Heliyon 11 (2025) e42158

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Potential effects of induced focal ischemia in the motor cortex of rats undergoing experimental periodontitis

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ARTICLE INFO

Keywords:
Motor cortex
Alveolar bone
Motor performance
Periodontitis
Behavior
Brain ischemia

ABSTRACT

Stroke is a severe medical condition resulting from an interruption in the blood supply to the brain, ultimately compromising tissue homeostasis. Currently, stroke stands as the second leading cause of death worldwide and the third leading cause when considering both mortality and disability together. Periodontitis is characterized by persistent inflammation in hard and soft tissues which support the teeth, primarily caused by bacterial biofilms, and is one of the most common causes of tooth loss in adults and can contribute to a systemic inflammatory burden. In the light of this, the present study investigated the effects of inducing focal ischemia in the motor cortex in rats undergoing experimental periodontitis. Adult Wistar rats were divided into four groups (control, ischemia, periodontitis, and periodontitis + ischemia) and were evaluated for motor performance, basic histology, and the volume and microarchitecture of alveolar bone. The results showed that the comorbidity between ischemia and periodontitis aggravates the spontaneous locomotion of rats, although the motor performance of adult rats had not been altered. Nonetheless, they revealed significant tissue impairment in the motor cortex. Additionally, there was a meaningful alteration in both the volume and microarchitecture of alveolar bone in this group. Our results indicate that the model of comorbidity between ligature-induced experimental periodontitis and focal ischemia was capable of inducing greater neurological impairment and alveolar bone loss in rats, attributable to diminished bone quality, when compared to each condition individually.

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https://doi.org/10.1016/j.heliyon.2025.e42158
Received 4 August 2024; Received in revised form 20 January 2025; Accepted 20 January 2025
Available online 25 January 2025

1. Introduction

Stroke is a severe condition caused by disrupted blood flow to the brain, leading to tissue damage from nutrient and oxygen deprivation and impaired homeostasis. It is classified into ischemic (blood vessel blockage) and hemorrhagic (vessel rupture) types [1]. Stroke is the second leading global cause of death and ranks third for combined mortality and disability [2,3], with varying incidence worldwide, placing a heavy strain on health and economic systems [4].

Low- and middle-income countries generally face higher stroke rates due to the widespread prevalence of uncontrolled cardiovascular risk factors, while developed nations have seen reduced incidence, likely due to better prevention and medical care [3,5]. Risk factors such as age, gender, race, and lifestyle are closely linked to stroke. Age is particularly significant, as stroke incidence rises with the aging population [6]. Nonetheless, strokes in younger individuals are also reported, often associated with low physical activity, diabetes, hypertension, smoking, and obesity [7,8].

Stroke symptoms can vary depending both on the brain region affected and the category. According to the National Institute of Neurological Disorders and Stroke (NINDS), the major signs include sudden weakness on one side of the body, trouble walking, difficulty speaking, loss of vision, sudden confusion, dizziness, and severe headache. In addition, tooth loss has been described as an event underlying of stroke [9]. Early diagnosis is crucial, as immediate treatment can help minimize brain damage and improve prognosis.

Inflammation, both local and systemic, is central to stroke and its outcomes [10]. In the acute phase, brain injury triggers a local inflammatory response with pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interferon-γ, interleukin-6 (IL-6), interleukin-1β (IL-1β), chemokines interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and growth factors [11] that attract macrophages and lymphocytes, worsening tissue damage, cerebral edema, and blood-brain barrier disruption. Systemic inflammation, marked by elevated inflammatory markers like TNF-α, IL-1β, and IL-6 in circulation, contribute to the progression of atherosclerosis, atheroma formation, and platelet instability, increasing stroke risk [10]. Immune cells like T lymphocytes and dendritic cells further amplify local and systemic inflammation, exacerbating damage and influencing recovery [12].

Periodontitis is a chronic inflammatory condition affecting the tissues supporting the teeth, primarily caused by bacterial biofilms, and a major cause of adult tooth loss [13]. It accounts for 30–35 % of adult tooth extractions, with prevalence increasing with age due to cumulative damage [14–16]. This localized inflammation can create a systemic inflammatory burden, as cytokines like TNF-α, IL-1β, and IL-6 may enter the bloodstream, impacting distant organs. Periodontitis is linked to systemic conditions such as cardiovascular diseases, rheumatoid arthritis, respiratory issues, and cerebrovascular events [17–20].

Damage to the motor cortex, a key brain region for planning, initiating, and controlling voluntary movements, can profoundly impact daily life after a stroke [21]. Beyond movement control, the motor cortex plays roles in attention, motor observation, learning, somatosensory feedback, and motor imagery [22,23]. After a stroke, the motor cortex undergoes adaptive changes, including neuroplasticity, where undamaged neurons form new synaptic connections to compensate for lost functions, aiding motor recovery [24]. Adjacent or opposite hemisphere areas may also activate to offset motor deficits [25]. Changes in cortical excitability can enhance or impair movement generation [26], and functional reorganization allows regions to take on new motor tasks [27]. Additionally, metabolic changes in neuronal-glial interactions reflect stroke severity in the ipsilesional cortex and stroke duration in the contralesional cortex [28].

Following a motor cortex stroke, patients often experience significant motor impairments, which depend on the severity of the injury [29]. These impairments commonly include a decrease in postural balance, difficulty walking, and limitations in upper limb movement [30]. Observing the reduction in the quality of life of a person who has suffered a stroke, as well as the physical, emotional, and financial challenges that are entailed, there must be scientific work engaged in discovering which factors contribute to a worse outcome. For the reasons already explained, periodontal disease could be one of such factors.

The link between vascular issues and periodontitis has been documented [31,32], with both periodontitis and stroke involving inflammatory processes, raising interest in whether chronic periodontitis may influence cerebrovascular diseases. Inflammation plays a critical role in atherosclerosis [20], a major contributor to stroke, and systemic inflammation from periodontitis may exacerbate this process [33]. A meta-analysis of 30 studies found that individuals with periodontal disease face a 24 % higher stroke risk, highlighting the importance of oral health in reducing vascular events. However, this link is influenced by confounding factors like age, smoking, and other health conditions [34].

