseli Mitsuy Kayahara[1], Rosani Belzunces Pereira[1], Mylena Fernanda Oliveira Santos[1], Glauco Issamu Miyahara[1,2], Éder Ricardo Biasoli[1,3], Sandra Helena Penha de Oliveira[4] and Daniel Galera Bernabé[1,3].

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1.1 Abstract

Aim To evaluate the influence of early life stress (ELS) on severity of the apical periodontitis (AP) in Wistar rats.

Methodology Forty male Wistar rats were divided into 4 groups (n=10): Control - rats not subject to ELS and AP induction; AP - rats with AP; ELS - rats subject to ELS; ELS+AP - rats exposed to ELS and subject to AP. ELS was induced by maternal separation (MS) for a period of 3 hours for 21 consecutive days. AP was induced via pulp exposure of the first and second right maxillary molars to the oral environment for 40 days until euthanasia. Three days before euthanasia, all rats underwent behavioral analysis to measure anxiety levels. Then, the rats were euthanized and the maxillas were removed to assess the occurrence and progression of AP. The periapical region was evaluated for the intensity of the inflammatory infiltrate and the extent of bone loss. The Mann–Whitney test was performed for nonparametric data, and the Tukey's or Student's t-test was performed for parametric data ($P < 0.05$).

Results The intensity of the inflammatory infiltrate was significantly larger in the ELS+AP group when compared with AP group ($P < 0.05$). The ELS+AP group exhibited significantly greater alveolar bone loss, with a periapical lesion size of 103,5 ± 29,88, compared with 72,3 ± 22,28 in the AP group ($P < 0.05$). Rats with AP displayed higher anxiety-like behavior in relation to control group ($P < 0.05$). However, the exposition to ELS abolished the AP-induced increased anxiety-like behavior, since that rats from ELS+AP group attended more the open arms than non-stressed rats with AP ($p<0.05$).

Conclusion Early life stress is predictive for the severity of apical periodontitis exacerbating the inflammatory process and increasing periapical bone resorption.

Keywords: early life stress; maternal separation; apical periodontitis; anxiety.

1.2 Introduction

In 2010 the World Health Organization (WHO) estimated that over 39% of the world's population was exposed to stressors in childhood and that this number is increasing (Kessler *et al*. 2010). The stress that occurs in the neonatal and infantile periods, called early life stress (ELS), has also been associated with the occurrence of diseases in later stages of life (Danese *et al*. 2007; Liu & Nusslock 2017). The response to physical and social stress is controlled by neurobiological systems such as the hypothalamic-pituitary-adrenal axis (HPA), the Sympathetic Nervous System (SNS), and its pro-inflammatory, immunological, and metabolic components (Godoy *et al*. 2018).

Stressful events that occur early, such as sexual abuse, abandonment, physical and emotional neglect are biologically incorporated because they happen in the period when the pathways for neurobiological regulation of stress response are being formed (Shonkoff *et al*. 2009; McEwen *et al*. 2015; King & Bures 2017). For this reason, individuals exposed to adversity during childhood have a greater susceptibility to disease development when adults (Shonkoff *et al*. 2009; Nusslock & Miller 2016). In bone tissue, for example, recent evidence has shown that adults with a history of ELS have changes in bone homeostasis which are converted into structural changes in adulthood (Wuertz-Kozak *et al*. 2020).

Apical periodontitis (AP) is a disease of inflammatory nature that occurs in response to an infectious insult in the pulp tissue (Nair 2004). The development of AP is dependent on the immune response of the host, with the release of antibodies, cytokines, and chemokines (Stashenko 1990; Howait *et al*. 2019). The intensity of the inflammatory process involved in AP influences bone biology inducing destruction of periapical tissues (Nair 2004). This unbalance between the immune system and bone biology acts as an important cofactor in the progression of AP, and can also be promoted by systemic conditions such as stress (Wippert *et al*. 2017).

Pre-clinical studies show that stress acts on the processes of bone remodeling and resorption making bone healing more difficult (Khoury *et al*. 2020; Wuertz-Kozak *et al*. 2020). Increased periapical bone resorption in rats with induced AP was observed in stressed animals when compared to unstressed animals (Gomes *et al*. 2019). However, the number of investigations seeking to understand the influence of stress

on AP pathogenesis is still limited. Moreover, there are no studies that have investigated the influence of stress in early life phases on AP. Therefore, the objective of this in vivo study was to investigate the relationship between ELS on AP progression. The null hypothesis is that there is no relationship between apical periodontitis and early life stress.

1.3 Materials and Methods

1.3.1 Animals and maintenance conditions

All experiments were performed according to the guideline of principles for the use of animals in the laboratory and the study was approved by the Animal Welfare Committee at the Araçatuba Dental School (São Paulo State University – Unesp, Araçatuba, São Paulo, Brazil) (Protocol Number: 00506-2019). For the study, 40 male rats (Rattus novergicus albinus, Wistar) were used. Sample size was estimated based on data from previous studies (Azuma *et al*. 2017a; Azuma *et al*. 2017b; Dal-Fabbro *et al*. 2019). Considering an alpha error of 0.05% and 95% power to recognize a significant difference of 1 in the median scores, a minimum of seven animals per group was necessary. Considering possible animal deaths, three more animals were added in each group, resulting in ten rats per group (Cosme-Silva *et al*. 2019). The animals were group-housed (4 per cage) (25.9 × 47.6 ×20.9 cm, polypropylene), under standardized conditions (22 ± 2 °C, 12/12-h light-dark cycle; lights on at 7:00h).

1.3.2 Experimental design

In order to evaluate whether ELS affect the progression of apical periodontitis (AP), the study was composed of four experimental groups: 1) Control: 10 male Wistar rats not subjected to ELS and not induced for AP; 2) ELS: 10 male Wistar rats subjected to ELS; 3) AP: 10 male Wistar rats subjected to AP, and 4) AP+ELS: 10 male Wistar rats submitted to ELS followed by AP induction in adulthood. After 21 days of induction or not of ELS in the postnatal period, all animals were weaned and separated by sex on day 22 after birth. Only male rats from each group were used for the later phases of the experiment. On completing 90 days of age, the rats from AP and AP+ELS groups were subjected to AP induction for 40 days until euthanasia. Three days before

euthanasia, all rats underwent behavioral analysis to measure anxiety levels. Then, the rats were euthanized and the maxillas were removed to assess the occurrence and progression of AP.

1.3.3 Early life stress induction

ELS was induced through maternal separation (MS). After birth (1st day post-birth - PBD), the litter was randomly divided among the experimental groups. The control rats were not disturbed during this period, except for cleaning of the cages. The ELS rats were separated from their mothers for 3 hours a day, from day PBD 1 to PBD 21. The MS protocol consisted in transferring the litters to another box, containing a thermal blanket with regulated temperature (32ºC) to compensate the maternal heat (Roman *et al.* 2006; Nishi *et al.* 2013). To avoid any maternal contact, the boxes containing the litters were taken to a separate room and after 3 hours were returned to the box with the mother. The weaning of all rats was made on the PBD 22, when they were grouped (4 rats per box) until they were 90 days old. In this age, the rats were subjected to anxiety-like behavioral test (Figueira 2018).

