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Diazepam reverses the alveolar bone loss and hippocampal interleukin-1beta and interleukin-6 enhanced by conditioned fear stress in ligature-induced periodontal disease in rats

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Background and Objective: Stress and anxiety have been associated with chronic periodontitis, but few studies examining the effects of psychotropic drugs on periodontal health have been performed. Therefore, we aimed to investigate the effects of diazepam on the progression of periodontitis in chronically stressed rats.

Material and Methods: Fourteen Wistar rats were submitted to ligature-induced periodontal disease and were divided into four groups . Two groups were not stressed, whereas two groups were submitted to a conditioned fear stress paradigm for 38 d. Daily diazepam treatment (2 mg/kg, orally) was administered to one unstressed group and to one group submitted to a conditioned fear stress paradigm from day 2 to the day 39, at which point the rats were submitted to an open field test and were killed on day 40. Brains and mandibles were removed for histological and immunohistochemical analyses.

Results: Animals exposed to conditioned fear stress presented an increase in freezing behavior, a decrease in locomotor activity, enhanced alveolar bone loss and higher levels of hippocampal interleukin-1beta (IL-1β) and interleukin-6 (IL-6), compared with the control group. Diazepam, at the dose used in the current study, had no effect on freezing behavior but reversed the decrease in locomotor activity provoked by stress. Additionally, the treatment reduced the levels of hippocampal IL-1β and IL-6 and alveolar bone loss in Wistar rats. Neither conditioned fear stress nor diazepam treatment had an effect on periodontal IL-1β or IL-6 levels in animals.

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Conclusion: Our results suggest that diazepam treatment reduces bone loss in rats submitted to conditioned fear stress. In addition, diazepam treatment led to decreased IL-1β and IL-6 levels in the hippocampus.

Periodontal disease is the main cause of dental loss in adults worldwide, and advanced periodontitis affects about 10% of adults in developed countries (1). It is well known that variations in the severity of periodontal disease, as well as in its response to therapy, are influenced by many individual factors, including genetics, smoking, oral hygiene and older age (2–4). Recent studies have investigated the influence of psychosocial factors on periodontal disease and have reported that stress, anxiety and depression are strongly associated with this disease (3,5). In the case of stress, its association with periodontal disease could be explained, at least in part, by the detrimental behaviors and physiological changes in the individuals (6).

Considering impairing behavioral changes, evidence suggests that increased neglect of oral care (7), smoking (8) and poor nutritional intake (9) induced by stress might be responsible for attachment loss, even after controlling for well-known confounding variables (10). Alternatively, physiological changes caused by many types of stressors can induce the production of cytokines by the brain and the consequent secretion of these cytokines (especially of interleukin (IL)-1,with subsequent secretion of IL6 and tumor necrosis factor-α) (TNF-α)) followed by activation of all components of the hypothalamic–pituitary–adrenal (HPA) axis (11,12). Stimulation of the HPA axis leads to the secretion of glucocorticoid hormones, which are released from the adrenal cortex (13). Stress-related stimulation of the HPA axis leads to the suppression of immune and inflammatory responses and facilitates the appearance and/or progression of periodontal disease. The hippocampus, in particular, represents an important target of circulating adrenal cortical hormones because target receptors for these hormones are abundantly expressed in this structure. Hence, chronic exposure to high corticosteroid levels during prolonged stress may have a detrimental effect on hippocampal integrity as well as function (14) and raise the levels of proinflammatory cytokines (15). Diazepam is a classic gamma-aminobutyric acid-benzodiazepine that is widely used in the treatment of anxiety disorders and has anxiolytic, sedative, anticonvulsant and myorelaxant properties (16). To the best of our knowledge, no study has been carried out in an attempt to investigate the role of diazepam in periodontal disease in stressed animals. Given the strict relationship between psychosocial factors and periodontal disease, we aimed to investigate the effects of diazepam on experimental periodontal disease progression in rats submitted to conditioned fear stress (CFS).