In like of the above, the main goal of the present study was to evaluate the effects of inducing focal ischemia in the motor cortex in rats undergoing experimental periodontitis. We sought to characterize motor performance, general aspects of nervous tissue in the region of interest, and the volume and microarchitecture of alveolar bone.

2. Methods

2.1. Ethical statement and experimental animals

Forty Wistar male albino rats, *Rattus norvegicus*, weighing between 200 and 300 g and with approximately 70 days old, were utilized in the present study. The animals were supplied by the Central Animal Facility of UFPA under the Ethics Committee on Experimental Animal Research (CEUA) (ID # 8203010119), in accordance with NIH Guidelines for Use and Care of Experimental Animals. The animals were housed in standard polypropylene vivarium cages (33 × 40 × 17 cm) (four animals per cage) and provided with access to food and water *ad libitum*, with the intake assessed twice daily, aligning with the circadian rhythms of the animals, and kept in a 12:12 h light-dark cycle in a climate-controlled room (25 ± 2 °C, relative humidity of air kept between 65 and 75 %), with lights on at 7:00 a.m.

The animals were randomly assigned, based on their body weight, to four experimental groups: group control without ischemia and without experimental periodontitis; group with ischemia and without experimental periodontitis; group without ischemia with experimental periodontitis; and group with ischemia and with experimental periodontitis. Animals from distinct groups were not mixed in the cages. Fig. 1 summarizes the methodological stages of the study.

2.2. Induction of experimental periodontitis

To ensure consistent stress conditions, all animals underwent anesthesia with xylazine hydrochloride (9 mg/kg) and ketamine hydrochloride (90 mg/kg) (i.p.), on the first day of the experiment. Once corneal and paw reflexes were no longer present in animals from the groups with experimental periodontitis, they were positioned on an operating table designed to maintain the oral opening of the rats, facilitating access to the posterior teeth of the mandible. This procedure was carried out to ensure sufficient surgical and visualization space of the oral cavity. The placement of the ligature was performed with the aid of tweezers. A cotton ligature (Coats Corrente Ltda., São Paulo, SP, Brazil; catalog # BA65601; diameter 0.1 mm) approximately 10 mm in length was carefully passed around the cervical regions of the mandibular first molars (M1) on both sides and securely tied with a double knot to ensure it remained in position without damaging the gingiva. After tying, the position of the ligature was checked to ensure it was correctly positioned at the gingival margin and had not slipped into the interdental space. Non-experimental periodontitis rats underwent the same handling and anesthesia procedures to create consistent stress conditions across all groups. The induction of experimental periodontitis started on the 1st day of the experiment and persisted until the 15th day, concluding with euthanasia [35–37].

Throughout the experiment, ligature retention was checked daily to ensure it remained correctly positioned and effective in inducing periodontitis. This daily check was conducted at the same time each day to maintain consistency. During these checks, rats were gently restrained manually by two examiners using a magnifying glass and focused lighting, without requiring anesthesia. This method facilitated quick and minimally stressful inspections. The number of times ligatures were lost and replaced was recorded. On average, ligatures were lost and replaced in 15 % of the subjects (3 out of 20 rats) over the 14-day period. If a ligature was found to be dislodged during these checks, the rat underwent re-anesthesia using the same protocol and surgical conditions as during the initial ligature placement to ensure consistent handling and experimental conditions [35–37].

2.3. Ischemic stroke model

The stereotaxically guided surgical procedure was conducted seven days after the induction of periodontitis surgery (Fig. 1). To

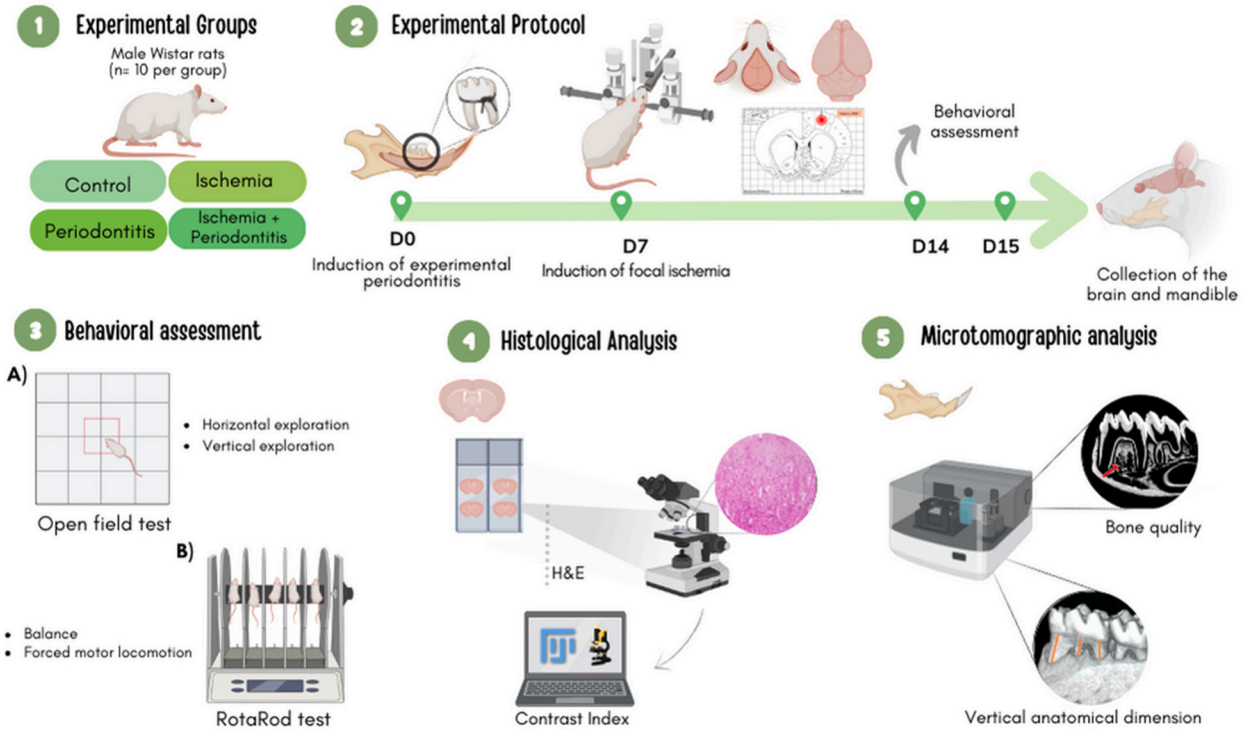

Fig. 1. Animal experimentation and analyses in the comorbidity model. Methodology of animal experimentation and analyses in the comorbidity model, involving the induction of experimental periodontitis and focal ischemia over a 15-day period. The subsequent collection includes both brain and jaw specimens. Statistical analysis was conducted using one-way ANOVA with Tukey's *post hoc* test for intergroup comparisons.