1.3.4 Induction of apical periodontitis

The pulp exposure for induction of periapical lesions was performed when the animals were 90 days old. The dental pulps of the first and second right maxillary and mandibular molars of rats from the AP and rats from the ELS+AP groups, were exposed by means of a carbon steel drill - Ln Long Neck (Dentsply/Maillefer, Ballaigues, Switzerland). The pulps got exposed for 40 days, allowing the periapical lesion formation (Cintra *et al.* 2016).

1.3.5 Anxiety-like behavior test

To assess whether ELS and AP induction promote changes in the anxiety-like behavior, the rats were subjected to the elevated zero maze (EZM) test. Before being tested, the rats were kept undisturbed over a one hour in a separate room for adaptation to the environment. EZM is an annular platform elevated 50 cm above the floor and 105 cm of diameter divided in two open arms and two closed arms opposite.

Rats were tested in the EZM apparatus (Insight LTDA, Ribeirão Preto, Brazil) under low light (15 lux). Individually, the rats were placed within one of the closed arms of apparatus and its activity was recorded for 10 minutes by a video camera mounted above the maze (Kayahara *et al.* 2020). The activity of each mouse was recorded and analyzed in real time using the ANY-maze software (Stoelting Co., Wood Dale, IL, USA). Time spent and number of entries in the open arm was later scored and used to quantify anxiety levels.

1.3.6 Sample obtaining and processing

Three days after the behavioral test (PBD 130), the rats were euthanized by decapitation. After euthanasia, the jaws containing maxillary molars were collected and fixed during the first 24 hours in a buffered solution with 4% formalin at neutral pH. The specimens were washed in running water for a period of 12 hours and placed in EDTA 10% for decalcified (Sigma-Aldrich St. Louis - Missouri, USA). Subsequently the tissues were paraffin-embedded and the first molar was sectioned at 4 μm along it longitudinal axis and stained with Hematoxylin and Eosin (H&E) (Cintra *et al.* 2014).

1.3.7 Histological and histometric analysis

The histologic analysis was performed to check if there were differences in the intensity of the inflammatory infiltrate in all groups. The intensity of periapical inflammation was analyzed in H&E staining by assigning scores graded as follows: no inflammation (score 1: 0 or few inflammatory cells), mild inflammation (score 2: <25 inflammatory cells), moderate inflammation (score 3: 25–125 inflammatory cells), and severe inflammation (score 4: >125 inflammatory cells) (Dal-Fabbro *et al*. 2019).

Histometric analysis was performed in the AP and ELS+AP groups to check for periapical bone loss when AP was associated with early life stress. The Leica LAS X (Leica Microsystems, Nussloch - Germany) was used to perform the historical analysis and the values were expressed in square micrometers (μm^2) obtained in area measurements (Azuma *et al.,* 2017a).

1.3.8 Statistical analysis

All data collected were tabulated in Microsoft Office Excel 2016 and GraphPad Prism 8.02 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform the statistical analysis. The data were tested for normality through specific tests. To evaluate whether ELS affects the severity of apical periodontitis, *Student's t*-test was used. The data from the evaluation of the intensity of the inflammatory infiltrate were analyzed by performing multiple comparisons with the *Mann-Whitney U* test. For data from behavioral test, we performed the analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons and *Student's t*-test. The results were presented in mean ± standard error of mean (SEM). The critical level was set at 5% ($p<0.05$) to admit differences in values as statistically significant.

1.4 Results

1.4.1 Histological and histometric analysis of Apical Periodontitis

The histological analysis revealed that the rats not subjected to AP induction (control and ELS groups) showed no signs of pulp and periapical inflammatory response (Table 1; Figure 1A,a1,a2,B,b1,b2). However, the evaluation of AP and ELS+AP groups showed the presence of inflammatory infiltrate with necrosis areas after 40 days of pulp exposure, confirming the AP induction (Figure 1C,c1,c2,D,d1,d2). The ELS+AP group showed a more intense chronic inflammatory infiltrate when compared to the AP group ($p = 0.0461$) (Table 1, Figure 1C,c1,c2,D,d1,d2). The histometric analysis revealed that the intense chronic inflammatory infiltrate observed in the periapical region of rats with AP exposed to ELS was accompanied by a more extensive periapical lesion, with a larger area of bone resorption in relation to the AP group ($p = 0.0164$) (Table 1, Figure 1C,c1,c2,D,d1,d2).

Table 1 Scores, median, mean, and standard deviation of the histological findings

Histological parameters		**Groups**					**Statistical analysis**
		Healthy rats			Stress rats		
		Control	AP		ELS	ELS+AP	
Intensity of inflammatory cell infiltrate in AP	1 - Absent	10/10	0/10		10/10	0/10	Mann Whitney AP-h × AP-s: p = .0461
	2 - Mild	0/10	7/10		0/10	3/10	
	3 - Moderate	0/10	3/10		0/10	3/10	
	4 - Severe	0/10	0/10		0/10	4/10	
	Median	1	3		1	4	
Periapical lesions (×10.000 μm^2)		-	72.3 ± 22.28^a		-	103.5 ± 29.88^b	T test (Student) AP-h × ELS-s: p = .0164

*Same letters on the line indicate no statistical difference among the groups (p > 0.05).

