Periodontitis can lead to an Initial Process of Neuroinflammation in Experimental Models

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Recent evidence shows a possible causal relationship between periodontitis and neurodegenerative diseases. Changes in the hippocampus can result in reduced cognitive functions such as behaviors associated with memory and emotions.
Objectives: Evaluate the influence of ligature-induced experimental periodontitis on the inflammation of the hippocampus of rats.
Materials and Methods: Twenty male Wistar rats, divided into Control Group (GC) and Periodontal Disease Group (GDP). GDP was induced to experimental periodontitis by placing a ligature on the lower molars for 30 days. The study performed three behavioral tests during the

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experiment in two stages: before and after the induction of periodontitis; at the end, the rats underwent euthanasia and the collection of the hippocampus (histological and immunological analysis) and hemimandibles (histological and radiographic analysis), with subsequent performance of Student's T-tests and Two-Way ANOVA to analyze the results.

Results: GDP showed a higher level of anxiety, less habituation and reduced time to explore the new object compared to GC ($p < 0.05$). Besides, GDP had a lower number of osteocytes and osteoblasts, and higher osteoclastic activity, as well as more significant alveolar bone loss compared to GC ($p < 0.05$). Senile plaques were noted in the hippocampus in addition to positive beta-amyloid, tau, and CD68 markings on GDP.

Conclusions: Therefore, it can be concluded that periodontitis triggered the presence of senile plaques, beta-amyloid, tau, and CD68 markings, which, together, manifested an initial neuroinflammation process in these animals. Clinical Relevance: Periodontitis can be a risk factor in neuroinflammation process

1. INTRODUCTION

Periodontal disease (PD) characterizes as a chronic, progressive and destructive disease initiated predominantly by gram-negative bacteria that colonizes the subgingival area and trigger a peripheral immune-inflammatory response, which leads to damage the dental apparatus [1-3].

In humans and in rodents, periodontal microorganisms can enter in circulation; which leads to bacteremia and systemic spread [2,4]. Therefore, an initial condition, such as PD, if not stabilized, can generate changes, such as the release of pro-inflammatory cytokines [5,6], which may cause systemic disorders [7,8]; in contact with the central nervous system, it may induce a neuroinflammatory response [9]. Thus, a connection between oral health and cognitive function may exist [10].

According to Saito et al. [11] and Stein et al. [12], the reduction of teeth induced by PD is associated with an increased risk of developing dementia / cognitive impairment in humans [13], which is reaffirmed by the evidence that certain bacterial and viral infections may increase the risk dementia [14].

Dementia is a degenerative brain condition with progressive decline in cognitive and emotional functions [15], such as memory loss, mental confusion and behavioral changes [16]. The individual feels the dementia's impact in social and professional performance [17]. There is neurodegeneration induced by an increase in the production of pro-inflammatory cytokines among the associated causes [18]. This condition mainly affects elderly individuals; Alzheimer's disease (AD) is the most common cause, with 60% -70% of all types of dementias [16].

Synaptic loss and neuronal death in certain regions cause the AD [19]; it starts in the entorhinal cortex and hippocampus [20]. Among the neuropathological changes, it is worth mentioning the presence of plaques produced by extracellular deposition of β-amyloid (Aβ) and the intraneuronal accumulation of hyperphosphorylated tau, which form the neurofibrillary tangles [21]. Aβ is responsible for neurotoxicity and for blocking the formation of new memories [22]. Thus, AD initially affects short-term memory [23].

Hence, this study aimed to evaluate the influence of ligature-induced experimental periodontitis on the inflammation of the hippocampus of rats.

2. MATERIALS AND METHODS

2.1 Sampling

The sample selected was composed by twenty adult Wistar rats, 12 weeks old, weighing an average of 250-300 grams, from the central vivarium of the Western Paraná State University (UNIOESTE). The experiment was carried out at the Cell Biology Laboratory (BIOCEL) and the Physiology and Endocrinology Laboratory (LAFEM) at UNIOESTE. The animals were kept in the LAFEM sector vivarium under controlled conditions of temperature ($23 \pm 2°$ C) and light (12-hour light-dark cycle). The sample received water and commercial feed at will. Furthermore, they were randomly distributed in two groups with ten animals in each, where the entire sample calculation was based on the use of the T-Test, with a power of 90% and a significance level of 5%, as well as in previous studies by the group of researchers [24,25].

- Control Group (GC): did not undergo any intervention, being euthanized on the 30th day of the experiment.
- Periodontal Disease Group (GDP): inducted to PD, being euthanized on the 30th day of the experiment.

2.2 Periodontal Disease Induction Protocol

The animals were anesthetized with 0.08ml / 100g of ketamine (Dopalen, Sespo Indústria and Comércio, Paulínia-SP) and 0.04ml / 100g of xylazine (Anasedan, Sespo Indústria and Comércio, Paulínia-SP) intraperitoneally and positioned on an appropriate operating table. With the aid of a modified clamp and an explorer probe, a cotton thread size 40 was placed around the lower first molars bilateral. This ligature acted as a gingival irritant for 30 days, which favoreded the accumulation of bacterial plaque and the development of PD [26].

2.3 Histological Analysis of the Mandible

After 30 days of the experiment, the animals were euthanized. The collected right hemimandibles were fixed in a 10% formaldehyde solution for 48 hours that underwent to a process of descaling and histological processing. The pieces were cut into 7 micrometers (µm) and stained in Hematoxylin and Eosin (H&E).