Material and methods

Subjects

Male adult Wistar rats ($n = 14$) were obtained from the animal facilities of Faculdades Integradas Pitágoras (Montes Claros, Brazil). Animals weighing approximately 260 g each were maintained at a controlled temperature of 21 ± 2°C with a 12-h light/12-h dark cycle (lights on from 7 AM–7 PM) with food and water provided *ad libitum*. All experiments were approved by the local Animal Ethics Committee (process 151/2008).

Induction of experimental periodontitis

Experimental periodontal disease was induced in rats under ketamine (60 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally) anesthesia by placement of a sterile cotton (000) thread ligature around the neck of the maxillary left first molar tooth. The ligatures retained oral microorganisms and remained fixed until the end of the experiment (day 40) when the rats were killed. The contralateral right side was used as the unligated control (17). The animals were divided into four experimental groups: no shock + vehicle ($n = 3$); no shock + diazepam ($n = 4$); shock + vehicle ($n = 3$); and shock + diazepam ($n = 4$). Animals were weighed daily, and the survival rate was also monitored daily.

CFS protocol

CFS-induced freezing behavior has previously been proposed as an animal model of anxiety (18). Briefly, 1 d after placement of the ligature, the rats were subjected to CFS sessions for 38 d. Initially, rats were individually placed for 3 min in a chamber to habituate to the apparatus (37 × 25 × 21 cm, Skinner Box, ELT-02; Eltrones, Joinvile, Santa Catarina, Brazil). During this period, the number of crossings across an imaginary line that divided the box floor into two equal segments was counted. Thereafter, rats received one presentation of a neutral conditioned stimulus (90 dB sound at 1000 Hz) for 5 s followed immediately by a noxious unconditioned stimulus (1.10 mA foot shock), six times, for 5 s each, with an intershock interval of 20 s, totaling 3 min. Animals in the no-shock groups were also placed individually in the chamber and submitted to the same experimental conditions but did not received the unconditioned stimulus. An inescapable electric foot shock through a stainless steel grid floor was delivered to the rats via a shock generator. Stimulus strength and number of training conditioned stimulus/unconditioned stimulus pairings were chosen based on a pilot experiment. After the last conditioned stimulus/

unconditioned stimulus pairing, rats were maintained in the chamber for 3 min before being returned to their home cages. The number of freezing behaviors (yes/no) was recorded by an observer, and the sum of freezing behaviors was used for comparison during the analyses. The chamber was cleaned with 70% ethanol before and after each rat. Freezing was defined as the absence of all observable movement, except for those related to respiration, and was measured by an observer who was blind to the experimental groups of the animals.

Drug treatment

Diazepam (Compaz; Laboratório Cristália, Itapira, São Paulo, Brazil) was suspended in 12% bidistilled glycerin and administered daily by intragastric gavage (2 mg/kg) (19,20), 30 min before the CFS sessions, starting on day 21 after the induction of periodontitis until an open field test was performed on day 39. Control groups received the same volume of vehicle per body weight.

Open field test

In order to assess spontaneous locomotor activity, the open field test was performed. The apparatus consisted of a round open field (56 cm in diameter), which had its floor divided into 10 equal areas. The test was performed 30 min after drug administration on day 39. Rats were placed in the central area and were allowed to explore the area freely for 5 min. The number of lines crossed with the four paws was manually counted for 5 min by an observer who was not aware of the subject's conditions. The apparatus was cleaned after each trial with 70% ethanol. Immediately afterwards, the rats were returned to the home cages.