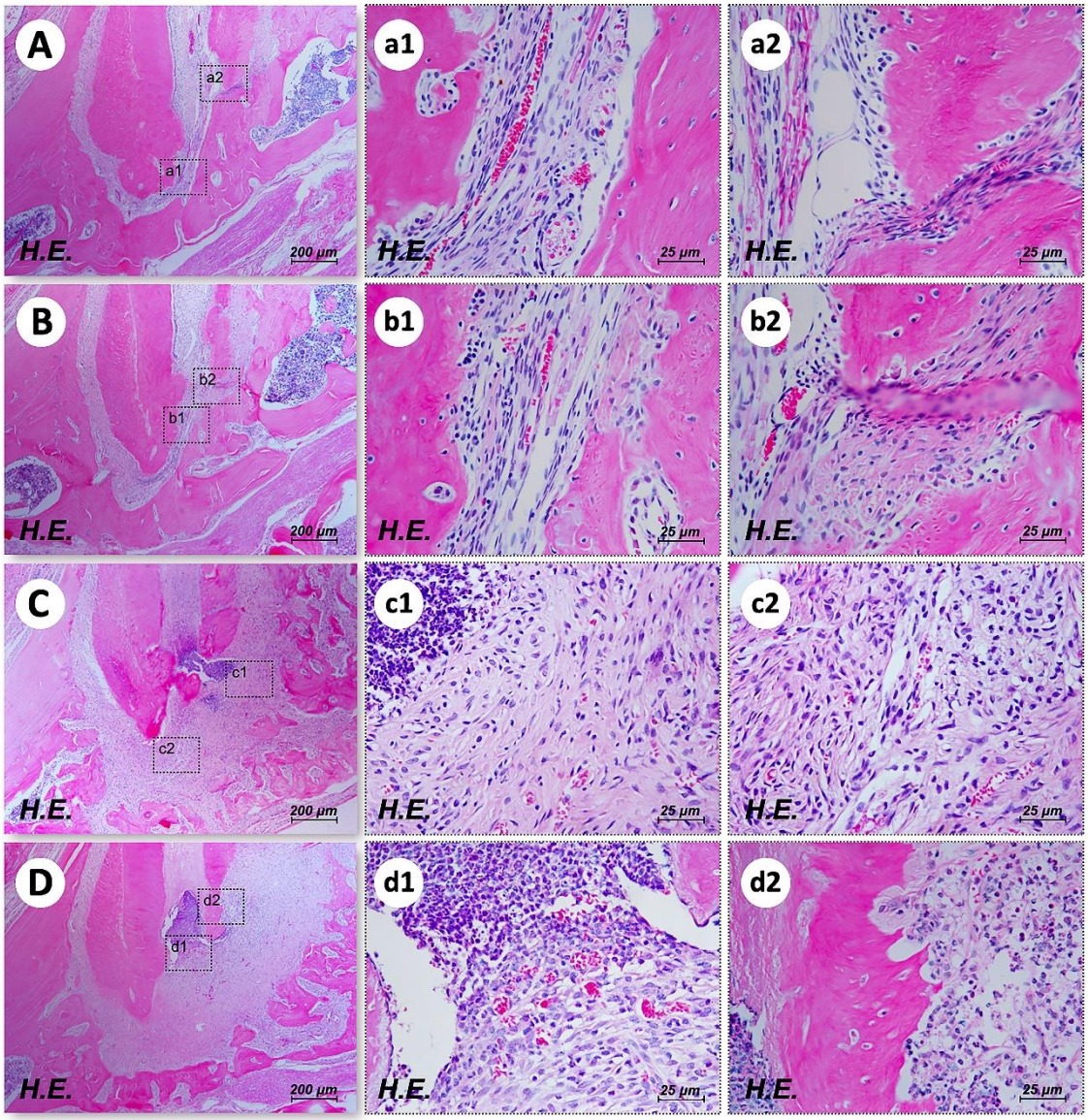

Figure 1. Photomicrographs of periapical region after 40 days of pulp exposure. H&E staining in control rats **(A, a1, a2)** showed periapical region free of inflammatory infiltrate; ELS rats **(B, b1, b2)** showed similar aspects of control rats with periapical region free of inflammatory infiltrate; Rats with AP **(C, c1, c2)** showed a moderate inflammatory infiltrate around the apex, disruption of periodontal ligament and bone resorption area; Rats with AP previously subjected to ELS **(D, d1, d2)** had intense inflammatory infiltrate, as well as extensive periapical bone resorption area. (A-D, 50x; a1-d1 and a2-d2, 400x increase).

1.4.2 Anxiety levels in rats subject to ELS and AP

In order to assess if there was a difference in anxiety levels among the experimental groups, all animals were subjected to the EZM test. Analysis of variance followed by the *Tukey test* for multiple comparisons did not indicate significant differences in anxiety-like behavior among the groups ($p>0.05$). However, when the groups were compared to each other using *Student's t*-test the results showed that rats with experimentally induced AP spent less time in the open arms (32.63 ± 5.80 sec) compared to the control rats (52.50 ± 6.89 sec) ($p = 0.0408$) (Figure 2A). However, exposition to ELS abolished the AP-induced increased anxiety-like behavior, since that rats from ELS+AP group attended more the open arms than non-stressed rats with AP ($p<0.05$) (Figure 2A). Rats exposed only to ELS attended less the open arms of EZM, but this result did not reach statistical significance ($p>0.05$) (Figure 2A). Regarding the number of entries in the open arms, the stressed rats had a lower number of entries (10 ± 1.11 entries) when compared to the control rats (6.6 ± 0.96 entries) ($p = 0.033$) (Figure 2B). The non-stressed rats with AP displayed lower number of entries in the open arms (7.30 ± 0.91 entries) in relation to AP-stressed rats (12.10 ± 1.84 entries) ($p = 0.035$; 0.031) (Figure 2B). AP rats previously exposed to ELS had more entries on the open arms (12.10 ± 1.84 entries) compared with non-stressed AP rats (6.60 ± 0.96 entries) ($p = 0.016$) (Figure 2B).

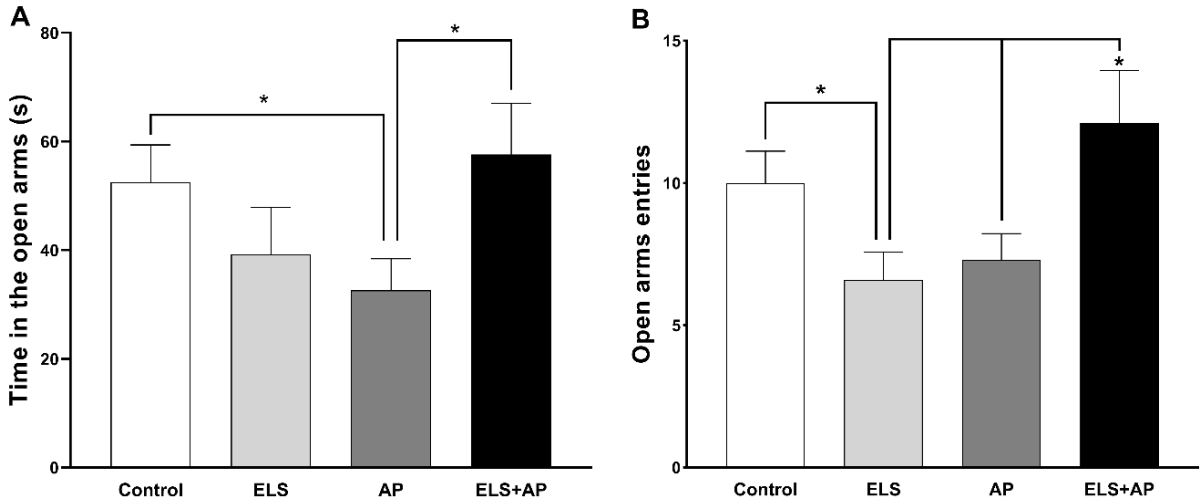

Figure 2. Comparison of anxiety-like behavior measured by EZM among experimental groups. **A**. There were no significant differences between the control and ELS groups. AP rats attended for less time the open arms of the apparatus in relation to control rats, showing more anxiety in the rats subjected to AP induction. The same was observed in the AP rats when comparing to AP-stressed rats, where AP rats attended for less time the open arms when compared to the animals stressed with the disease. **B.** ELS rats had less entries in the open areas than control rats. ELS rats also had less entries in the open arms of the apparatus when compared with AP-stressed rats. When comparing the AP and AP-stressed groups, we notice that the AP rats tried less often to enter the open arms. Bar graphs represent mean ± SEM. Test *t* de *student*. *p < 0.05.