2.4 Radiographic Analysis of the Mandible

After 30 days of the experiment, the animals were euthanized, and the hemimandibles on the left side of each animal were removed and fixed in buffered formaldehyde (pH 7.2) for 48 hours. The hemimandibles were placed with the lingual face on the Kodak RVG 6100 digital radiographic sensor with image resolution 20 pl/mm, theoretical resolution of the sensor 27.03 pl/mm, optical fiber 1, dimensions of active surface of 22x30 mm and matrix dimensions (pixels) 1200x1600 (1.92 million); the hemimandibule was positioned so that the buccal and lingual cusps of the first molars were in the same vertical plane. A GE-1000 X-ray apparatus was used regulated to 15mA, 65Vp, 18 pulses, 50 cm focus/film distance with an X-ray incidence perpendicular to the pieces. The digitized images were analyzed in three measurements in the Image Tools 3.0 software (University of Texas Health Science Center, San Antonio, TX, USA);

and an average was taken through a linear measurement of the distance from the cementoenamel junction to the alveolar bone crest on the mesial side of the lower-left first molar [26].

2.5 Bone Histomorphometry

A measurement of the shortest distance between the apex of the vestibular alveolar bone crest and the cement-enamel junction was performed in the GC and GDP, with a 40x objective lens and with the Image Tools 3.0 software (University of Texas Health Science Center, San Antonio, TX, USA). The measurements were repeated once a day, on three different days, and then an average was performed between the values [25].

Manual cell quantification (osteocytes, osteoclasts, and osteoblasts) was performed on the ImageJ 1.80 software (National Institute of Health, NIH, USA), in the GC and GDP, with one cut per animal in 4 consecutive fields of the vestibular alveolar bone crest, with the 40x objective lens [26].

2.6 Behavior Tests

2.6.1 Elevated plus maze test (EPM)

The EPM test was performed in two stages with each animal in GC and GDP (pre- and post-induction (on the 28th day of the experiment) groups. The animal was placed in the center of the EPM and left for exploration for 5 minutes for assessing locomotor and exploratory activity and anxiety, which was recorded by a Sony digital camcorder (Sony Corporation, Tokyo, Japan), model DCR- SR68 60 GB.

The following motor parameters were checked manually:

a) The elevation frequency;
b) The grooming frequency;
c) The freezing frequency;
d) The number of fecal lumps;
e) The time spent in the open arms, in seconds;
f) The time spent in the closed arms, in seconds.

2.6.2 Open field test

The Open Field test was performed in two stages with each animal in GC and GDP (pre- and post-induction (on the 29th day of the experiment) groups. The animal was placed in the center of the arena and left for exploration for 5 minutes

for assessing locomotor and exploratory activity and anxiety [27], which was recorded by a Sony digital camcorder (Sony Corporation, Tokyo, Japan), model DCR-SR68 60 GB.

The following motor parameters were checked manually:

a) The elevation frequency;
b) The grooming frequency;
c) The freezing frequency;
d) The number of fecal lumps;
e) The time spent in the center, in seconds;
f) The time spent in the periphery, in seconds.

2.6.3 Object recognition test

The Object Recognition Test was performed in two stages with each animal in GC and GDP (pre- and post-induction) groups: before induction and after it, on the 27th, 28th, and 29th days of the experiment, as described by Bevins and Besheer [28], which consists int three phases: habituation, training, and testing.

In the first session, the animal was placed in the box and left for 10 minutes; on the second day (24 hours later), two identical objects (green cubes) were placed next to the animal for 5 minutes. On the third day (24 hours later), the rat was placed again with a familiar object (A: green cube) and a new object (B: black cylinder) for 5 minutes [29]. The session was registered by a Sony digital camcorder (Sony Corporation, Tokyo, Japan), model DCR-SR68 60 GB.

The time (seconds) that the rat explored each object was timed and the relationship between the time spent exploring the familiar object (A) and the new object (B) was calculated; the discrimination ratio (DR) was calculated with the formula: $DR = \{ [A / B + A \times 100] \}$ [30].

2.7 Histological Analysis of the Nervous Tissue

The hippocampi on the right side were randomly chosen and collected and fixed in Metacarn for 24 hours. After, it passed through histological processing and it has been cut to 5 μm and stained in H&E and Congo red.

2.8 Immunohistochemistry of the Hippocampus

The slides were made as described for histological analysis in order to perform the technique. They contained two sections per slide per experimental group. The paraffin was removed by a thermal removed carried out for16 hours in an oven at 60°C (Celsius); and by a chemical removel, which was carried out with two immersions: the slides were immersed in a solution of Sodium Citrate, after a water bath, in a closed container and placed in a water bath for 30 minutes. Field marking was performed as described by Panis et al. [31]. The sections were delimited with a Dako Pen® hydrophobic pen, and the endogenous peroxidases were blocked in 10% hydrogen peroxide solution for 30 minutes that was followed by a non-specific binding block by incubation in 0.1% fetal serum for 1 hour. Below, the sections were negative with the primary Sigma Aldrich antibodies, anti-beta-amyloid (1: 300), anti-tau (1: 300), and anti-CD68 (1: 300) in a humid chamber overnight at 4°C. After incubation, the slides were submitted to 3 baths (5 minutes) in PBS (phosphate-buffered saline) and supplemented with secondary antibody; it was maintained for 15 minutes; after, it was washed again with PBS in a jet and three more washes with a drop of PBS. The staining was revealed by incubation with 3,3'-diaminobenzidine (DAB) for 15 minutes that was followed by two washes with PBS: the first with a jet, and the second with a drop. In the last step, the sections were slightly counter-colored with Harry's hematoxylin (Merck) for 30 seconds, and then it was washed in running water; subsequently, they were incubated in 70% alcohol for 5 minutes in an immersion bath, incubated in 95% alcohol for 5 minutes in an immersion bath, incubated in xylol for 5 minutes and again in xylol for 10 minutes. After draining all the liquid, the slides were assembled with Canadian balsam and coverslip. For the semi-quantitative score, the images, totaling 10 for each cut of each animal, were evaluated using the color deconvolution tool in Image-ProPlus 4.0.

2.9 Statistical Analysis

The results were displayed in spreadsheets and analyzed on the GraphPad software. The normality of the data was analyzed by the Shapiro-Wilk test; after checking the normality distribution with the GraphPad Prism, the Student's T-test was performed with $p < 0.05$ to assess the difference between the groups in analysis radiographic, morpfometric and immunohistochemistry. The Two-Way ANOVA test analyzed the data referring to the behavioral tests.