Brain tissue preparation and immunohistochemistry

At the end of the experimental procedures (day 40) the animals were anesthetized and subjected to aortic perfusion with 0.6 M phosphate buffer, pH 7.4, followed by 4% paraformaldehyde. The brains were removed and fixed for 4 h in 4% paraformaldehyde and then cryoprotected for 48 h in 30% sucrose/phosphate-buffered saline at 4°C. Then, the samples were embedded in cryoprotectant medium and frozen at −70°C. The brains were sectioned coronally using a cryostat and 20-μm slices were obtained from the hippocampal region. For immunohistochemistry (IHC) using the avidin–biotin technique, the sections were pre-incubated for 30 min with ethanol containing 0.03% H_2O_2 to block endogenous peroxidase and were then incubated for 18 h at 4°C with the primary antibodies anti-IL-1β (1:100 dilution, rabbit polyclonal anti-IL-1β, clone H-153; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-IL-6 (1:100 dilution, goat polyclonal anti-IL-6, clone M-19; Santa Cruz Biotechnology, Inc.). The signals were developed by exposure to 3,3′-diaminobenzidine-tetrahydrochloride for 5 min and then the slides were dehydrated in an alcohol series before counterstaining for 30 s with Mayer's hematoxylin.

The immunohistochemical expression of biomarkers was evaluated using an Olympus® BH2 microscope (Olympus, Center Valley, PA, USA) (fitted with 10× ocular and 40× objective lenses), and an ocular lattice (area 0.092 mm^2), with 100 points composed of 10 horizontal and 10 vertical test lines, was superimposed on the test field to be measured. A total area of 1.84-mm^2 of hippocampus was evaluated for each sample. The areas showing the strongest staining were selected. Immunohistochemical analyses of all antigens investigated in the hippocampus were performed by determining the positively stained cells divided by the total number of cells in all fields counted (10 fields for each specimen).

Measurement of alveolar bone loss and IHC analyses

After perfusion, maxillae halves were immediately excised, fixed for 48 h in 10% buffered formalin solution (pH 7.4) and demineralized in 10% EDTA for 30 d. At the end of the demineralization, each hemi-maxilla was washed in running water, dehydrated in an alcohol series, and embedded in paraffin. The blocks were cut serially into 3-μm sections in the mesiodistal plane, mounted on glass, deparaffinized in xylene and rehydrated using a series of alcohol dilutions. Staining with hematoxylin and eosin was performed to show the most central section of each tooth (i.e. the section in which the center of the dental pulp was clearly observable). Images from experimental (ligated) and control (unligated) sites were obtained for comparison at 200× magnification through a camera adapted to an optical microscope. The bone-loss analysis was performed in ligated sites by measuring the distance, in mm^2, from the cemento–enamel junction to the alveolar bone crest, and the results were analyzed using the software IMAGE J (National Institutes of Health, Bethesda, MD, USA). The measurements were made by an observer who was unaware of the nature of the tissue sample.

Following this, the mandible sections were subjected to IHC analysis. The paraffin sections (3 μm) mounted on glass were deparaffinized in xylene and rehydrated through an alcohol series. For antigen retrieval, sections were heated in 1.0 mM citrate buffer (pH 8.0) in a steam cooker for 5 min at 121°C. The procedures of endogenous peroxidase block, incubation with IL-1β and IL-6 primary antibodies, development and counterstaining were exactly the same as those described earlier for brain tissue. The immunohistochemical expression of biomarkers was evaluated using an Olympus® BH2 microscope (fitted with 10× ocular and 40× objective lenses), and an ocular lattice (area 0.092 mm^2), with 100 points composed of 10 horizontal and 10 vertical test lines, was superimposed onto the test field to be measured. A total area of 1.84 mm^2 of periodontal ligament was evaluated for each sample and the areas showing the strongest staining were selected. Immunohistochemical analyses of all antigens investigated were performed by determining the positively stained

cells in all fields counted (10 fields for each specimen). Samples of acute inflammatory processes collected at the University Dental Clinic and samples of mouse heart were used as positive controls for IL-1β and IL-6, respectively. Negative control staining was performed by replacing the primary antibodies with a universal negative control (DakoCytomation, Glostrup, Denmark).

Statistical analysis

Results are presented as mean ± standard error. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test, where appropriate. Statistical analysis showing a confidence above 95% ($p < 0.05$) was considered significant. All analyses were performed using a statistical software program (SPSS, version 18.0; SPSS Inc., Chicago, IL, USA).

