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Hypertension favors the inflammatory process in rats with experimentally induced periodontitis

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Background and Objective: Cardiovascular diseases are significantly correlated with chronic periodontitis. The aim of this study was to evaluate bone-loss level, neutrophil migration, CXCL2/CINC-2α, CXCL5/LIX, CCL20/MIP-3α and tumor necrosis factor-α (TNF-α) production, inducible nitric oxide synthase (iNOS) expression and C-reactive protein (CRP) release in spontaneously hypertensive rats (SHRs) and normotensive (WTK) rats after experimental induction of periodontal disease.

Material and Methods: Periodontitis was induced by placement of silk yarn ligatures around the first molar counterparts. The levels of CRP, CCL20/MIP-3α and CXCL5/LIX were evaluated in the peripheral blood, and bone-loss level, neutrophil recruitment, the production of myeloperoxidase, CXCL2, CXCL5, CCL20 and TNF-α, and the expression of iNOS were evaluated in the gingival tissue. Histological sections were taken to evaluate and measure bone resorption and neutrophil recruitment in the furcation region.

Results: Rats with periodontitis had alveolar bone resorption. SHRs with periodontitis showed marked bone loss and increased neutrophil infiltration in comparison with WTK rats. SHRs with periodontitis showed increased levels of TNF-α and CXCL2, and a slight tendency for increased levels of CXCL5, in the gingival tissue but no increase in the level of CCL20. In SHRs, even without periodontitis, the levels of TNF-α, CXCL2, CXCL5 and CCL20 showed a slight tendency to increase. In the WTK rats, TNF-α, CXCL2 and CXCL5 levels were increased with periodontitis, but the level of CCL20 was not. iNOS was expressed in the gingival tissue of WTK rats and SHRs with periodontitis; however, SHRs appeared to express a higher level of iNOS than did WKT rats. The CRP level was elevated in both types of rats with periodontitis; however, the CRP level was higher in SHRs with periodontitis than in WTK rats with periodontitis.

Conclusion: In SHRs, the hypertensive condition per se seems to favor the inflammatory processes that become potentiated with periodontitis, when compared with WKT rats.

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Periodontal disease is amongst the most common infectious diseases of humans and is characterized by bacterial-induced inflammatory destruction of tooth-supporting tissues, including alveolar bone. These manifestations are mediated by periodontal pathogens in the biofilm, which can induce changes in lipid metabolism and increases in C-reactive protein (CRP), fibrinogen, cytokines and chemokines, and trigger the coagulation system (1). Although the primary cause of periodontitis is bacterial infection, several systemic diseases seem to be initiated with the development of destructive periodontal disease, or the presence of systemic disease may favor the development of periodontitis (2).

Cardiovascular disease, the leading cause of death in most countries, is significantly correlated with chronic periodontitis (3,4). Although this correlation is probably because the two diseases have various common risk factors, most epidemiological studies have interpreted poor oral health, and mainly periodontal disease, as being a risk factor for coronary heart disease and stroke (5). On the other hand, hypertension can affect periodontal membrane vessels, inducing malfunction of small arterioles and gingival bleeding, causing alteration of the tooth-supporting alveolar bone (6–8). In this context, a number of hypotheses have been put forward; these include common susceptibility, inflammation via increased levels of circulating cytokines and inflammatory mediators in both diseases, direct infection of the blood vessels and the possibility of cross-reactivity interfering with innate immunity and the adaptive response.

Spontaneously hypertensive rats (SHRs) were originally inbred from Wistar rats and their Wistar–Kyoto inbred nonhypertensive controls (9). These rats develop hypertension at about 4–6 wk of age without physiological, pharmacological or surgical intervention (10); however, environmental factors also affect the development of hypertension (11). SHRs have an increased cardiac output with normal total peripheral resistance. As the SHR progresses into the established hypertension state, the cardiac output returns to normal and the hypertrophied blood vessels produce an increase in the total peripheral resistance (12). The importance of this model has been attributed to the similarity of its pathophysiology with essential hypertension in humans (11) and it can be used to investigate the interaction of hypertension conditions and periodontitis. The relationship between hypertension and periodontitis was previously evaluated in different studies (7,13–16). However, few studies have focused on the impact of hypertension on inflammatory parameters observed in periodontal local tissue (7,13,14,17). Considering the availability of arterial-hypertension experimental models and the hypothesis that hypertension increases the risk of development of periodontal disease, the present study was conducted to examine the influence of hypertension and/or periodontitis in the inflammatory response in the periodontium and to systemically compare hypertensive and normotensive animals. For this purpose, bone-loss level, neutrophil recruitment, production of tumor necrosis factor-α (TNF-α), CXCL2/CINC-2α, CXCL5/LIX and CCL20/MIP-3α, expression of inducible nitric oxide synthase (iNOS) mRNA and release of CRP were evaluated in SHRs and the results were compared with those obtained for normotensive (WTK) rats.