3. RESULTS

3.1 Histological Analysis of the Hemimandible

3.1.1 Control group

The periodontium morphology in the GC showed healthy tissue aspects without the presence of an inflammatory process. The alveolar bone was intact, compact and regular; with the bone crest showing height in the portion of the coronal third of the root. The presence of osteoclasts, osteocytes, and osteoblasts was observed; incremental lines in the typical pattern of bone remodeling (Figs 1A and 1C).

3.1.2 Periodontal disease group

There were morphological changes in the periodontium for the GDP. There was tissue retraction and disorganization of part of the collagen fibers in the oral, junctional, and sulcular gingival epithelium. The periodontal ligament showed decreased fibers, disorganized cells, and inflammatory infiltrate. The alveolar bone maintained the morphological characteristics of the GC, except in the bone crest, which presented irregular shape with a bone resorption aspect in addition to a higher number of osteoclasts (Figs 1B and 1D).

3.2 Radiographic and Histomorphometric Analysis of the Measurement of the Cementum-Enamel Junction to the Alveolar Bone Crest

According to the radiographic evaluation, more significant alveolar bone loss was found in the GDP compared to the GC (p = 0.0017) (Fig 1E). The same result was observed (p = 0.0025) in the histomorphometric analysis (Fig 1F). According to the radiographic images, the alveolar bone crest maintained the radiographic characteristics of the GC (Fig 1G), and the

radiographic image of the reabsorption of the alveolar bone crest in the GDP (Fig 1H).

3.3 Histomorphometric Analysis for Quantification of Osteocytes, Osteoblasts, and Osteoclasts

According to Table 1, the GDP had a lower number of osteocytes and osteoblasts compared to the GC. Besides, the GDP had higher osteoclastic activity compared to the GC (p<0.05).

3.4 Behavioral Analysis

3.4.1 Elevated plus maze

In the freezing and grooming variables, there was no difference when comparing groups and times, whereas, in the elevation variable, an increase was observed in the post compared to the pre in the GDP (p = 0.0447) (Table 2).

The time spent in the open and closed arms of the GC were not different; however, there was a reduction in the time spent in the open arm (p = 0.0068) and an increasing in the time spent in the closed arm (p = 0.0090) for the GDP (Table 2).

The number of fecal lumps did not change for both groups. However, it was lower in the post stage, both in the GC group (p = 0.0047) and in the GDP (p = 0.0160) (Table 2).

3.4.2 Open field

In the freezing, grooming, elevations variables, there were no difference in the time spent in the periphery and the center when comparing the groups and the pre and post stages (Table 3).

In the feces variable, there was a difference in the GC with a reduction in the number of fecal lumps in the post period compared to the pre period (p = 0.0016) (Table 3).

Table 1. Morphometric analysis of the right hemimandible of rats in the experimental groups to quantify osteocytes, osteoblasts, and osteoclasts. The values represent the mean ± standard deviation from the mean, and units are expressed

	Osteoblasts	Osteocytes	Osteoclastic
GC	61.6±14.59[A]	539.20±106.46[A]	1.1±0.87[A]
GDP	42.22±17.13[B]	438.55±55.44[B]	4.22±3.07[B]

The different letters refer to the significant differences between the groups (p<0.05). Abbreviations: GC, Control Group; GDP, Periodontal Disease Group

Table 2. Analysis of behavioral parameters in the Elevated Plus Maze. The values represent the mean ± standard deviation of the mean. The variables freezing, grooming, elevations, and fecal lumps are expressed in frequency, and the time spent variables for both closed and open arms are expressed in seconds

	Means ± standard desviation			
Groups	**Before GC**	**After GC**	**Before GDP**	**Afetr GDP**
Freezing	0.85±0.74 [A]	1.55±1.42 [A]	1.25±0.79 [A]	2.05±1.35 [A]
Grooming	2.25±1.63 [A]	1.55±0.84 [A]	2.15±2.55 [A]	2.12±1.32 [A]
Elevation	20.1±2.33 [AB]	24.55±5.12 [B]	18.50±5.01 [A]	24.11±4.80 [B]
TOA	94.05±43.78 [AB]	55.61±33.12 [B]	132.05±40.65 [A]	64.94±47.65 [B]
TCA	119.38±31.32 [AB]	127.75±30.76 [AB]	81.38±27.23 [A]	151.61±69.18[B]
Fecal lumps	1.50±1.17 [A]	0.00±0.00 [B]	1.60±1.17 [A]	0.55±0.46 [B]

The different letters refer to the significant differences between the groups (p <0.05). Abbreviations: GC, Control Group; GDP, Periodontal Disease Group; TOA, time spent in the open arm; TCA, time spent in the closed arm.

Table 3. Analysis of behavioral parameters in the Open Field; The values represent the mean ± standard deviation of the mean. The variables freezing, grooming, elevations, and fecal lumps are expressed in frequency, and the time spent variables for both center and periphery are expressed in seconds

	Means ± standard desviation			
Groups	**Before GC**	**After GC**	**Before GDP**	**After GDP**
Freezing	1.30±1.63 [A]	1.00±0.43 [A]	1.45±1.30 [A]	0.77±0.44 [A]
Grooming	3.15±2.00 [A]	3.22±1.43 [A]	2.50±1.58 [A]	4.61±3.34 [A]
Elevation	41.90±6.19 [A]	40.70±10.06 [A]	34.90±9.17 [A]	34.44±10.26 [A]
TC	13.30±5.14 [AB]	16.90±8.65 [B]	7.40±3.47 [A]	11.22±10.59 [AB]
TP	273.05±14.04 [A]	269.15±14.54 [A]	282.80±10.93 [A]	279.94±17.33 [A]
Fecal lumps	3.40±2.22 [A]	0.11±0.33 [B]	3.30±1.70 [A]	2.00±2.12 [AB]

The different letters refer to the significant differences between the groups (p<0.05). Abbreviations: GC, Control Group; GDP, Periodontal Disease Group; TC, time spent in the center; TP, time spent in the periphery.