Results

As depicted in Fig. 1A, our results revealed that the CFS paradigm was effective in producing stress in animals ($F_{1,10} = 441.58$, $p = 0.00$), as seen by increased freezing behavior in the groups submitted to shock sessions. The effect of diazepam treatment on the freezing behavior induced by CFS is also shown in Fig. 1A. Statistical analysis (two-way analysis of variance) revealed that diazepam, at 2 mg/kg, had no effect on freezing in the stressed animals ($F_{1,10} = 0.14$, $p = 0.715$).

The number of crossing responses was monitored (Fig. 1B) and the effect of intragastric administration of diazepam on the crossing mean was analyzed. Statistical analysis (two-way anova) revealed a significant main effect of chronic shock sessions ($F_{1,10} = 5.10$, $p = 0.048$), indicating that the CFS decreased locomotion in animals. However, treatment with diazepam reversed the stress-induced locomotor alteration, as observed by an increase in the crossing number, because there was no difference between stressed rats treated with diazepam and the nonstressed groups ($F_{1,10} = 2.77$, $p = 0.127$).

The effects of CFS and the influence of diazepam treatment on alveolar bone loss in rats submitted to experimental periodontal disease are shown in Fig. 2. Histological evaluation of dental sections demonstrated that CFS augmented alveolar bone loss ($F_{1,10} = 5.98$, $p = 0.034$) compared with the control group. Additionally, statistical analysis (two-way anova) showed a significant main effect of diazepam treatment ($F_{1,10} = 7.85$, $p = 0.019$). *Post-hoc* comparison (Student–Newman–Keuls test) revealed that diazepam reversed the alveolar bone loss induced by CFS. This was confirmed by the representative images of bone loss, showing a larger distance between the cemento–enamel junction and the alveolar bone crest in the shock + vehicle group (Fig. 2D) compared with the control group (Fig. 2B), providing evidence of the effect of stress on bone resorption. Additionally, the effect of diazepam treatment on alveolar bone loss can be observed in Fig. 2E, which shows restored alveolar bone integrity, even under stressful conditions.

In order to verify the effect of diazepam on brain cytokines, we investigated the levels of hippocampal IL-1β (Fig. 3A) and IL-6 (Fig. 3B) in rats with periodontal disease and submitted to CFS. The IHC analyses of hippocampal sections revealed that CFS produced an increase in both IL-1β ($F_{1,10} = 13.44$, $p = 0.004$) and IL-6 ($F_{1,10} = 30.73$, $p = 0.000$). Interestingly, intragastric administration of diazepam (2 mg/kg) was able to reverse the stress-induced increase in the levels of these hippocampal cytokines (IL-1β: $F_{1,10} = 9.40$, $p = 0.012$; and IL-6: $F_{1,10} = 15.47$, $p = 0.003$). Representative IHC images of hippocampal sections showed increased expression of IL-1β and IL-6 in shock + vehicle-treated rats (Fig. 3E) compared with control rats (no shock + vehicle) (Fig. 3C). Additionally, the images reveal that diazepam was able to reverse increased levels of cytokines, as evidenced by a reduction in staining intensity (Fig. 3F).

As illustrated in Fig. 4, IHC analysis of the mandible sections showed that CFS had no effect on either periodontal IL-1β ($F_{1,10} = 2.59$, $p = 0.139$; Fig. 4A) or IL-6 levels ($F_{1,10} = 0.04$, $p = 0.845$; Fig. 4B). In turn, treatment with diazepam had no effect on periodontal cytokine levels (IL-1β: $F_{1,10} = 3.59$, $p = 0.088$; and IL-6: $F_{1,10} = 0.02$, $p = 0.890$). The representative images of IHC staining for IL-1β and IL-6 in the periodontal ligament confirm that no differences in cytokine levels are present between