Material and methods

Animals and ethical aspects

All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Welfare Committee of the School of Dentistry, of Araçatuba, Universidade Estadual Paulista (Araçatuba, SP, Brazil) (protocol #52/06). A total of 30 male WTK rats and 30 male SHRs, weighing 200–250 g, were used. These animals were housed in temperature-controlled rooms and received water and food *ad libitum*. In order to obtain all the gingival samples from the first-molar perimeter used in this study, the rats were killed with an overdose of halothane (Tanohalo; Cristália, Campinas, SP, Brazil).

Experimental design

The rats were anesthetized with 80 mg/mL of ketamine hydrochloride (Dopalen; Vetbrands, Paulínia, SP, Brazil) and with 10 mg/mL of xylazine hydrochloride (Anasedan; Vetbrands), and a silk thread ligature was placed bilaterally around the mandibular first molars in a submarginal position to induce experimental periodontitis. The rats were divided into four groups: SHRs with periodontal disease (n = 15); SHRs without periodontal disease (n = 15); WTK rats with periodontal disease (n = 15); and WTK rats without periodontal disease (n = 15). SHRs and WTK rats were killed 14 d after ligature placement by halothane inhalation. The fragments of gingival tissue adjacent to the first molar were removed with scalpel blades, frozen in liquid nitrogen and then stored at −80°C until use. The jaw was dissected and stored in alcohol. Five rats of each group were randomly selected, and their mandibles were processed for histological analysis.

Histologic procedures and histometric analysis

The mandibles were removed and fixed in 4% neutral formalin solution for 48 h, then demineralized by immersion for 45 d in a solution containing 10% ethylenediaminetetracetic acid. After this procedure, the mandibles were dehydrated in an ascending series of ethanol solutions, xylene and embedded in paraffin. Serial sections (6 μm) were obtained in a mesio-distal direction, stained with hematoxylin and eosin, then analyzed by light microscopy to establish bone loss and characteristics of periodontal ligament in the furcation region of the mandibular first molars. After excluding the first and last sections in which the furcation area was totally evident, five equally distant sections of each tooth were selected for histometric analysis and captured by a digital camera connected to a light microscope. The area (mm^2) of periodontal ligament for unligated

teeth and the area of bone loss for ligated teeth in the furcation region were determined histometrically (18), as the area between the inter-radicular bone crest and the furcation roof. Another masked, trained and calibrated examiner conducted the histometric analysis using image-analysis software (AxioVision Rel. 4.8; Carl Zeiss, Frankfurt, Germany). The area between the inter-radicular bone crest and the furcation roof was also determined from a radiograph of using Digora (Software Digora, SOREDEX, Milwaukee, WI, USA).

Histological analysis of neutrophil recruitment

Whole mandibules were removed and fixed in 4% neutral formalin. The mandibules were maintained in formalin for a further 24 h before being embedded in paraffin using standard histological techniques. Six-micrometer mandibular tissue sections were stained with hematoxylin and eosin for analysis of neutrophil accumulation. Slides were blinded and coded. Neutrophil counts were analyzed by counting the number of neutrophils surrounding the area between the inter-radicular bone crest and the furcation roof in 10 high-power fields (× 1000).