achieve this, all animals underwent intraperitoneal anesthesia with xylazine hydrochloride (9 mg/kg) and ketamine hydrochloride (90 mg/kg), ensuring the absence of corneal and paw withdrawal reflexes as a sign of adequate anesthesia. After anesthetic induction, trichotomy of the cranial calotte region was performed. The animals were then positioned in a stereotaxic frame (Model EFF 331, Insight Scientific Equipments Ltda., Ribeirão Preto, SP, Brazil), with their heads firmly fixed to ensure immobility during the procedure. A sagittal incision was made to expose the cranial region. Following the stereotaxic coordinates of Paxinos and Watson [38] - in millimeters relative to bregma - the access point to the motor cortex (2.3 medio-lateral; 1.2 anteroposterior; 0.4 dorsoventral) below the pial surface was identified, and punctual access was achieved using a long-shank spherical carbide drill bit, No. 7, with a tip diameter of 7 mm (Invicta®, American Burrs, Palhoça, SC, Brazil). This access lies within the M1 area, precisely between layers III and IV (pyramidal neuron areas) [38].

All animals in the ischemia groups were administered an injection of 40 pmol of the vasoconstrictor peptide endothelin-1 (ET-1; Sigma Company, St Louis, MO, USA) diluted in 1 μl of sterile 0.9 % saline solution using a Hirschmann® microcapillary pipette (Sigma-Aldrich Co., Burlington, MA, USA; #Z611239 - 250 EA) (Fig. 1). Following injection, the pipette was left stationary for 3 min to avoid reflux of the solution, being slowly withdrawn. All animals in the non-ischemia groups received an equal volume of 0.9 % saline sterile solution, following the same approach [39–43], so all 40 animals had some sort of brain manipulation.

The exposed region was sutured with nylon thread No. 4, the surgical area was cleaned with a sterile 0.9 % NaCl solution, and a topical healing ointment (Vetaglos®, Vetnil, Louveira, São Paulo, Brazil) was applied for three consecutive days. After surgery, the animals were returned to the animal housing, kept in appropriate cages with balanced water and food (Labina Presence, #5001; Purina, São Paulo, Brazil), and observed daily for the condition of the lesion area. In the present ischemic model, the mortality rate is less than 5 %. Specifically, one animal from the periodontitis group was lost after the induction of periodontitis, and another one from the periodontitis + ischemia group was lost following the induction of ischemia. These animals were promptly replaced, in accordance with the approved number of animals by the ethics committee, ensuring the integrity of the experimental groups.

2.4. Behavioral assays

Behavioral motor tests were conducted on the 7th day following the induction of cerebral ischemia. Subsequently, all animals (n = 10 per group) were carried to the assay room, where noise levels were attenuated, and low illumination (12 lux) was maintained. The animals were acclimated for at least 1 h before the beginning of the behavioral tests. Each animal was assigned sequential numbers by one of the researchers, and the researchers responsible for the behavioral assessment were kept blind to this information.

2.4.1. Spontaneous locomotor activity - open field test

The open field test aimed to evaluate both horizontal and vertical exploration based on the animal's spontaneous locomotion. A wooden arena with a floor measuring 100 × 100 cm and walls 40 cm high, covered with waterproof formica and colored black, was utilized for this study. The arena was divided into 25 equal segments (20 × 20 cm) by a video monitoring system ANY-maze™ version 4.99 (Stoelting Co., Wood Dale, IL, USA). Thigmotaxis evaluation included two zones: central (36 %) and peripheral (64 %) [44,45].

At the beginning of the session, rats were placed in the center of the arena, and their movements were recorded and analyzed using the video tracking system. An experimenter, unaware of the treatment group being tested, observed the animals for a duration of 5 min. The behavioral assessment included measurements of the number of rearing behavior and the total distance traveled in meters.

2.4.2. Rotarod test

The rotarod test was employed to evaluate motor coordination, balance, and forced motor locomotion by assessing the animals' ability to adapt and remain on a rotating cylindrical base. Animals were positioned in the rotarod apparatus (Insight Scientific Equipments Ltda., Ribeirão Preto, SP, Brazil), consisting of an acrylic box containing a transversely installed grooved rotating cylinder metal roller, 8 cm in diameter, situated approximately 20 cm above the ground and driven by a motor. The box was partitioned into four compartments, each around 10 cm wide, allowing simultaneous analysis of four animals. The test involved placing the animal on the rotating cylinder, and the number and latency of falls were recorded. Firstly, animals were submitted to stay on the rotating rod at 16 revolutions per minute (rpm) for 3 min (training session). Then, we assessed the animal's ability to remain on the rod for three successive sessions of 3 min each at 16 rpm with an interval of 60 s [46,47].

2.5. Euthanasia and sample collection

At the conclusion of the experimental period, animals were anesthetized with a mixture of ketamine hydrochloride (180 mg/kg) and xylazine hydrochloride (30 mg/kg), i.p. Following the complete loss of reflexes, euthanasia was carried out via exsanguination, drawing whole blood into a syringe through cardiac puncture. Subsequently, both the motor cortex and mandibles were collected from each animal. The mandible extraction procedure began with an incision made using sharp scissors, gently cutting from the labial commissure to the region of the mandibular condyle. This initial step exposed the necessary areas for subsequent dissection. Using a No. 5 scalpel, all the soft tissues around the buccal mucosa and the muscles adjacent to the mandible were separated. During this stage, pliers were used to assist in manipulating and separating the tissues, ensuring a clear and complete exposure of the temporomandibular joint (TMJ). With precision, all the soft tissues and muscles were fully removed to expose the TMJ, which was crucial for performing the disarticulation without damaging the bony structures. After the complete exposure of the joint, the mandibles were manually disarticulated to gently separate the bones at the joint.