1.5 Discussion

This appears to be the first experimental study analyzing the relationship between apical periodontitis and early life stress. We evaluated the impact of ELS on induced AP progression using a model of maternal separation (MS) which consists of the separation of litter from their mothers for a certain time (Ishikawa *et al.* 2015). This method is strongly consolidated because it is performed in the first days of the litter's life, a period of which the close contact with the mother is responsible for the cognitive and emotional development of the offspring (Arborelius & Eklund, 2007; Nishi *et al*. 2013; Ishikawa *et al*. 2015). Our results showed that rats exposed to ELS by maternal separation displayed more destructive periapical lesions with an intense chronic inflammatory process compared to the rats not stressed in neonatal period. Thus, the null hypothesis was rejected.

Adversities that occur during childhood remain "under the skin" during the development of the individual, directly influencing their neuroendocrine and immune systems (Nusslock & Miller, 2016; Liu and Nusslock, 2017). From there, a negative

feedback loop begins, conferring vulnerability to the development of diseases in adulthood (Nusslock & Miller, 2016; Liu & Nusslock, 2017). In addition, ELS can promote neurobiological and behavioral changes that predispose to physical and psychological disorders (McEwen *et al*. 2015; Nusslock & Miller, 2016).The most interest finding of our study was the larger size of the periapical lesions in rats exposed to ELS in relation to non-stressed AP rats. We used a AP induction methodology based on previous studies that also investigated possible relationships between AP and systemic changes (Cintra *et al*. 2014; Azuma *et al*. 2018; Samuel *et al*. 2019, Conti *et al*. 2020). Studies exploring the influence of stress on AP pathogenesis are scarce. Our finding is consistent with that of a previous study that observed a greater extent of periapical bone loss in animals subjected to conditioned fear stress (Gomes *et al*. 2019). In a recent study Khoury *et al.* (2020) observed no differences in the aggressiveness of periapical lesions between control and stressed animals. However, we cannot make comparisons of our study with that of Khoury *et al.* (2020), because they used a chronic mild stress model, which consists of the chronic sequential application of different stress stimuli widely used to study depression (Willner *et al*. 1992; Duda *et al*. 2019).

In our study we used a model of ELS induced by MS, a method of stress that causes permanent effects on the functioning of the innate and adaptive immune system, enabling the progression of the inflammatory phenotype (Elwenspoek *et al*. 2017). There is a bidirectional pathway between the immune system and bone tissue, to the detriment of a common origin of inflammatory and bone cells in bone marrow (Dar *et al*. 2018). The process of bone remodeling and resorption is a constant balance between immune factors such as cytokines, hormones, and neurotransmitters (Grässel. 2014; Alves *et al*. 2016; Dar *et al*. 2018). For this reason, imbalances in the remodeling process are usually related to the occurrence of inflammatory diseases such as periodontal disease and apical periodontitis (Dar *et al*. 2018). A recent study revealed that ELS, both in animals and humans, promotes a negative adaptive response of bone homeostasis (Wuertz-Kozak *et al*. 2020). ELS can induce an alteration in bone innervation with a reduced remodeling process and lower bone healing capacity (Wuertz-Kozak *et al*. 2020). As previously mentioned, we can infer that stress can exacerbate the imbalance in bone biology, since it affects directly the neuroendocrine and immune systems.

In our results the rats with AP previously exposed to ELS had a more intense chronic inflammatory phenotype. Khoury *et al*. (2020) did not observe differences in the severity of inflammation after pulp exposure in stressed rats. Curiously, there was a higher number of stressed rats with lower scores for the inflammatory infiltrate (Khoury *et al*. 2020). It is not surprising the difference in results between our study and that of Khoury *et al*. (2020). The effects of chronic stress on the inflammatory phenotype of a particular disease depend directly on the experimental circumstances and age of the animals (Sagiyama *et al*. 2004; Ostrander *et al*. 2006; Dumitrescu 2016). The main effects of ELS on the immune system are the hyperresponsiveness of inflammatory cells with constant low-grade activation and accelerated immunosenescence process (Elwenspoek *et al*. 2017). Evidences have indicate an increase in the levels of pro-inflammatory cytokines and a disparity in sensitivity to glucocorticoids in children exposed to different adversities during childhood (Miller & Chen 2010; Nusslock & Miller 2016). Inflammation has an important role in the AP pathogenesis with the releasing of cytokines and other inflammatory promoting the maintenance and progression of periapical lesion (Stashenko 1990). It is plausible to consider that the AP-associated inflammation has been exacerbated in animals exposed to ELS by them already having an altered inflammatory profile.

Our results also showed that rats with AP displayed increased anxiety-like behavior, represented by spending less time in the open arms of the EZM. This may indicate that AP alone is a potential stressor agent, capable of significantly altering behavior. Interestingly, the AP-induced anxiety-like behavior was abolished when the rats were previously exposed to ELS. Although we did not measure the pain behavior in rats with AP, a recent study showed that rats subjected to AP induction developed mechanical allodynia and hyperalgesia after pulpal exposure (S.B. *et al*. 2020). Studies have suggested that ELS is a potent mediator of pain nociception, altering sensitivity to painful stimuli derived from the inflammatory conditions in later life. Genty *et al.* (2018) observed that ELS reduced the hyperalgesia caused by the injection of Complete Freunds Adjuvant (CFA) an important immunostimulatory substance (Genty *et al*. 2018). The same hyporesponsiveness to pain caused by the ELS was observed when rats exposed to stress in early stages developed lower sensitivity to pain from a second stressor stimulus in adulthood (Post *et al*. 2014). Therefore, we assume that

exposure to early stress may has made the rats hyporesponsive to painful stimuli possibly caused by AP.

The current study has some limitations. First, we did not perform the characterization (e.g., by immunohistochemistry) of the inflammatory cells in the infiltrate of the periapical lesion. Second, as mentioned before the measurement of pain in rats induced for AP was not performed. In the current investigation we sought to identify whether there was an interrelation between ELS and AP and secondarily its impacts on anxiety-like behavior. In view of our findings, further studies are needed to explore the mechanisms involved in the association between periapical disease and anxiety. In conclusion, our results demonstrate for the first time that early life stress affect the severity of induced apical periodontitis promoting an exacerbated inflammatory response and increased periapical bone resorption. Clinically, these findings suggest that stress experienced in childhood can be predictive for a phenotype of greater severity of apical periodontitis.