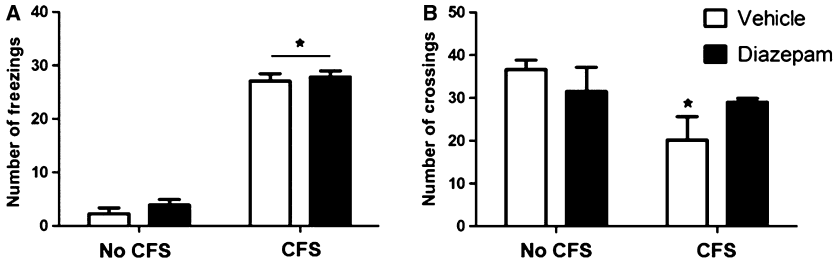

Fig. 1. Effect of conditioned fear stress (CFS) and diazepam treatment on freezing behavior (A) and crossing (B). CFS increased the freezing behavior and decreased the locomotor activity in the open field test. Diazepam treatment did not reduce the freezing behavior but reversed the stress-induced locomotor deficit. Bars represent mean ± standard error. *$p < 0.05$.

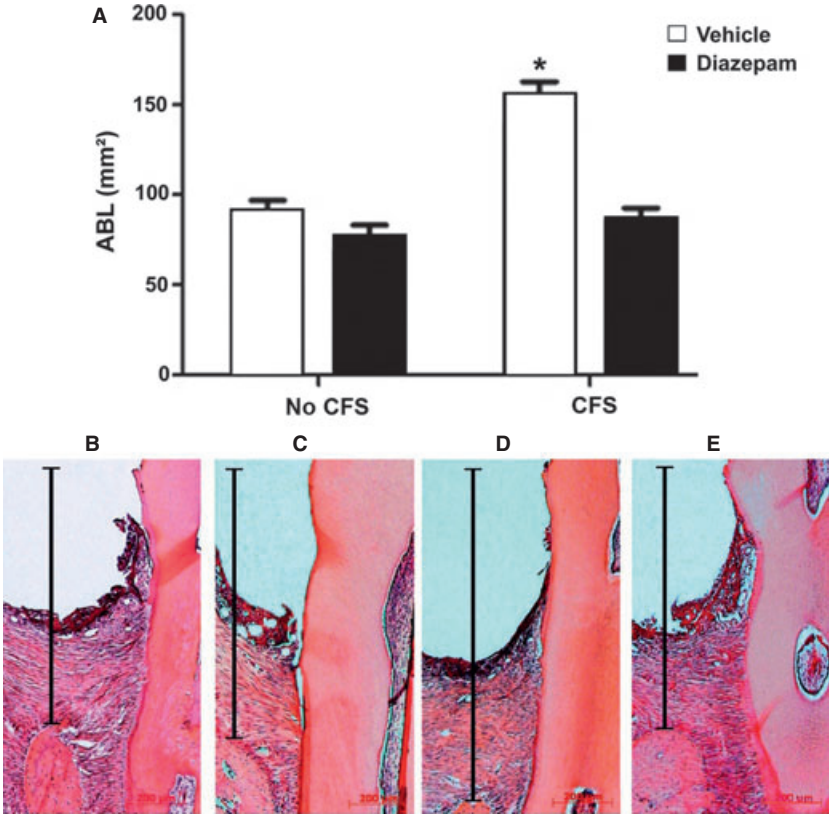

Fig. 2. Effect of conditioned fear stress (CFS) and diazepam treatment on alveolar bone loss (ABL) (A). CFS enhanced ABL and diazepam reversed this stress-induced bone loss in rats Bars represent mean ± standard error. *$p < 0.05$. Histological assessment revealed a greater distance between the cemento–enamel junction and the alveolar bone crest in the shock (measurement bar) + vehicle group (D) compared with the no shock + vehicle group (B); the bone integrity is evident in the shock + diazepam group (E) compared with the shock + vehicle group (D), even under stressful conditions. (C) No shock + diazepam group. Hematoxylin and eosin stain, magnification 200×.

the shock + vehicle group (Fig. 4E) and the control group (no shock + vehicle) (Fig. 4C). The same was observed in the shock + diazepam group (Fig. 4F) compared with the control group (shock + vehicle) (Fig. 4E). This suggests that there is no association between diazepam treatment and periodontal IL-1β and IL-6 levels.