The animals underwent perfusion through the left ventricle of the heart using 0.9 % saline solution, heparinized, followed by 4 %

paraformaldehyde in 0.1M phosphate buffer, for morphological analyses in the motor cortex. The mandibles were fixed in 4 % formalin for 48 h for subsequent microtomographic and 3D bone loss analysis.

2.6. Tissue processing

Brain samples were collected and post-fixed in Bouin's solution for 6 h. Subsequently, they were dehydrated using increasing concentrations of ethanol (70 %, 80 %, 90 %, absolute I, and absolute II), diaphanized in xylol I and xylol II, and embedded in Paraplast (McCormick Scientific, Baltimore, MD, USA). Following embedding, blocks were sectioned by a microtome to obtain 5 μm-thick sections. All histological analyses were conducted in the M1 area of the motor cortex, specifically between layers III and IV (pyramidal neurons areas), with coordinates at 2.5 mm lateral, 1.2 mm posterior, and 4.5 mm below from the pial surface (at Bregma 0.20 mm and interaural 9.20 mm) [38]. Sections were stained with hematoxylin and eosin (HE) for tissue evaluation, being then dehydrated, cleared with xylol and coverslipped with Entellan (Merck, Darmstadt, Germany) [48]. Photomicrographs were captured of the most representative fields for each group using an Axioscope microscope equipped with a CCD AxioCam HRC color camera (Carl Zeiss Inc., Oberkochen, Germany), as described previously [46,49].

Intensity assessment of HE staining was conducted via densitometric analysis using ImageJ software, version 1.53 (http://rsb.info.nih.gov/ij/), in a blinded fashion. A 0.25 mm^2 square window was defined in ImageJ and positioned over the motor cortex, with three samples per section and two sections per animal. To address within-group variability, a normalized scale based on the cortical white matter beneath the motor cortex was applied. For each animal, the average optical density (OD) was defined as Ctx, the white matter as Wm, and a contrast index (C) was calculated using the formula: $C = (Ctx - Wm)/(Ctx + Wm)$ [50].

2.7. Bone microstructural analysis

The samples underwent evaluation using X-ray microcomputed tomography (MicroCT.SMX-90 CT; Shimadzu Corp., Kyoto, Japan). Each hemimandible was positioned on a rotating platform within the device, capturing images in a 360° rotation with an intensity of 70 kV and 100 mA. Reconstruction of images was performed using inspeXio SMX-90CT software (Shimadzu Corp., Kyoto, Japan) with a voxel size of 10 μm.

Bone images were specifically captured in the interradicular region, close to the furcation region of the mandibular first molar. A standardized area was established to create the region of interest (ROI), focusing on the interradicular region of the mandibular first molar from the apical third to the cervical third, with an average area of 0.200 mm^2. To segment different gray values in the image, a threshold was applied, and measurements were conducted using the NIH ImageJ software program (National Institutes of Health, Bethesda, MD, USA). Gray levels of bone and other structures in the images were considered to determine the threshold, which was set from 120 to 255. Trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), and bone volume to tissue volume ratio (BV/TV) were quantified using the BoneJ plugin [35–37].

2.8. Analysis of vertical bone loss through 3D reconstruction

To evaluate vertical bone loss, 3D reconstructions of the hemimandibles were performed using RadiAnt DICOM Viewer 5.0.1 software (Medixant, Poznan, Poland). The three-dimensional models were manipulated to rotate and position them in a standard orientation, enabling observation of the buccal and lingual aspects of the teeth. Vertical bone loss was quantified by measuring the distance between the cemento-enamel junction (CEJ) and the alveolar bone crest at six specified points around the lower first molar: mesio-lingual, mid-lingual, disto-lingual, mesio-buccal, mid-buccal, and disto-buccal. The average of these measurements was subsequently calculated [35,37].

2.9. Statistical analyses

Statistical analyses were performed by using Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA). The data from the four experimental groups were tabulated, and the normality (Gaussian distribution) of each group was assessed using the Shapiro-Wilk method. Subsequently, one-way ANOVA was applied, and Tukey's post-test was conducted for group comparisons, with a significance level set at $p < 0.05$. We also used a two-way ANOVA with Tukey's post-test to analyze the significant difference between groups in body weight register during the experimental period. The results are presented as the mean ± standard error of the mean (SEM).

3. Results

3.1. The comorbidity between ischemia and periodontitis influences initial body weight changes but stabilizes over time

The analysis of body weight variations over time was performed by calculating the individual changes in body weight relative to the initial weight at D0 (Δ weight) and comparing these values for each animal at D7 and D15. At D7, significant differences were observed between the stroke group (15.05 ± 4.42) and the control group (9.57 ± 7.07; $p < 0.01$) and between the stroke and periodontitis groups (1.54 ± 9.12; $p < 0.05$). Specifically, the stroke group showed a significant increase in body weight as compared with the control group and a significant decrease as compared to the periodontitis group. In contrast, at D15, no statistically significant differences in body weight were observed among the groups ($p > 0.05$).

3.2. The comorbidity between ischemia and periodontitis aggravates the spontaneous locomotion of rats

Spontaneous locomotor activity was evaluated through the open field paradigm (Fig. 2). Fifteen days post-ischemia and post-periodontitis was not able to change the horizontal ambulation related to control group (Fig. 2A). Also, the co-occurrence of periodontitis and ischemia did not result in change in the total distance traveled compared with all groups tested (Fig. 2A).

Vertical exploration was evaluated through the number of rearing parameter (Fig. 2B). The periodontitis and periodontitis + ischemia exhibited the worst performance on the apparatus, reducing the rearing frequency related to all groups tested (Fig. 2B).