Conflict of Interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Capítulo 2

Early life stress aggravates the inflammation of periodontal disease induced in rats

Early life stress aggravates the inflammation of periodontal disease induced in rats *

Running Title: Early life stress and periodontal disease

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2.1 Abstract

Early life stress (ELS) negatively affects physiological mechanisms that increase the susceptibility to various diseases in adulthood. This study aimed to evaluate the influence of ELS on experimental periodontitis in a pre-clinical model. Forty male Wistar rats were randomly divided into four groups: control, periodontal disease (PD), early life stress (ELS) and periodontal disease exposed to early life stress (ELS+PD). ELS was induced by using a maternal separation (MS) model in neonatal period. On completing 90 days of age, induction of PD was performed with a ligature on the second left maxillary molar for five weeks. The elevated zero maze test (EZM) was performed to evaluate anxiety-like behavior. Histological analysis was performed in the jaws to evaluate the periodontal inflammatory infiltrate and alveolar bone loss. The Mann–Whitney test was performed for nonparametric data, and the Tukey's or Student's t-test was performed for parametric data ($P < 0.05$). Histological evaluation revealed a greater intensity of inflammatory infiltrate in PD rats exposed to ELS compared to unstressed rats ($p<0.05$). However, no difference was observed among the experimental groups for the measurement of alveolar bone loss ($p>0.05$). Behavioral analysis showed reduced anxiety-like behavior in the PD group in relation to control group ($p<0.05$). The same can be observed in the ELS+PD group compared to non-stressed PD rats ($p<0.05$). Thus, we can conclude that early life stress aggravates the intensity of inflammatory response in chronic periodontal disease but does not affect alveolar bone resorption.

Keywords: Early life stress. Psychological stress. Anxiety. Periodontal disease. Inflammation.

2.2 Introduction

Periodontitis is a chronic inflammatory process that results in an evolution of gingivitis and reaches the dental support tissues, such as the periodontal ligament, cement and alveolar bone.[1] The disease is a consequence of the reaction between bacterial antigens from the dental biofilm and the host's defense cells.[1,2] The epithelial cells of the gingival tissue, trigger immune responses as a result of the bacterial challenge.[1,2] The release of chemokines and cytokines promotes a cascade of pro-inflammatory events and the persistence of bacterial biofilm prolongs this response and generates damage to dental support tissues.[2] It is known that systemic health such as diabetes and environmental factors such as smoking play a critical role in the maintenance and progression of periodontitis.[3]

Recent evidences suggest that psychosocial factors such as stress, anxiety and depression may also influence the pathogenesis of the disease.[4,5] Stress can act on behavioral factors such as poor nutrition and inadequate oral hygiene that directly influence the development of periodontitis.[6,7] At the same time, chronic stress can favor the progression of periodontitis through the promotion of neural circuit maladjustments, immunological and endocrine changes.[8] Modifications in the immune system generate a cascade of negative events, such as an increase in the inflammatory response and a consequent imbalance in bone tissue that loses its ability to remodel.[9,10] These effects can be observed in some studies where the alveolar bone loss (ABL) was higher in rats induced to periodontal disease (PD) and exposed to physical stress by immobilization[11] or restriction[12].

In the last few decades, there has been a growing number of investigations demonstrating the negative impact of early life stress (ELS) on health in adulthood.[13,14] ELS consists of a broad spectrum of adversities that occur during the early stages of life, a critical period for neurobiological, endocrine and immune development.[14–18] Because it occurs at an important stage in the development of the neuronal circuits, ELS promotes allostatic overload that results not only in neurological but also somatic consequences.[14,19] A number of researchers have found an association between the occurrence of adversities during childhood and predisposition for diseases throughout the lifespan.[13,14,16,20] Individuals exposed to ELS have a susceptibility to psychiatric disorders such as depression, anxiety and schizophrenia.[21–23] In addition, the

occurrence of metabolic, cardiovascular and inflammatory diseases in patients exposed to potentially stressful events during childhood have also been demonstrated.[24–28] In this context, a study showed a high ABL in animals exposed to ELS induced by maternal separation (MS) and experimental PD.[29] This impact was explored in a recent investigation, which shows the deleterious effects of ELS on homeostasis and bone structure.[10]

Classically renowned in science, the MS is a well-established ELS model that mimics in a pre-clinical model, adversities like abandonment and neglect.[30,31] Studies using MS have shown that this model can negatively alter the immune system as well as promote changes in neuronal stress response circuits.[31] In the current investigation, we used the MS model to investigate the impact of ELS on the progression of periodontal disease in rats. The animals' behavior was also evaluated to verify whether the effects of ELS on periodontal disease were accompanied by changes in anxiety behavior. The null hypothesis is that there is no relationship between periodontal disease and early life stress.

2.3 Material and Methods

2.3.1 Animals and maintenance conditions

All experiments were performed according to the guideline of principles for the use of animals in the laboratory and the study was approved by the Animal Welfare Committee at the Araçatuba Dental School (São Paulo State University – Unesp, Araçatuba, São Paulo, Brazil) (Protocol Number: 00569-2019). For the study, 40 male rats (Rattus novergicus albinus, Wistar) were used. The animals were group-housed (4 per cage) (25.9 × 47.6 ×20.9 cm, polypropylene), under standardized conditions (22 ± 2 °C, 12/12-h light-dark cycle; lights on at 7:00h).

2.3.2 Experimental design

In order to evaluate whether ELS affect the progression of periodontal disease (PD), the study was composed of four experimental groups: 1) Control: 10 male Wistar rats not exposed to ELS and not induced for PD; 2) PD: 10 male Wistar rats subjected

only to PD; 3) ELS: 10 male Wistar rats subjected only to ELS; and 4) PD+ELS: 10 male Wistar rats exposed to ELS followed by PD induction in adulthood. After 21 days of induction or not of ELS in the postnatal period, all animals (only male rats) were weaned and separated by sex on day 22 after birth. Rats from the PD and ELS+PD groups were subjected to PD induction at 90 days of age. Posteriorly, after 37 days of PD induction, all rats were tested in the elevated zero maze (EZM) to evaluate anxiety-like behavior. After three days, the rats were euthanized, and the maxilla of each rat was removed to assess the occurrence and progression of PD.