Discussion

In the present study, we observed that animals submitted to CFS present an increase in the number of freezing behaviors, suggesting that the shocks were successfully delivered. We also observed that CFS induced changes in anxiety-like behavior and this was characterized by a decrease in locomotor activity observed in the open field test, a behavioral test commonly used to assess locomotor performance (21–23). Diazepam reversed CFS-induced locomotor alterations in the open field test as indicated by an increase in the crossing number. No differences were observed between stressed rats treated with diazepam and nonstressed animals.

An earlier study reported that enhanced hippocampal levels of IL-1β and IL-6 correlate with anxiety and locomotor impairment in rats with arthritis (24). Furthermore, Miller *et al.* (25) suggested that increased IL-1β levels lead to greater gamma-aminobutyric $acid_A$ receptor function, which might contribute to a reduction in locomotion. In agreement with these findings, we observed an increase in the levels of stress-induced hippocampal IL-1β and IL-6 and this was accompanied by a reduction in locomotor activity. In the current study, diazepam reversed stress-induced locomotor impairment, which may be a result of the effects of this benzodiazepine on hippocampal cytokine levels (Fig. 3F). It can also be speculated that diazepam treatment decreased the levels of stress in the animals in the open field test and this led to greater motor performance.

Fixation of a ligature around the teeth is a widely used model for periodontal disease in animals as a result of its role in plaque retention (26). The inflammatory response of periodontal disease is characterized by infiltration of gingival tissues by neutrophils, macrophages and lymphocytes, and the generation of high concentrations of cytokines, eicosanoids and MMPs locally (27). Our results showed that CFS enhanced alveolar bone loss in animals, an observation that is in agreement with the findings of other studies. Nakajima *et al.* (28) and Takada *et al.* (29)