3.3. Experimental periodontitis and ischemia, in comorbidity, did not alter the motor performance of adult rats

The impact of concurrent induction of periodontitis and ischemia on the motor coordination and balance of adult rats was assessed in the Rotarod test (Fig. 3).

The latency time of the animals, evaluated in three sessions, did not exhibited significantly values in the groups exposed to damages (periodontitis and/or ischemia) compared with control group. There was no difference in the training session between the groups.

In addition, the number of falls during training showed no statistical change among the groups. In the three sessions, no changes were observed between these groups in the Rotarod test.

3.4. The comorbidity between ischemia and experimental periodontitis modifies the volume and microarchitecture of alveolar bone in rats

The data indicate that, in all evaluation parameters, the control group and ischemia group did not exhibit statistically significant differences ($p > 0.05$) (Fig. 4). However, when comparing the control group with the periodontitis group, a decrease was observed in trabecular thickness (Tb.Th), bone volume (BV/TV), and trabecular number (Tb.N), whilst trabecular spacing (Tb.Sp) demonstrates an increase in the periodontitis group when compared to the control group. Additionally, it was observed that in the periodontitis plus ischemia group, there was an even greater decrease in these mentioned parameters when compared with the other groups, except for trabecular spacing.

3.5. Histological disturbance induced by ischemia and periodontitis

Periodontitis did not induce alteration of tissue in the motor cortex, showing no difference as compared with the control group. Injection of ET-1, in turn, induced a conspicuous loss of tissue in the motor cortex as compared with the control group. Such pattern was amplified in the periodontitis + ischemia group, revealed by a significant cell loss in the region of lesion (Fig. 5).

3.6. Ischemia associated with experimental periodontitis induction aggravates vertical alveolar bone loss

The evaluation of vertical bone loss, measured by the distance from the cementoenamel junction to the alveolar bone crest in 3D, revealed that both the control group and ischemia group did not exhibit statistically significant differences (Fig. 6). However, when

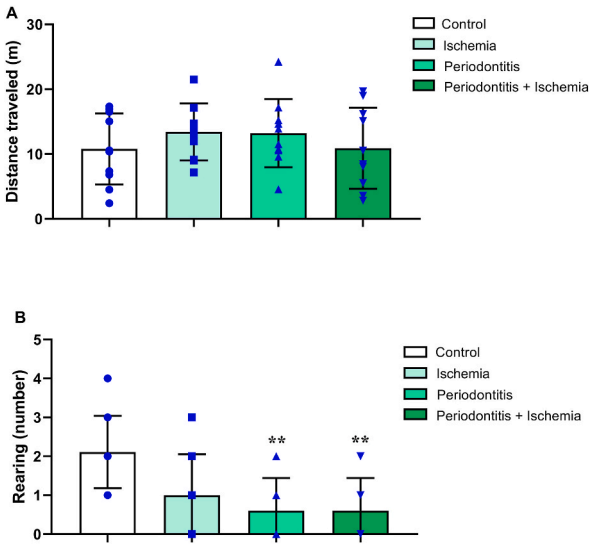

Fig. 2. Effects of experimental periodontitis associated with the ischemia model, over a 15-day period, on the spontaneous locomotion in the motor cortex of adult rats. Total distance traveled (A) and rearing behavior (B). Results were expressed as mean ± S.E.M. **$p < 0.01$, ***$p < 0.001$ compared to the control group. One-way ANOVA, Tukey's *post hoc* test.

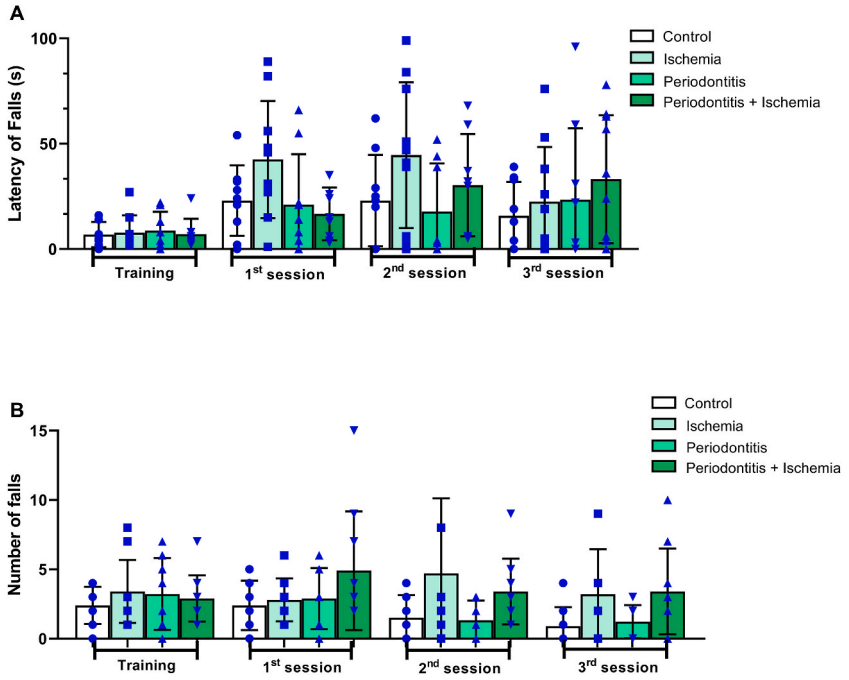

Fig. 3. Effects of experimental periodontitis induction associated with the ischemia model over a 15-day period, on spontaneous locomotion in the motor cortex of adult rats. Latency of falls, in seconds (A), and number of falls (B). Results were expressed as mean ± S.E.M. No significant statistical differences were observed ($p > 0.05$). One-way ANOVA, Tukey's *post hoc* test.