2.3.3 Early life stress model

ELS was induced through maternal separation (MS). After birth (1st day post-birth - PBD), the litters were randomly divided among the experimental groups. The control rats were not disturbed during this period, except for cleaning of cages. The ELS and ELS+PD rats were separated from their mothers for 3 hours daily, from PBD 1 to PBD 21. The MS protocol consisted in transferring the litters to another box, containing a thermal blanket with regulated temperature (32ºC) to compensate the maternal heat.[31] To avoid any maternal contact, the boxes containing the litters were taken to a separate room and after 3 hours they were returned to the box with the mother. The weaning of all rats was made on the PBD 22, when they were grouped (4 rats per box).[32]

2.3.4 Induction of periodontal disease

For induction of periodontal disease, the rats from the PD and PD+ELS groups were anesthetized and the left upper second molar was selected for induction of periodontal disease. Induction was performed with a ligature as described by Cintra et al.[33] A number 24 cotton ligature was made around the upper second molar. The ligatures remained for the entire post-operative period (40 days).[34]

2.3.5 Anxiety-like behavior test

To assess whether PD induction (associated or not to ELS) promotes changes in the anxiety levels, the rats were tested in the EZM test. Rats were tested in the EZM apparatus (Insight LTDA, Ribeirão Preto, Brazil) under low light (15 lux). EZM is an annular platform elevated 50 cm above the floor and 105 cm of diameter divided in two open arms and two closed arms opposite. Before being tested for anxiety-like behavior, the rats were kept undisturbed over a one hour in a separate room for adaptation to the environment. After, the rat was placed within one of the closed arms of apparatus and its activity was recorded for 10 minutes by a video camera mounted above the maze. Activity of each rat was video recorded and analyzed using the ANY-maze software (Stoelting Co., Wood Dale, IL, USA). Time spent and number of entries in the open arm was later scored and used to quantify anxiety level.[35]

2.3.6 Sample obtaining and processing

The rats were euthanized by decapitation three days after the behavioral test (PBD 130). After euthanasia, the jaws containing maxillary molars were collected and fixed during the first 24 hours in a buffered solution with 4% formalin at neutral pH. The specimens were washed in running water for a period of 12 hours and placed in EDTA 10% for decalcified (SIGMA-ALDRICH St. Louis - Missouri, USA). Subsequently the tissues were paraffin-embedded and the first and second molars was sectioned at 4 µm along it longitudinal axis and stained with Hematoxylin and Eosin (H&E).[36]

2.3.7 Histological and histometric analysis

Histological analysis was performed in the H&E sections to verify if there were differences in the intensity of the PD-associated inflammatory infiltrate, by assigning scores graded as follows: no inflammation (score 1: 0 or few inflammatory cells), mild inflammation (score 2: <25 inflammatory cells), moderate inflammation (score 3: 25–125 inflammatory cells), and severe inflammation (score 4: >125 inflammatory cells). Histometric analysis was performed in the PD and ELS+PD groups to verify alveolar bone loss (ABL) when PD was associated with stress. This evaluation was done by measuring the distance between the cementoenamel junction and the vestibular

alveolar bone crest. Leica's LAS X (Leica Microsystems, Nussloch - Germany) was used to perform the histometric analysis and the values were expressed in square micrometers (μm^2) obtained in area measure.[37]

2.3.8 Statistical analysis

All data collected were tabulated in Microsoft Office Excel 2016 and GraphPad Prism 8.02 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform the statistical analysis. The data were tested for normality through specific tests. Nonparametric data, representing the analysis of the intensity of the inflammatory infiltrate, were analyzed by performing with the Mann-Whitney *U* test applied. To evaluate whether ELS exposition influenced the severity of alveolar bone loss and anxiety-like behavior, *Student's t*-test and analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons were used. The results were presented in mean ± standard error of mean (SEM). The critical level was set at 5% ($p<0.05$) to admit differences in values as statistically significant.

2.4 Results

2.4.1 Histological and histometric analysis of Periodontal Disease

Histological images of PD in different experimental groups are shown in Figure 1. Analysis of the sections stained in H&E revealed absence of inflammation in periodontal region in the control and ELS groups (Table 1; Fig. A, a1; C, c1). However, rats from PD and ELS+PD groups displayed a large tissue destruction, proving the effectiveness of the inductor method. The histological evaluation revealed a higher intensity of inflammatory infiltrate associated with PD in rats exposed to ELS compared to non-stressed PD rats ($p = 0.0186$) (Table 1; Fig. B, b1-b5; D, d1-d5) (Table 1). Although the bone resorption in the stressed rats had characteristics of greater aggressiveness in relation to the PD rats, the result of the histometric analysis did not reach statistical significance ($p > 0.05$) (Table 1; Fig. B, b1-b5; D, d1-d5).

Table 1 Scores, median, mean, and standard deviation of the histological findings

Histological parameters		Groups				Statistical analysis
		Healthy rats		Stress rats		
		Control	PD	ELS	ELS+PD	
Intensity of inflammatory cell infiltrate in PD	1 - Absent	10/10	0/10	10/10	0/10	Mann Whitney PD × ELS+PD: $p = 0.0186$
	2 - Mild	0/10	1/10	0/10	0/10	
	3 - Moderate	0/10	7/10	0/10	2/10	
	4 - Severe	0/10	2/10	0/10	8/10	
	Median	1	3	1	4	
Alveolar Bone Loss (×10 µm)		482.6 ± 591.3[a]	157.9 ± 7.372[a]	582.6 ± 591.3[a]	158 ± 800.9[a]	T test (Student) C × ELS: $p = 0.2594$ PD × ELS+PD: $p = 0.994$

*Same letters on the line indicate no statistical difference among the groups ($p > 0.05$).