3.4.3 Object recognition test

As for the exploration time of object A (familiar), the GC means in the pre and post times were 19.33s (seconds) and 20.4s, and the GDP pre and post means were 20.4s and 15s, respectively (p>0.05).

As for the exploration time of object B (new), the GC means in the pre and post times were 15.44s and 13.5s, and the GDP means pre and post were 15.3s and 9.77s, respectively. There was a statistical difference in the GDP with less exploration of object B in the post time when compared to the pre period (p = 0.0023).

For the discrimination ratio, the means of the GC in the pre and post times were 0.45 and 0.45, and the means of the GDP pre and post were 0.43 and 0.44, respectively. There was no statistical difference.

3.5 Histological Analysis of the Hippocampus

3.5.1 H&E stain

In Fig. 2, images of histological sections of the hippocampus are displayed with the 4x and 40x objective lenses of both the GC and the GDP. In Figs 2A and 2B, the regions of the hippocampus and of the dentate gyrus (DG) are represented by the letters AH, where neurons with regular features and designs are observed. In Fig 2D, the black arrows show a pyramidal neuron (PN*), and the same aspects that represent the deposition of senile plaque can be seen around it. In Fig. 2C, the black arrows indicate the disposition of several neuronal cells without the presence of senile plaques; the presence of blood vessels can be noted. In Figs 2C and 2D, sensory neurons, astrocytes, and oligodendrocytes with functional aspects can be observed.

3.5.2 Congo red stain

In Fig 2, images of histological sections of the hippocampus are displayed with the 4x and 40x objective lenses of both the GC and the GDP. In Figs 2E and 2F, the regions of the hippocampus and dentate gyrus (DG) are represented by the letters AH, where neurons with regular features and designs are observed. In Fig 2H, the black arrows show a pyramidal neuron (PN) with a senile plaque aspect; no neurofibrillary aspects

are observed. In Fig 2G, several neuronal cells are available without the presence of senile plaques, in which few neurons are also found in the cerebral parenchyma of the hippocampus, whereas in Fig 2H, a higher number of neurons can be observed in the brain parenchyma.
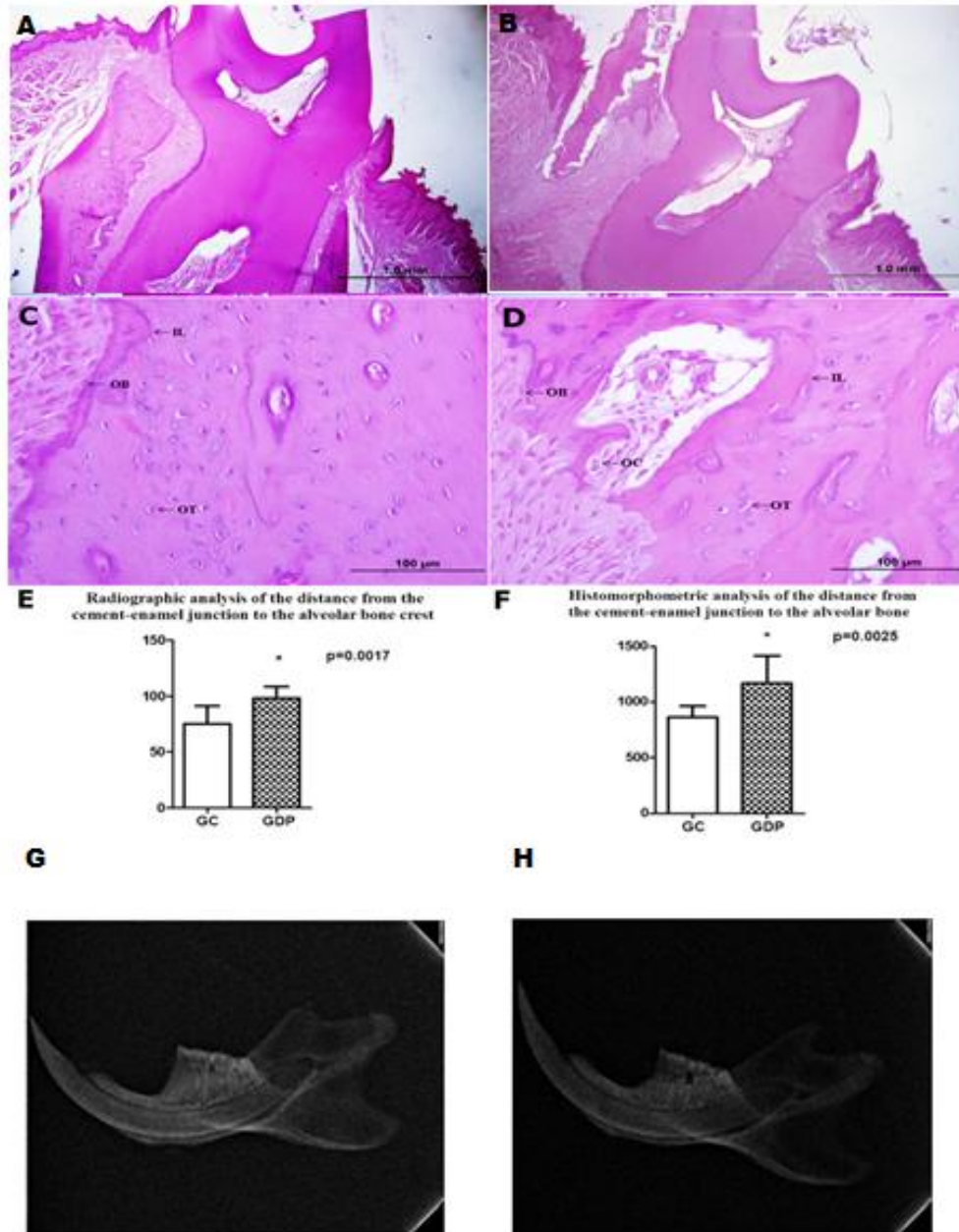