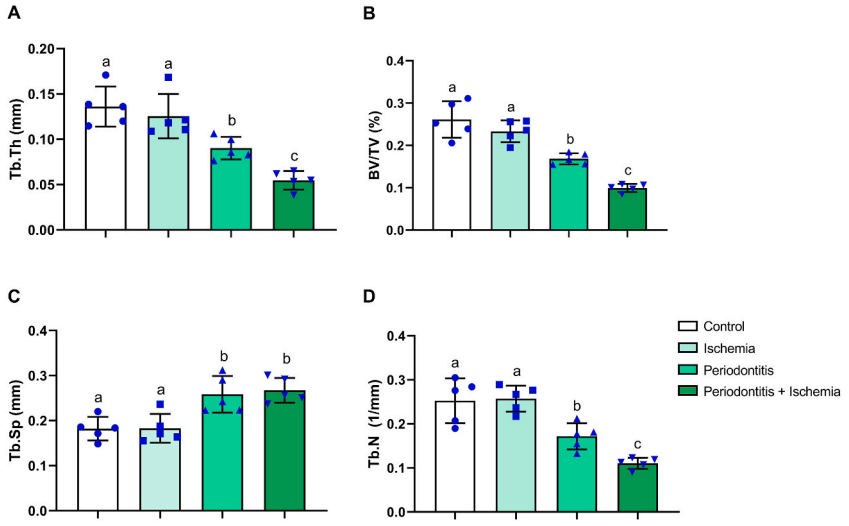

Fig. 4. Effects of experimental periodontitis induction associated with the ischemia model, over a 15-day period, on bone microstructure in adult rats. Results were expressed as mean ± standard error. Identical superscript letters (a, a) do not indicate statistical difference ($p > 0.05$), and different letters (a, b, c) indicate statistical significance after One-way ANOVA and Tukey's *post hoc* test ($p < 0.05$).

comparing the control group with the periodontitis group, significant bone loss was observed. Additionally, it was noted that in the periodontitis plus ischemia group, the bone loss was greater compared to the control (Control group: 0.72 ± 0.02; ischemia: 0.76 ± 0.02; $p = 0.90$; periodontitis: 0.93 ± 0.06; $p = 0.03$; periodontitis + ischemia: 1.16 ± 0.07; $p = 0.0004$).

4. Discussion

In the present study, we evaluated the relationship between periodontitis and ischemia in a rat model. Our novel findings demonstrate that the comorbidity between ischemia and periodontitis leads to more pronounced body weight changes in the initial

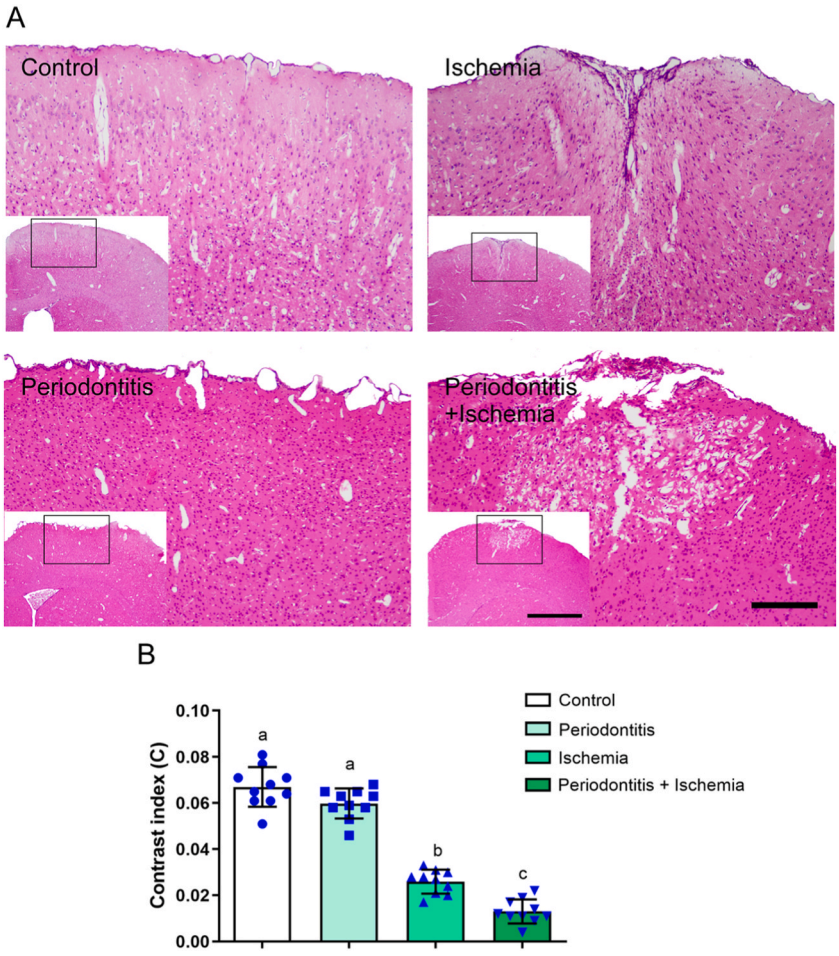

Fig. 5. A. Basic histology following endothelin-1 (ET-1) injection and periodontitis. It is possible to notice a tissue rarefaction in the ischemia group, amplified in the periodontitis + ischemia group, as compared with the control group. Periodontitis group, conversely, presented no difference in the motor cortex as compared with the control group. Scale bars: 1 mm (lower magnification); 200 μm (enlargements). B. Effects of experimental periodontitis induction associated with the ischemia model, over 15 days, on nervous tissue. Results were expressed as mean ± standard error. Identical superscript letters (a, a) do not indicate statistical difference ($p > 0.05$), and different letters (a, b, c) indicate statistical significance with the control group after One-way ANOVA and Tukey's *post hoc* test ($p < 0.05$).

days following treatment induction, with these changes stabilizing over time. Additionally, this comorbidity attenuated rearing behavior in rats. Despite inducing histological disturbances, however, experimental periodontitis and ischemia did not affect the motor performance of animals. Moreover, this comorbidity significantly altered the volume and microarchitecture of alveolar bone, with ischemia combined with experimental periodontitis induction exacerbating vertical alveolar bone loss.

There is a relationship between tooth loss/periodontitis and the onset and exacerbation of stroke [51]. In line with this, our study shows similar tissue alterations as described in other studies [52–55] as well as in humans who have suffered a stroke [56]. However, in the periodontitis + ischemia group, tissue degeneration was distinctly increased. This phenomenon can be explained by the increase in neuroinflammation caused by periodontitis [57]. As observed by Wang et al. [58] mice with periodontitis present higher levels of TNF-α compared with the control group. In addition, a notable gliosis was observed in the periodontitis group [58].