Determination of the myeloperoxidase levels in gingival tissues of SHR and WTK animals

Samples of gingival tissue of SHRs and WTK rats, with or without periodontal disease, were collected 14 d after surgery. The samples were placed in buffer 1 (0.1 M NaCl + 0.015 M Na_2EDTA in 0.02 M $NaPO_4$, pH 4.7), at 2 mL/1 g of tissue sample, then homogenized three times using a Polytron® (Omni International, Marietta, GA, USA) at 15,700 *g*, centrifuged for 15 min at 15,700 *g* and the supernatant discarded. The same volume of buffer 1, as used initially, was added to the pellet and the solution was agitated. Then, 800 μL of 0.2% NaCl was added for 30 s, followed by 800 μL of 1.6% NaCl + 5% glucose. The samples were centrifuged for 15 min at 15,700 g, and the supernatant was again discarded. Buffer 2 (0.5% H-TAB in 200 mL of 0.05 M $NaPO_4$, pH 5.4) was added, at the same volume as initially used for buffer 1, to the pellet the samples were homogenized using a vortex, subjected to two freeze–thaw cycles in liquid nitrogen, then centrifuged for 15 min at 9300 *g*. The supernatant was collected for myeloperoxidase determination. In a 96-well plate, 5 μL of sample was incubated with 45 μL of 0.08 M $NaPO_4$. In the next step, 25 μL of 3, 3′,5, 5′-Tetramethylbenzidine (Sigma Chemical, St Louis, MO, USA) solution was added to each well for 5 min. Then, 100 μL of 30% H_2O_2 was added to each well, and after 5 min, 50 μL of H_2SO_4 was added to each well to stop the reaction. The readout was taken in an ELISA plate-reader spectrophotometer (Spectramax; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm. The results were expressed as weight (g) of gingival tissue divided by amount of neutrophils recruted to the inflammatory focus and the results were correlated with a standard curve created previously with 30,000 neutrophils.

Determination of cytokine TNF-α and chemokines CXCL2/CINC-2α, CXCL5/LIX and CCL20/MIP-3α by ELISA

Gingival tissues were collected, weighed and kept in frozen liquid nitrogen in order to measure the amounts produced of TNF-α, CXCL2/CINC-2α, CXCL5/LIX and CCL20/MIP-3α. One day before the experiment, the samples were homogenized in phosphate-buffered saline plus protease-inhibitor tablets using a Polytron®. After centrifugation, the supernatant was collected and kept at −80°C until use. The 96-well plate was coated with monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) to TNF-α, CXCL2/CINC-2α, CXCL5/LIX and CCL20/MIP-3α. Samples and recombinant chemokines were added to the wells and, after 2 h, unbound proteins were washed away and an enzyme-linked polyclonal antibody was added to the wells. This antibody acted as a link between the cytokines or chemokines and a dye agent. A color change proportional to the amount of TNF-α, CXCL2/CINC-2α, CXCL5/LIX and CCL20/MIP-3α was observed. This was quantified by comparing the absorbance of the samples, measured using a plate reader at 450 nm, with those of known dilutions. The concentrations of TNF-α, CXCL2/CINC-2α, CXCL5/LIX and CCL20/MIP-3α were calculated (in pg/mL) by comparison with a standard curve.

Evaluation of CRP

CRP levels were measured in peripheral blood plasma using a specific, commercially available ELISA kit (DSL, Genese, Brazil) in accordance with the manufacturer's instructions. The readout was taken in a spectrophotometer at a wavelength of 450 nm.

Determining expression of *iNOS* mRNA in gingival tissue from SHRs and WTK rats with experimentally induced periodontal disease

Total RNA was extracted from the gingival tissue. Then, *iNOS* expression was detected by RT-PCR.

RNA extraction

Total RNA was extracted from gingival tissue using the Trizol® reagent (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the protocol recommended by the manufacturer.

RT-PCR

RT-PCR was performed as described previously, with some modifications (19). Total RNA was extracted from gingival tissue using Trizol® reagent. First-strand cDNA was synthesized from 2 μg of total RNA. The resulting cDNA was amplified using the *Taq* polymerase (Invitrogen, Life Technologies). Sequences of the primers for amplification were as follows: β-actin, sense, 5′-GAA GCT GTG CTA TGT TGC CCT AGA-3′, and antisense, 5′-GTA CTC CTG CTT GCT GAT CCA CAT-3′; 445 bp (20); and iNOS, sense, 5′-ATC CCG AAA CGC TAC ACT T-3′, and antisense, 5′-TCT GGC GAA GAA CAA TCC-3′; 314 bp (21).