Fig. 1. Bone and connective tissue loss and increased distance from the cement-enamel junction to the alveolar bone crest occurred in rats with periodontitis; Representative photomicrographs of the sagittal section of the first mandibular molar of the GC and the GDP, in 4x and 40x, in H&E (A, B, C, and D). Radiographic analysis (E) and histomorphometric analysis (F) of the distance from the cement-enamel junction to the alveolar bone crest of all groups. Representative radiographic images of the GC (G) and the GDP (H); Values are expressed in pixels. Abbreviations: OB, osteoblast; OC, osteoclast; OT, osteocyte; IL, incremental lines; GC, Group control; GDP, Periodontal Disease Group
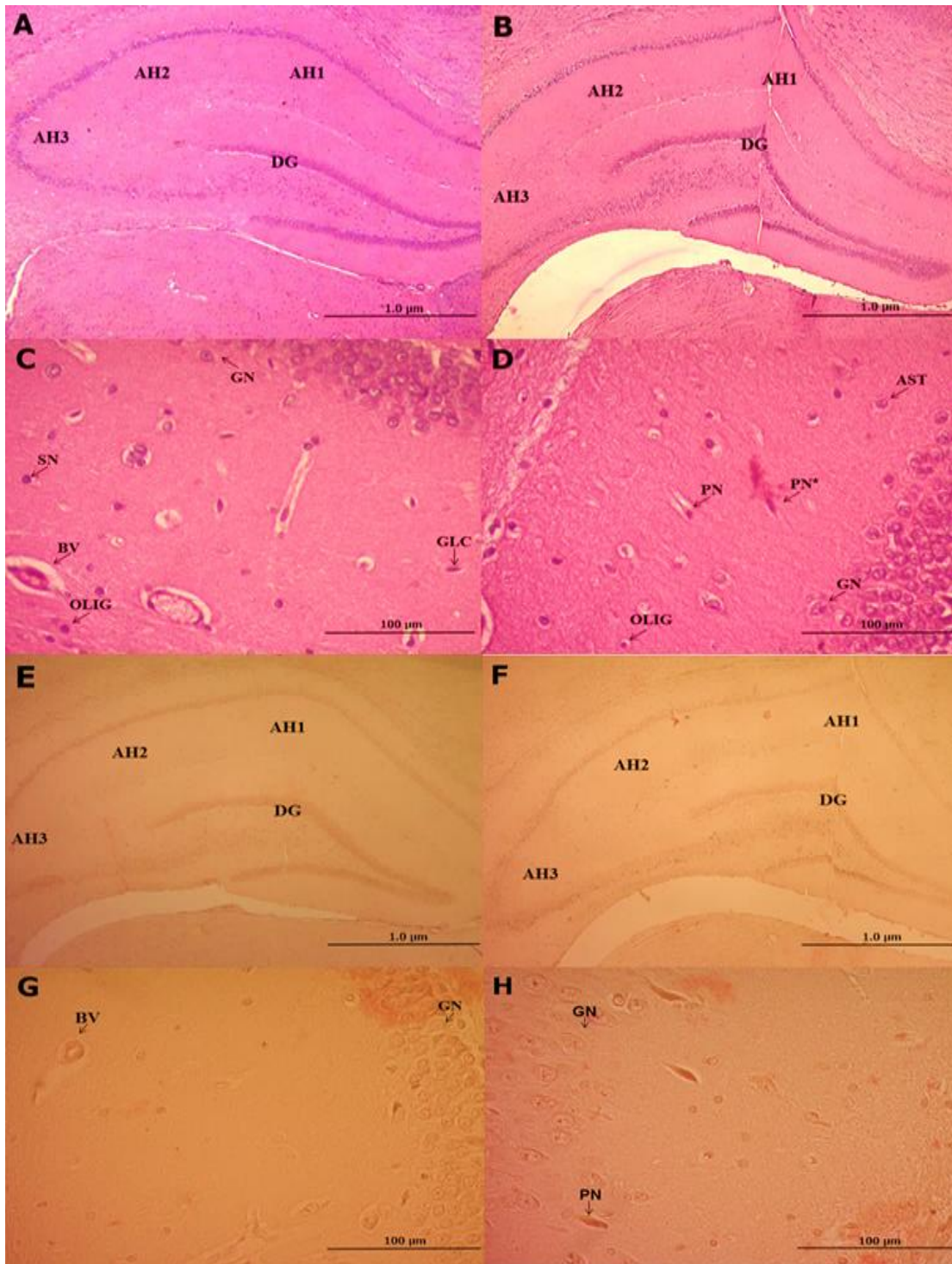
Fig. 2. The presence of senile plaques was found in rats with periodontitis; Representative photomicrographs of the coronal section of the hippocampus of GC and the GDP, in 4x and 40x, in H&E (A, B, C, and D) and Congo red (E, F, G, and H). Abbreviations: AH, Ammon's horn; DG, dentate gyrus; GN, granular neuron; AST, astrocyte; OLIG, oligodendrocyte; PN, pyramidal neuron; SN, sensitive neuron; BV, blood vessels; GLC, glial cell

3.6 Immunohistochemical Analysis of the Hippocampus

3.6.1 Histological analysis

In Fig 3, images of histological sections of the hippocampus with the 4x and 40x objective lenses of both the GC and the GDP are displayed. In Figs 3A and 3C, neurons with standard features and designs are observed. In Fig 3D, the black arrows demonstrate the deposition of Aβ.

In Fig 4, images of histological sections of the hippocampus are displayed with the 4x and 40x objective lenses of both the GC and the GDP. In Figs 4A and 4C, neurons with standard features and designs are observed. In Fig 4D, the black arrows demonstrate the deposition of tau.

In Fig 5, images of histological sections of the hippocampus with the 4x and 40x objective lenses of the GC and GDP are displayed. In Figs 5A and 5C, neurons with standard features and designs are observed. In Fig 5D, the black arrows demonstrate the presence of macrophages.