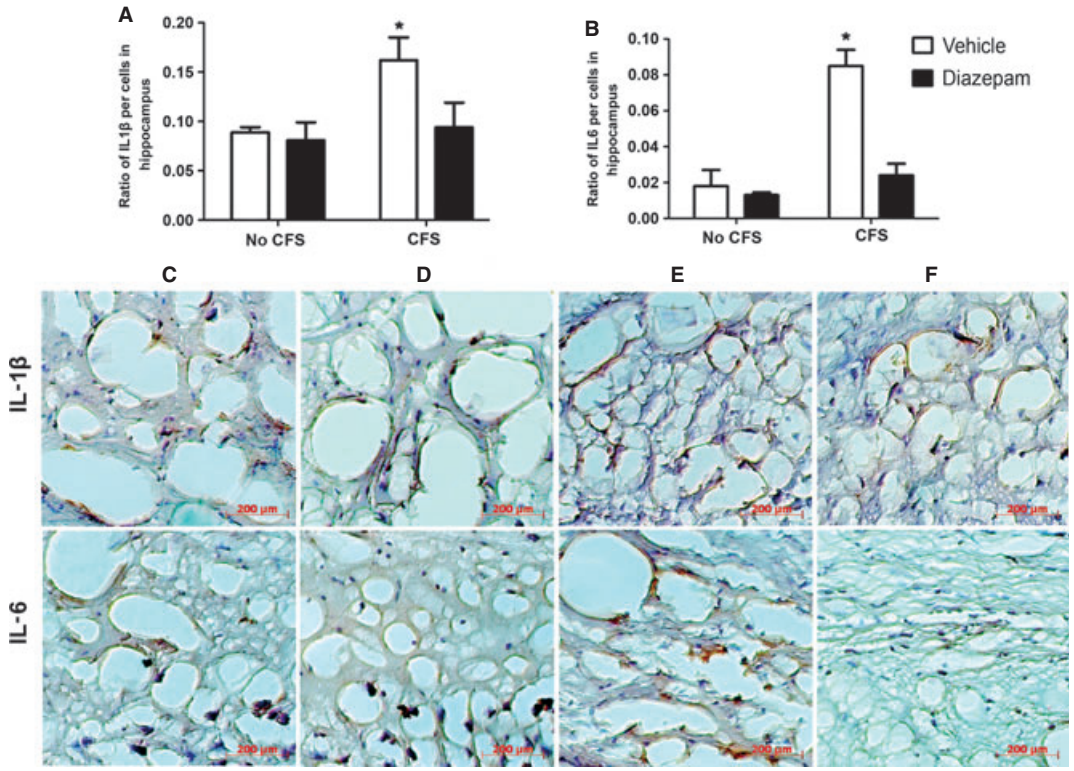

Fig. 3. Effect of conditioned fear stress (CFS) and diazepam treatment on the immunohistochemical detection of interleukin (IL)-1β (A) and IL-6 (B) in the hippocampus. The stress-induced increase in both IL-1β and IL-6 was reversed to control levels by diazepam treatment. Bars represent mean ± standard error. $*p < 0.05$. Photomicrographs of immunohistochemical analysis of IL-1β and IL-6 in the hippocampus show greater levels of these cytokines in the shock + vehicle group (E) compared with the no shock and vehicle group (C). Additionally, decreased cytokine levels were observed in animals treated with shock + diazepam (F). (D) No shock + diazepam. The immunolocalization of interleukins is evidenced by brown staining in brain tissue. The sections were stained with 3,3′-diaminobenzidine-tetrahydrochloride and were counterstained with Mayer's hematoxylin. Magnification 400×.

verified that rats submitted to ligature-induced periodontitis and under restrain stress showed enhanced alveolar bone loss. It is known that proinflammatory cytokines, especially IL-1, are produced following exposure to immunological and psychological challenges. Immune challenges, such as local inflammation, as well as viral and bacterial infections, were found to induce the production and secretion of IL-1 in the brain and this was followed by activation of all components of the HPA axis (30). In this study, not only were the animals submitted to ligature but they were also under CFS. Brain cytokines are an important link between stress-induced activation of the HPA axis and secretion of glucocorticoids, which mediate the effects of stress. Importantly, these cytokines are able to exert deleterious effects at high levels (30). Therefore, because of the high levels of stress-induced cytokines (IL-1β and IL-6) present in the hippocampus, more studies are necessary to test whether there is an association between hippocampal cytokine levels and bone loss. However, it is important to note that the neuroendocrine system does not operate independently but rather in close cooperation with the immune system. Thus, the decrease in immunological activity resulting from psychological stress (14,30) can also play a role in the development of periodontal disease because such factors facilitate increased colonization with pathogenic bacteria and the breakdown of periodontal attachment. This may be a protective mechanism as the animal attempts to decrease its pathogenic load by releasing the teeth where the pathogens are concentrated in the event that the immune system is unable to function properly (31).

We also observed that diazepam treatment led to a decrease in alveolar bone loss in stressed animals. Interestingly, treatment with diazepam reversed the increase in hippocampal IL-1β and IL-6. This interesting finding is supported by previous investigations which have shown that diazepam inhibits the release of IL-1β, IL-6 and TNF-α by glial cells following traumatic lesion in rats (32) and also suppresses IL-1β and IL-6 release from glioma cells (33). In addition, it has been demonstrated that central and peripheral benzodiazepine receptor agonists inhibit the production of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, by macrophages and monocytes in mice (34).