Furutama et al. [59] demonstrated that mice with periodontitis exhibited disruption of the blood-brain barrier (BBB) and increased expression of hippocampal levels of IL-6 and IL-1β mRNA. Alongside the upregulation of pro-inflammatory cytokines, there was a notable increase in microglial activation, a hallmark of neuroinflammation [60,61]. It is important to note that in cases of systemic neuroinflammation, such as periodontitis, the nervous system becomes more susceptible to the onset and exacerbation of neurodegeneration [62,63].

From a behavioral perspective, studies point out motor deficits in animals following brain ischemia, particularly affecting limbs contralateral to the injury [54,64–66]. Teixeira et al. [67] have described an association between cell loss in this region and motor deficits. It is noteworthy that in some instances, stroke can lead to degeneration of corticospinal motor fibers, which may affect long-term motor capabilities in addition to neuronal loss [68]. Studies have highlighted the potential link between oral health, specifically periodontitis, and worsened stroke outcomes. For instance, research has shown that individuals with periodontitis may

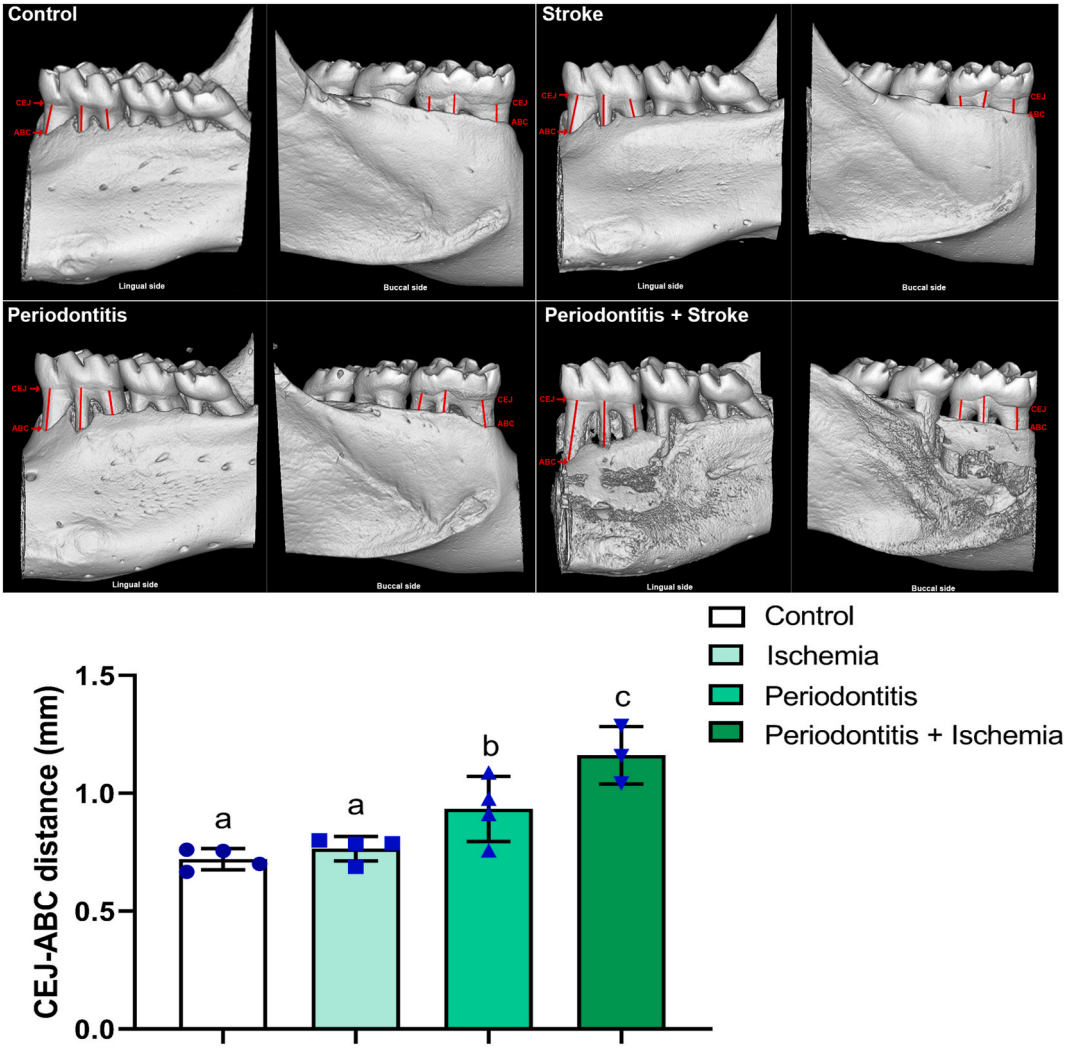

Fig. 6. Effects of experimental periodontitis and ischemia on vertical alveolar bone loss in rats. The figure shows the 3D reconstructions of the left mandibles from four groups of adult rats: control, stroke, periodontitis, and periodontitis + ischemia. The images display both the lingual and buccal sides of the mandibles. Lines indicate the cementum-enamel junction (CEJ) and the alveolar bone crest (ABC) distance. The graph below the reconstructions quantifies the CEJ-ABC distance (vertical alveolar bone loss) for each group. Results are expressed as mean ± standard error. Identical superscript letters (a, a) indicate no statistical difference ($p > 0.05$), whereas different letters (a, b, c) denote statistical significance according to One-way ANOVA and Tukey's *post hoc* test ($p < 0.05$). The data shows a significant increase in CEJ-ABC distance in the periodontitis and periodontitis + stroke groups compared to control and stroke groups, indicating greater bone loss in these conditions.

experience larger infarct sizes, increased neurological deficits, and higher mortality rates following a stroke [69,70]. Consistent with this, Slowik et al. [71] reported that patients with severe periodontitis experienced more significant neurological deficits after stroke, a finding paralleling with the present study. In the present study, we did not observe significant changes in the motor behavior of the animals, except for attenuated rearing behavior. This attenuation may be related to subtle changes in the motor cortex resulting from ischemic injury combined with systemic inflammation triggered by periodontitis. The absence of broader motor deficits could be attributed to the animals' survival time. Future studies assessing chronic progression of ischemia in the motor cortex and its interplay with periodontitis will be crucial to further elucidate this association.