3.6.2 Quantitative analysis of Beta-Amyloid, Tau, and CD68

According to Fig 3E, the GDP showed positive activity of Aβ when compared to the GC ($p = 0.0127$); the GC average was 486, with a standard deviation of 342.1, and the GDP average was 807.1, with a standard deviation of 133.3.
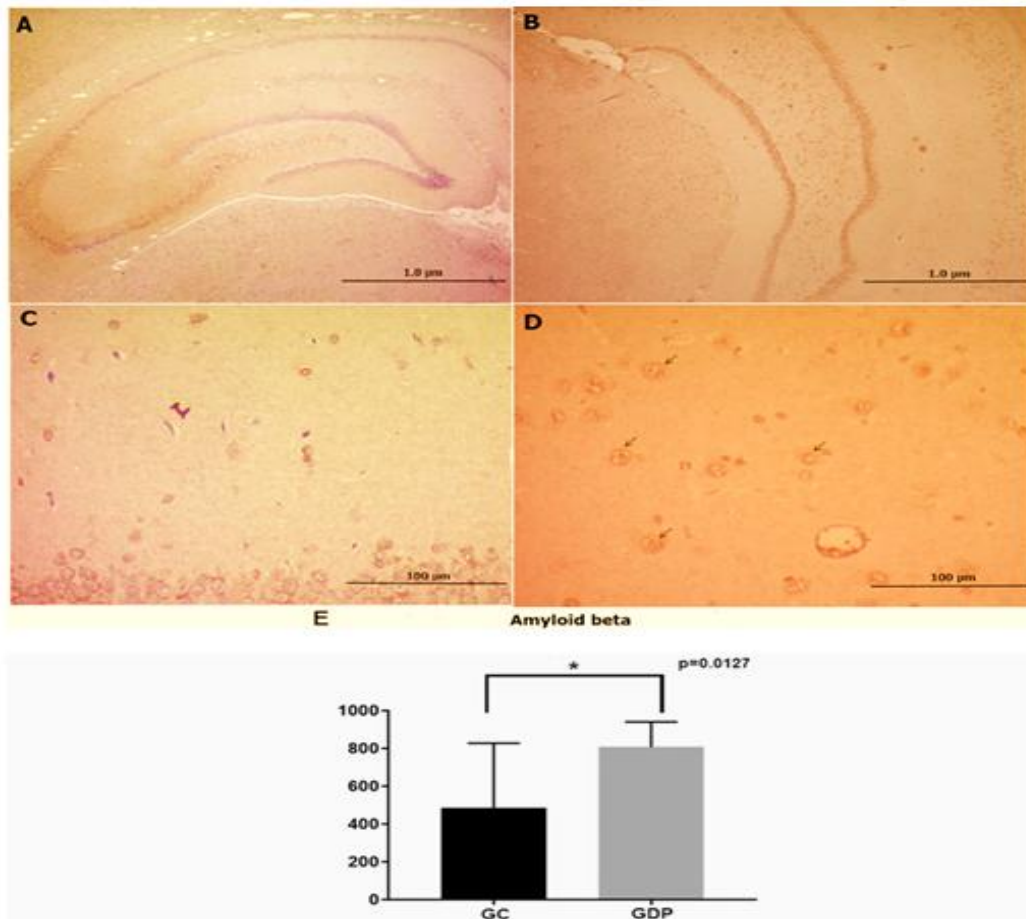

Fig. 3. The beta-amyloid marker was positive in rats with periodontitis; Representative photomicrographs of the coronal section of the hippocampus of the GC and the GDP, in 4 x and 40x (A, B, C, and D). Quantitative analysis of anti-beta-amyloid (E); Abbreviations: GC, Control Group; GDP, Periodontal Disease Group
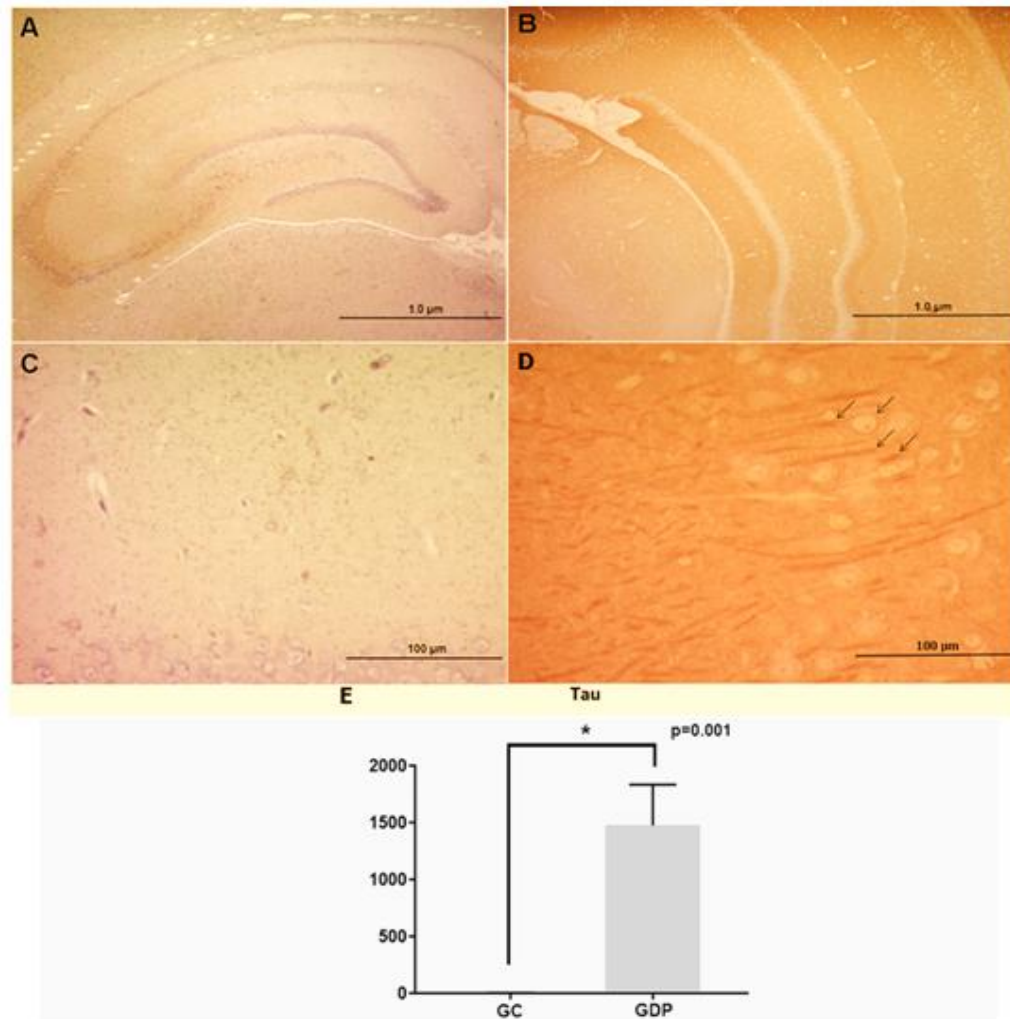
Fig. 4. The tau marker was positive in rats with periodontitis; Representative photomicrographs of the coronal section of the hippocampus of the GC and the GDP, in 4 x and 40x (A, B, C, and D). Quantitative analysis of tau (E); Abbreviations: GC, Control Group; GDP, Periodontal Disease Group