Interestingly, the CFS was not linked to high levels of proinflammatory

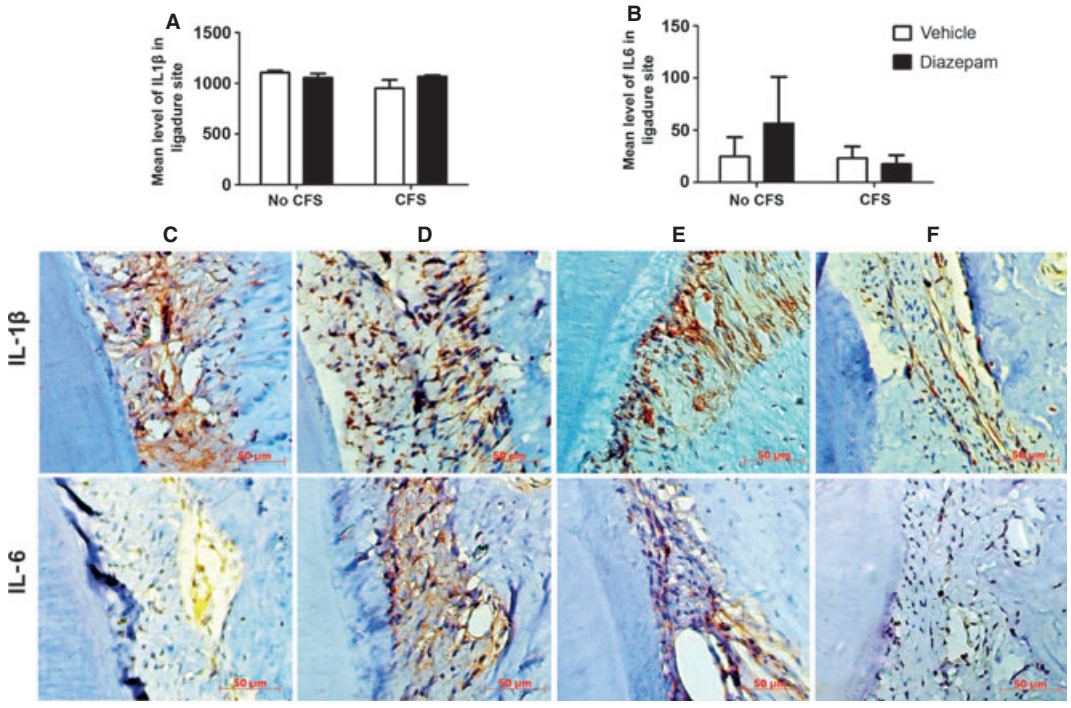

Fig. 4. Effects of conditioned fear stress (CFS) and diazepam treatment on the immunohistochemical detection of interleukin (IL)-1β (A) and IL-6 (B) in the periodontal ligament. CFS did not alter the levels of either IL-1β or IL-6 following the course of the study (i.e. 40 d). Diazepam also had no effect on IL-1β and IL-6 levels in the periodontal ligament of stressed Wistar rats. Bars represent mean ± standard error. $*p < 0.05$. Immunohistochemical analysis revealed no differences in the levels of IL-1β and IL-6 in the periodontal ligament in the shock + vehicle group (E) compared with the no shock + vehicle group (C). Diazepam treatment did not alter the levels of IL-1β and IL-6, and similar immunolocalization of IL-1β and IL-6 was observed in both shock + diazepam (F) and shock + vehicle groups (E) in the periodontal ligament of Wistar rats. The sections were stained with 3,3′-diaminobenzidine-tetrahydrochloride and were counterstained with Meyer's hematoxylin. Magnification 400×.

cytokines in the periodontium. Proinflammatory cytokines are known to act as bone resorption-stimulating factors via activation of RANKL expression (35–39). RANKL can bind to RANK on osteoclast precursors and trigger their differentiation, function and survival (37,40,41). However, the levels of proinflammatory cytokines in the periodontium were not increased in this study. This might be a result of the length of the study, an idea which is supported by a previous study (33) where higher bone loss and low proinflammatory cytokine levels (IL-1β, TNF-α and interferon-γ) were observed in the gingival tissue of animals submitted to 22 d of CFS.

In conclusion, our results suggest that diazepam treatment reduces bone loss in rats submitted to CFS. This effect may partly be a result of the decreased levels of IL-1β or IL-6 observed in the hippocampus following diazepam treatment.

Acknowledgements

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Conflict of interests

None declared.

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