In periodontitis, the exacerbated host inflammatory response is associated with a greater amount of tissue damage, exacerbating periodontal breakdown [72,73]. Periodontitis progression can result in changes in oxidative balance, bone metabolism with the disruption of osteoblast and osteoclast activity [74]. This stimulus leads junctional epithelial cells to produce cytokines, such as TNF-α, IL-1β, and IL-6, that trigger a cascade of events that activate innate immunity, culminating in damage to the alveolar bone and other structures of the periodontium and influence the inflammatory mediators into the systemic circulation, which may trigger inflammatory responses in other organs and systems of the body [75]. Further studies using our model can help to further elucidate the role of cytokines in following the association between periodontitis and stroke.

Our results showed that ischemia was able to reduce the volume and quality of alveolar bone affected by periodontitis, rather than

the disease itself. Furthermore, these results indicate that ischemia can potentiate induced bone loss. In cases of periodontitis, the application of antioxidant and anti-inflammatory compounds, as outlined by Lima et al. [42], has been linked to enhanced endothelial function and a reduction in inflammation markers, and an increase in the antioxidant capacity of the intrinsic glutathione system, thereby overcoming oxidative effects. Also, periodontitis tends to be linked to an increase in serum levels of inflammatory markers related to systemic diseases, such as C-reactive protein, leading to a chronic state of low-grade systemic inflammation and therefore causing endothelial dysfunction [32,70]. In this context, neuroinflammation, which plays a significant role in acute cerebrovascular events such as stroke, involves mechanisms that impact cerebral vascular function and neuronal integrity. Although the direct colonization of the brain by periodontal pathogens has not been demonstrated, bacterial vesicles, lipopolysaccharides and inflammatory mediators may cross the blood-brain barrier, contributing to the neuroinflammatory environment and potentially exacerbating ischemic injury.

Our present findings pointed out alterations in the motor cortex tissue due to ischemia and periodontitis, yet without a notable functional impact that exacerbates ischemia significantly. Conversely, the ischemic event prompted a systemic response intensifying periodontitis. These findings can be attributed to the experimental design chosen, which focused on acute injury (tissue evaluation after seven days of concomitant induction of periodontitis and ischemic injury). Considering the relationship between periodontitis and stroke in humans [32,51], future investigations in animal models evaluating longer injury durations should unveil a more direct association between chronic peripheral disruptions and tissue damage post-stroke induction. This is particularly relevant considering that periodontitis is a chronic disease that quietly unfolds over time, characterized by a persistent inflammatory reaction of the immune system to dysbiotic dental biofilm. This sets off a slow and steady degradation of periodontal tissues, often initially unnoticed in its early stages, potentially influenced by age-related pathophysiological changes that may similarly underlie their development and progression [76,77].

Given the findings of our study, another key factor to be considered is the extent of ischemic damage. In our lesion model, ischemia induction involves the injection of a small quantity of ET-1, which causes a focal injury [42]. Since focal ischemia typically results in smaller areas of damage compared to occlusion of the middle cerebral artery models [78,79], the low concentration of ET-1 used may not have been sufficient to induce a disturbance significant enough to be magnified by acute periodontitis. Further studies adopting a more extensive lesion induced by ET-1 through multiple injections or higher concentrations may offer a clearer draw of the association between periodontitis and ischemia in the proposed model, as well as the exploration of biomarkers such as S100B and GFAP [80–82] in our model.

In our study, we focused on contrast density due to several methodological limitations and considerations. Our available equipment and resources were optimized for imaging and analyzing contrast density within the central nervous system. Performing specific neuronal health indicators, such as cell counts or using neuronal-specific stains, requires specialized equipment and techniques that were not accessible to us during this study. Contrast density analysis has been widely used in previous studies of our group to infer changes in tissue structure and integrity in various models of central nervous system injury and disease [43,50,83]. By utilizing this method, we aimed to maintain consistency with established methodologies, allowing for better comparison and validation of our results against existing literature. Given these constraints, we prioritized a method that could provide reliable and reproducible data within the scope of our current capabilities.

5. Conclusions

In our animal model of comorbidity, the combination of periodontitis and cortical ischemia led to changes in motor cortex tissue, yet without a significant functional impact that worsened ischemia. Moreover, this combination exacerbates alterations in both the quality of tissue and vertical alveolar bone loss. Our findings highlight the need for further studies to understand the underlying mechanisms of this comorbidity. Such insights are crucial for developing effective therapeutic strategies for patients in dental and neurological clinics dealing with these conditions.

CRediT authorship contribution statement

Victória dos Santos Chemelo: Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marco Aurelio M. Freire:** Writing – review & editing, Writing – original draft, Formal analysis. **Leonardo Oliveira Bittencourt:** Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Deborah Ribeiro Frazão:** Resources, Investigation, Data curation, Conceptualization. **Deiweson Souza-Monteiro:** Resources, Methodology, Investigation, Data curation. **Sabrina C. Cartagenes:** Methodology, Investigation. **Walace Gomes-Leal:** Writing – review & editing. **Cristiane do Socorro Ferraz Maia:** Writing – original draft, Visualization, Formal analysis, Data curation. **Gabriel S. Rocha:** Writing – original draft. **Daniel Falcao:** Writing – review & editing. **Rafael Rodrigues Lima:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability statement

All data are available within the article.

Funding

Research funded by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Finance Code 001), and Fundação de Amparo à Pesquisa do Estado do Pará (FAPESPA) — Brazil. RRL and CSFM are researchers from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant numbers 312275/2021–8 and 303196/2022-0, respectively. The APC payment was supported by Pró-reitoria de Pesquisa e Pós-graduação (PROPESP) from Federal University of Pará (UFPA).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the two anonymous reviewers for their valuable comments and propositions for the study.

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