Regarding tau protein (Fig 4E), the GDP showed positive activity when compared to the GC (p = 0.001); the GC average was 1.1, with a standard deviation of 1,475, and the GDP average was 2.331, with a standard deviation of 357.4.

According to Fig 5E, the GDP showed positive CD68 activity (p = 0.003); the GC average was 1.1, with a standard deviation of 2.846, and the GDP average was 91.5, with a standard deviation of 63.03.

4. DISCUSSION

Periodontal infection can harm the systemic health status of the organism, which may be associated with systemic diseases, such as coronary heart disease, cerebrovascular disease, diabetes, chronic obstructive pulmonary disease, and endothelial dysfunction, the latter being the pathophysiological link between PD and erectile dysfunction [24,32,33]. According to Spolidorio et al. [34], the increase in the number of osteoclasts is associated with bone resorption; still, according to Kurikchy et al. [35], a more considerable amounts of osteocytes indicates bone maturity, as it is possible to estimate whether or not there is bone loss, with the increase in the distance between the cementoenamel junction and the alveolar bone crest [24,35].
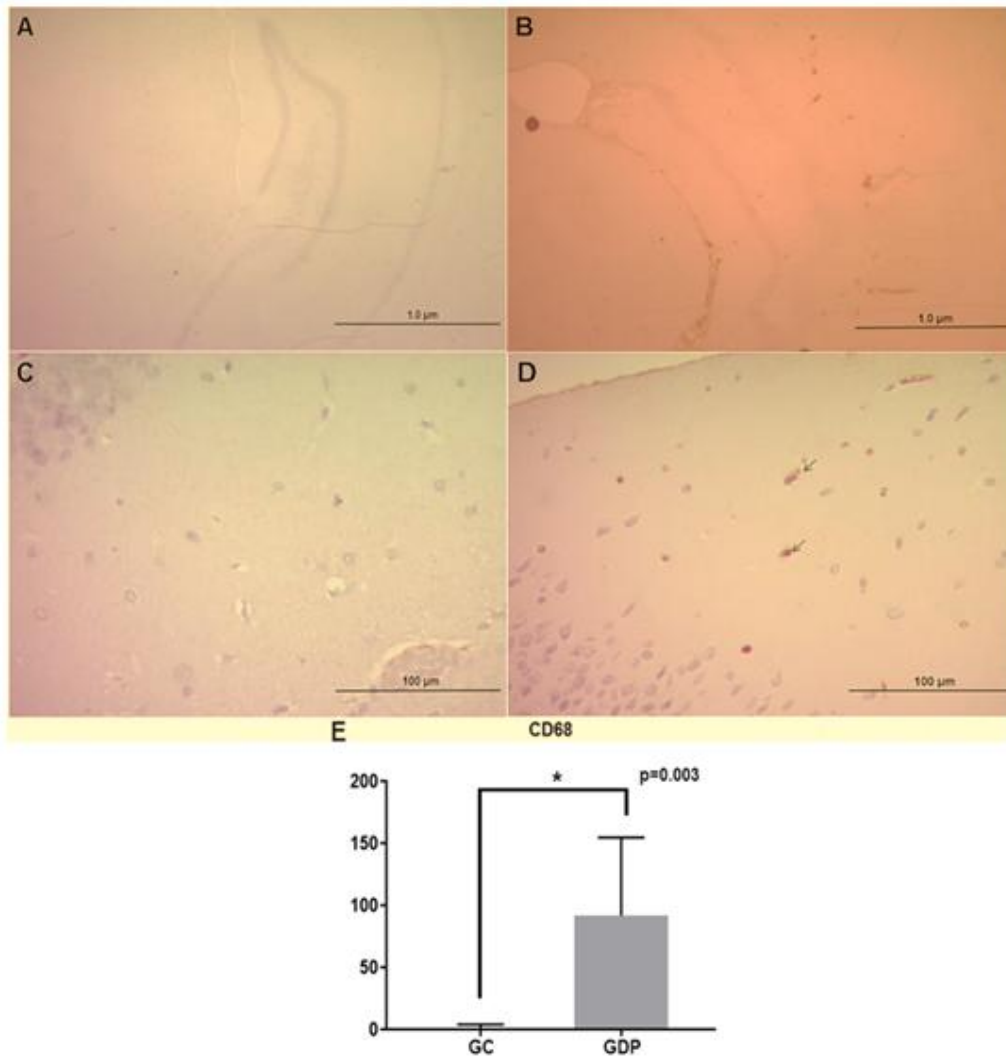
Fig. 5. The CD68 marker was positive in rats with periodontitis; Representative photomicrographs of the coronal section of the hippocampus of the GC and the GDP, in 4 x and 40x (A, B, C, D); Quantitative analysis of CD68 (E); Abbreviations: GC, Control Group; GDP, Periodontal Disease Group

This study used ligature-induced periodontitis methods because it is inexpensive, easy to place, and it has little tissue damage. The biofilm in contact with the tissues provides a continuous antigenic stimulus, which makes the inflammation initially acute, with tissue degradation in a chronic lesion, activation of fibroblasts, polymorphonuclear cells in the junctional epithelium, collagen destruction, inflammatory infiltration and bone resorption, they could be considered as chronic and progressive as the results showed by Nassar et al. [26] and by Pedrotti et al. [24].