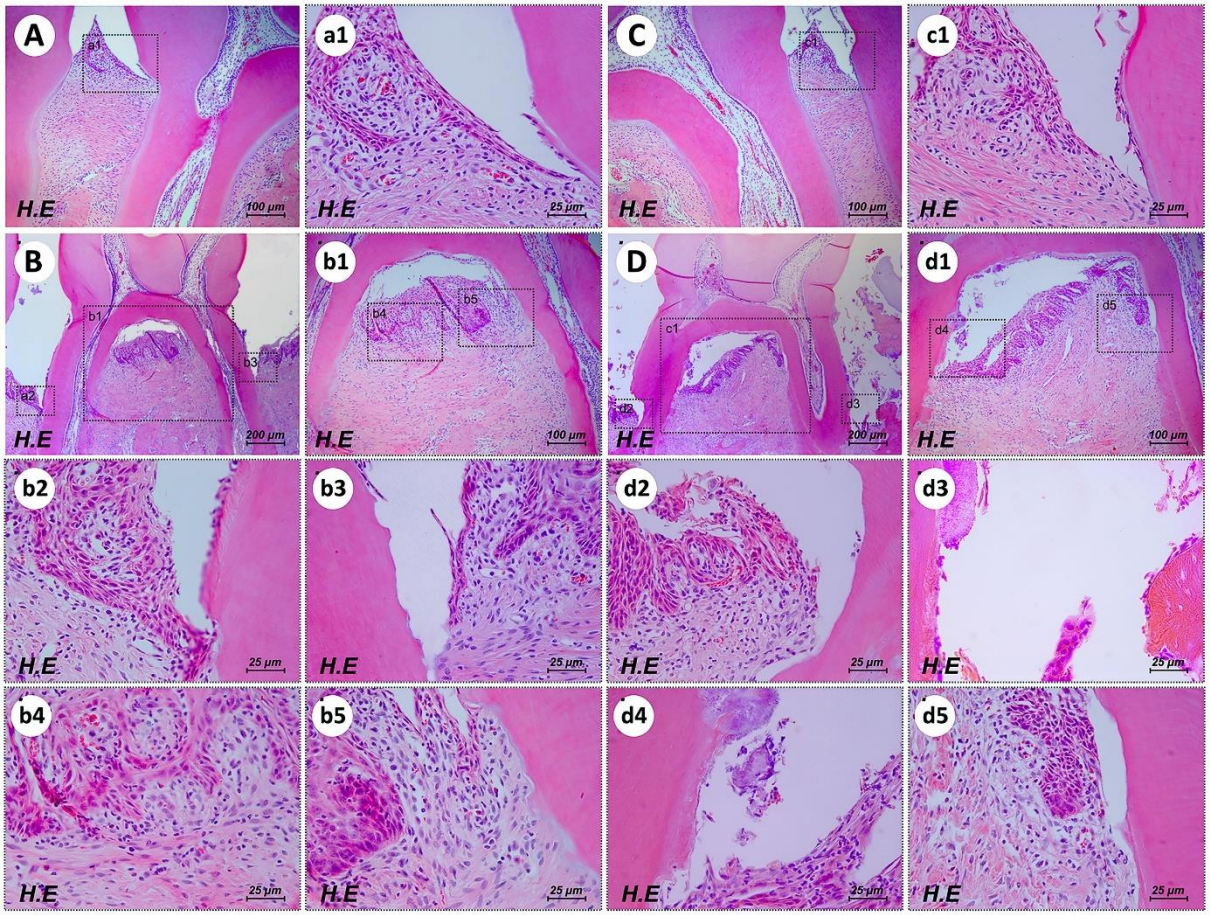

Figure 1. Representative images of histological analysis of periodontal region. H&E staining in control group **(A, a1)** showed periodontal region free of inflammatory infiltrate (x100 and x400, respectively); PD group showed a moderate bone resorption area with loss of conjunctive insertion **(B)** (x50), and moderate inflammatory infiltrate around the roots and in furca region **(b1-b5)** (x100 and x400, respectively); ELS group **(C, c1)** showed similar aspects of control group with periodontal region free of inflammatory infiltrate (x100 and x400, respectively); ELS+PD group showed a large bone resorption area with loss of conjunctive insertion **(D)** (x50), and moderate inflammatory infiltrate around the roots and in furca region **(d1-d5)** (x100 and x400, respectively).

2.4.2 Anxiety-like behavior in rats subjected to ELS and PD

To evaluate the behavioral alterations after PD induction associated or not with ELS exposition, all rats were tested for anxiety-like behavior through EZM test. Statistical differences between groups were determined by the analysis of variance followed by the *Tukey test for* multiple comparisons, where no statistical significance was observed. Then we performed the analysis using the *Student's t*-test. When comparing the ELS and control groups we observed that there were no differences for the time spent in the open arms ($p > 0.05$) (Figure 2A). In evaluating the anxiety-like behavior of the PD group, we observed an increase in the time spent in the open arms of the apparatus (85.08 ± 10.49 sec) compared to control rats (52.50 ± 6.89 sec) ($p = 0.0182$) (Figure 2A). In contrast, the stressed PD rats attended less the open arms of the apparatus (85.08 ± 10.49 sec) compared to non-stressed PD rats (37.94 ± 7.98 sec) ($p=0.0022$) (Figure 2A). The ELS rats entered less in the open arms (6.60 ± 0.96 entries) compared to the control rats (10 ± 1.11 entries) ($p = 0.035$) (Figure 2B). The same did not apply when comparing the PD and control groups ($p > 0.05$) (Figure 2B). However, non-stressed PD rats showed a higher number of entries (12.89 ± 1.38 entries) than PD rats exposed to ELS (8.30 ± 1.54 entries) ($p = 0.042$) (Figure 2B).

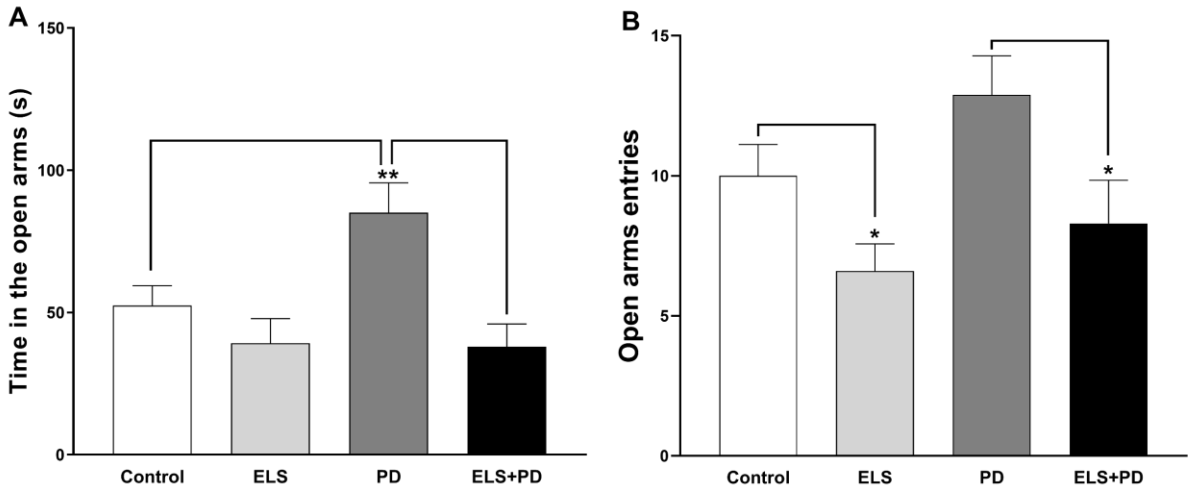

Figure 2. Anxiety-like behavior in the rats with PD exposed or not to ELS. A. There were significant differences between the PD and control groups. The same was observed when comparing PD-stressed and PD non-stressed rats. **B.** The ELS rats displayed a lower number of entries in relation to control rats. Likewise can be observed for the stressed group subjected to PD induction compared to the PD group not exposed to ELS. Bar graphs represent mean ± SEM. Test *t* de *student*. *p < 0.05.

2.5 Discussion

This study set out to explore the influence of early life stress on the progression of experimental periodontitis in Wistar rats. Our results showed that rats exposed to stress by maternal separation in childhood had higher intensity of inflammatory infiltrate in PD. However, this result was not accompanied by a greater ABL. Thus, the null hypothesis was partially rejected. Because of the great anatomical and histological similarities, pre-clinical models with rats are widely used to investigate the pathogenesis and progression of the periodontal disease.[38] In this context, there are several methods of induction that differ in the time of induction of the disease, location, and some sites also, to the material used.[38,39] The models using ligation, as in our study, stand out for being very effective for the retention of biofilm and bacterial colonization on the surface of the material, which accelerates the process of bone destruction.[40-44] Some doubts still pervade the time in which the ligature must be maintained. Studies have showed that bone loss begins with 7 days of induction, and with 15 days it is already possible to

have considerable bone damage.[45,46] However, models of more than 4 weeks have been frequently used.[35,46]

In our study, exposition to ELS did not induce significant impact on DP-induced ABL. A strong relationship between adversities that occur during childhood and the process of bone remodeling has been reported.[10,47] Wuertz-Kozak et al.[10] showed that animals subject to MS had a catabolic bone profile, with changes in innervation and bone mediators related to bone healing process. A decrease in bone density was also found in adults exposed to adversity during childhood.[10] Breivik et al.[29] showed that rats subjected to MS had greater bone resorption when compared to unstressed rats. In our study, although visually the periodontal lesions were more aggressive, there was no statistical significance when comparing the groups with PD with and without early life stress. In part, although our result does not corroborate with those observed by Breivik et al.[29], there are methodological differences between both studies. In our study, we performed MS for 21 consecutive days and the ligation time was 40 days for rats with PD, while the protocols by Breivik et al.[29], consisted of a 14-day period of MS and 21 days of PD induction. In prior studies, these researchers showed that ELS influences susceptibility to periodontal disease and that the HPA axis is strongly related to this impact.[8,48,49]

Our hypothesis for the stressed rats not to have displayed greater ABL in our study is the possible interference of the induction time of the disease. We believe that the period of 40 days has been sufficient for the alveolar bone loss of unstressed PD rats to be equated with that of PD rats exposed to ELS. As previously mentioned, the time of ligature use in PD induction models is still contradictory. However, some studies show that in the prolonged induction periods the disease progression can reach a plateau phase. As observed in the study by Molon et al.[50], who found that after 21 days there is the so-called "chronic phase" of induction, where no representative evolution of ABL is observed. Evidence was also found by Cavagni et al.[51], who observed an equivalence between ABL with 5 and 22 weeks of PD induction. Therefore, our hypothesis is that at the beginning of the experiment the ELS rats with PD may have displayed a more accentuated ABL, but that over the experimental period the ELS-induced bone loss has reached

a plateau phase making it at the end of the 40 days equalized with the unstressed rats.

Although we found no differences for ABL between groups with PD exposed to MS or not, early stressed animals had a PD with more intense inflammatory infiltrate than unstressed animals. Periodontitis consists of a chronic inflammatory process promoted by dysbiosis between oral microbiota and the release of pro-inflammatory factors involved in adaptive and innate immune responses.[2,3] The presence of cytokines, lymphocytes, mast cells and macrophages trigger a series of responses that destroy periodontal tissues.[52,53] As previously mentioned, some studies have shown that stress can act as an important co-factor in the development of PD through the two-way street that exists between the immune, endocrine and central nervous systems.[8,48,49] In this context, it is already known that ELS produces incisive and permanent effects on the stress regulation pathways, which can promote a constant inflammatory status.[13,22,31] Adverse family environments during childhood have been associated with inflammatory changes, which predispose the development of chronic diseases.[19,25,27,54] In addition, a recent study showed that adults exposed to socioeconomic difficulties during childhood have an increased risk for the development of moderate and severe periodontitis.[55] In our study, maternal separation possibly incited a phenotype in stressed rats which was predicted for an exacerbated inflammatory response resulting from PD.

Besides impacting the bone homeostasis and immune system, ELS is known for its neurological effects that trigger the occurrence of emotional disorders such as anxiety and depression.[22,56] In our behavioral results we observed this effect, since the rats with PD previously exposed to ELS displayed high levels of anxiety compared to PD rats non-stressed. When we evaluated the anxiety-like behavior in stressed rats subjected to the induction of PD, they attended less the open arms of the EZM after disease induction. Varotto et al. evaluated anxiety-like behavior and mechanical allodynia in the acute (14-day induction) and chronic (28-day induction) phases of induced PD in rats.[58] These researchers observed increased anxiety-like behavior in the acute phase, which was positively correlated with decreased allodynia. However, there were no differences in anxious behavior between the acute and chronic phases. In our study the ligature was maintained

for 40 days, i.e., we used the chronic phase protocol of the disease. Furthermore, when the PD rats were compared to control rats, the result was contrary to our initial hypothesis that the disease would elevate anxiety levels. This data evidenced that somehow the PD decreased the anxious behavior of the rats. Within this context, further studies are needed to investigate the influence that chronic periodontal disease has on anxiety-like behavior. The current study has some limitations. First, we did not carry out microscopical evaluation at earlier stages of PD. An evaluation of the jaws could also have been performed through computed microtomography to ensure a more accurate analysis of alveolar bone loss.

In conclusion, our results suggest that early life stress does not significant affect the alveolar bone loss but aggravates the local inflammatory response associated with chronic periodontal disease in rats.

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ANEXOS

ANEXO A – Normas de publicação da International Endodontic Journal

International Endodontic Journal

Online ISSN: 1365-2591

Editor in-Chief:

Dr. Paul MH Dummer

https://onlinelibrary.wiley.com/journal/13652591

Acesso em Novembro de 2020.

ANEXO B – Normas de publicação da Journal of Periodontology

Journal of Periodontology

Online ISSN: 1943-3670

Editor in-Chief:

Dr. Kenneth S. Kornman

https://aap.onlinelibrary.wiley.com/journal/19433670

Acesso em Dezembro de 2020.

ANEXO C – Comitê de Ética no Uso de Animais

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Araçatuba

FLS.....: 31
PROC. : 00569/2019
RUB.....:

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Processo.....: FOA-00569/2019
Interessado(a).....: **DANIEL GALERA BERNABÉ**
Assunto.....: Projeto de Pesquisa "Efeitos do estresse precoce de vida sobre o desenvolvimento de diferentes doenças bucais"

DESPACHO.....: n.º 789/2019-CEUA

Encaminhe-se ao Pesquisador Responsável, por intermédio do Departamento de Patologia e Propedêutica Clínica, para conhecimento da manifestação da CEUA, juntada às folhas 29.

Em seguida, devolva-se à CEUA para aguardar o relatório final que deverá ser apresentado em 17/01/2023.

CEUA, 19 de dezembro de 2019.

Profa. Associada Maria Cristina Rosifini Alves Rezende
Coordenadora da CEUA

ciente 22/01/2020

MCRAR/wbm.

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