Therefore, it is suggested that PD generated an alveolar bone loss as showed in Figs 1E and 1F, and Table 1. These results are corroborated by the detailed microscopic evaluation that revealed changes in the junctional epithelium and osteoclastic resorption activity in the GDP, which is morphological characteristics typical of periodontitis (Figs 1B and 1D).

There is a growing scientific interest in the hypothesis of an association between oral and systemic diseases based on the role of systemic inflammation [13], because if oral pathogens

reach the bloodstream and come into contact with the CNS or if there is stimulation of pro-inflammatory cytokine production in the CNS, there may be a neuroinflammatory response [9,36]. Thus, both periodontal infection and the immune-inflammatory response can increase the host's risk of developing systemic [7,8] and neuroinflammatory diseases; whether there is a genetic susceptibility and, consequently, this individual presents an exaggerated innate immune response in the CNS, making them prone to neurodegeneration [37].

Regarding the exploratory and locomotor activity evaluated in the EPM, it was observed in the GDP that the animals remained longer in the closed arms in the post-induction period (Table 2), which may suggest the presence of anxiety in this group concerning the GC. According to Handley and Mithani [38], it is a natural behavior of the animal in the EPM. In this situation, the animal makes more entries and stays longer in the closed arms., and the higher the levels of anxiety, the lower the percentage of entries in the open arms and the time spent in the same arms. According to Donovan et al. [39], elevated levels of Aβ in the brain are associated with an increase in anxiety-depressive symptoms in cognitively healthy older adults, and these emerging neuropsychiatric symptoms may be early predictors of preclinical AD. Also, the accumulation of Aβ can start in the brain of anxious individuals even before cognitive and behavioral limitations of dementia appear.

According to the fecal lumps variable, this was lower in the post stage; in both groups in the EPM (Table 2), whereas, in the Open Field, there was a decrease in the number of fecal lumps in the GC (Table 3), which may suggest a lower level of anxiety compared to the GDP, a fact that corroborates the study by Pellow et al. [27], who demonstrated that the lower the number of fecal lumps, the lower the anxiety rate.

Ishida et al. [40] observed impaired cognitive function through the object recognition test in female rats, five weeks after induction of PD. In disagreement, in our study, through the discrimination ratio, the object recognition test after four weeks of induction showed that there was no impairment of declarative memory in any of the groups, both of young rats (4 months). Ding et al. [41] found no cognitive impairment in the Morris water maze performed after six weeks of PD induction with *P. gingivalis* in young rats (4 weeks of age), while in middle-aged rats with PD

(12 months of age), learning and memory skills were impaired. Although in the present study the discrimination ratio was not different between the groups, there was a shorter time of exploration of object B (new) in the post stage when compared to the pre stage in the GDP, which may suggest that this was not enough to lead to a deficit in learning and memory, probably due to the age of these animals although there is a discriminatory change. According to Herrup [42], these differences in results between young and middle-aged rats can be attributed to chronic inflammation due to aging.

Matsumura et al. [43] observed in rats with AD, in addition to Aβ accumulation, early CD68 accumulation in Aβ deposition sites. They also found an increase in Aβ deposition after six months, followed by a gradual increase in microglial accumulation at Aβ deposition sites. Accordingly, in this work, the accumulation of deposits of Aβ (Fig 3D), tau (Fig 4D), and CD68 (Fig 5D) were also observed in the hippocampus of PD rats.

Ilievski et al. [44] detected, in rats with *P. gingivalis*-induced PD at seven months and two weeks, elevated levels of IL-6, TNF-α and IL-1β, presence of microgliosis and astrogliosis, neurodegeneration of hippocampal, elevated expression of the Aβ precursor protein (APP) gene and beta-secretase enzyme, presence of extracellular Aβ42 peptide, existence of tau protein and neurofibrillary tangles in the hippocampus. In our study, similar to Ilievski et al. [44], pathological changes were observed earlier (at 4 months of age) in the hippocampus of rats with PD, as previously mentioned about Aβ aggregates, tau protein, and CD68, in addition to histological changes (presence of senile plaques) observed in H&E (Fig 2D) and Congo red (Fig 2H) stains in the hippocampus of the GDP.

Although locomotor and cognitive changes were not observed in the present study (Tables 2 and 3), according to Yoshiyama et al. [45], the presence of Aβ along with neurofibrillary tangles is correlated with cognitive impairment, as well as Aβ and tau can act synergistically causing behavioral deficits.

As Aβ (Fig 3D), the tau markings (Fig 4D) and CD68 (Fig 5D) were positive in the GDP to the GC. This finding translates into a process of disorganization of the system's cytoarchitecture; in this case, hippocampal, together with

histological findings (Figs 2D and 2H), which aspects of senile plaque deposition were observed, characterizes neuroinflammation in process.

5. CONCLUSION

Therefore, it can be concluded that PD triggered the presence of senile plaques, beta-amyloid, tau, and CD68 markings, which together manifested an initial neuroinflammation process in these animals.

CONSENT

It is not applicable.

ETHICS APPROVAL

The Ethics Committee on the Use of Animals (CEUA) of UNIOESTE (with approval opinion of Aug 17th, 2018) approved the project, which followed the Ethical Principles in Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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