Detection of mRNA for β-actin was used as the internal control. The PCR products were electrophoresed and visualized under ultraviolet light on a 1.8% agarose gel (Invitrogen, Life Technologies) containing 10 mg/mL of ethidium bromide (Sigma Chemical, St Louis, MO, USA). The agarose gel was scanned and analyzed by a computer program to obtain a numeric value that permitted a semiquantitative comparison between target iNOS and the constitutive control β-actin.

Statistical analysis

Statistical analysis was performed using the independent-samples two-tailed Student's *t*-test to compare the means between the groups as well as the correlation within the group. A general linear analysis of variance test was used when testing the correlation coefficients between the hypertensive and normotensive rats with periodontal disease and the control rats. The statistical program GRAPHPAD PRISM 5.0 was used. Values of $p < 0.001$ were considered significant.

Results

Experimentally induced periodontitis in normotensive and hypertensive rats

Figure 1A shows a significant reduction in the proportion of mineralized tissue in rats with experimentally induced periodontitis. The evaluation demonstrated that rats with periodontitis had alveolar bone resorption, but that SHRs showed increased alveolar bone resorption compared with the WTK animals. In addition, SHRs showed marked bone loss in comparison with normal rats.

Histometric data

As shown in Fig. 1, in all groups without periodontal disease (Fig. 1C, D, I and J), the periodontal ligament, alveolar bone crest and furcation region were intact. The groups with experimentally induced periodontitis (Fig. 1F, G, L and M) demonstrated areas of bone resorption, indicating that ligatures are able to induce bone loss. The intergroup comparison of unligated groups did not show a statistically significant difference in the periodontal ligament area. The mean periodontal ligament or bone-loss areas of WTK rats without periodontal disease, of WTK rats with periodontal disease, of SHRs without periodontal disease and of SHRs with periodontal disease were 0.081 ± 0.012 mm^2, 0.842 ± 0.138 mm^2, 0.124 ± 0.024 mm^2 and 1.513 ± 0.519 mm^2, respectively.

Neutrophil recruitment into gingival tissue of SHRs and WTK rats

Figure 1B shows significant recruitment of neutrophils into the interradicular bone crest and furcation roof in SHRs and WKT rats with periodontitis. The SHRs without periodontal disease showed a slight, but nonsignificant, increase of neutrophil migration when compared with WKT rats. The numbers of lymphocytes, macrophages and eosinophils in the sites of inflammation were nonsignificant (data not shown).

Myeloperoxidase level in gingival tissue of SHRs and WTK rats

Figure 2 illustrates neutrophil migration into gingival tissue after periodontal disease induction in SHRs and WTK rats. SHRs with experimentally induced periodontitis showed a higher level of myeloperoxidase at the inflammation site than WTK rats, which was correlated with the number of neutrophils recruited into the inflammatory tissue.

TNF-α, CXCL2/CINC-2α, CXCL5/LIX and CCL20/MIP-3α production in gingival tissue of SHRs and WTK rats

Figure 3A illustrates the amount of TNF-α produced in gingival tissue after induction of periodontal disease in normotensive and hypertensive rats. SHRs and WTK rats with experimentally induced periodontal disease showed an increased level of TNF-α in the gingival inflammation tissue. In rats without periodontitis, the gingival tissue of SHRs showed a slight, but nonsignificant, tendency for an increased production of TNF-α when compared with WKT rats. Figure 3B–D illustrates the production of CXCL2, CXCL5 and CCL20 in normotensive and hypertensive rats. In SHRs, even without periodontal disease, the levels of CXCL2, CXCL5 and CCL20 were increased, and after periodontal disease induction, a further increase was observed of CXCL2 and CXCL5 chemokines. The CCL20 level was not statistically signifcant but it had a slight tendency to be lower in SHRs with periodontal disease than in SHRs without periodontal disease. In the WTK rats, the levels of CXCL2 and CXCL5 chemokines were increased with periodontal disease induction, but the level of CCL20 was not.

CCL20/MIP-3α and CXCL5/LIX in peripheral plasma from SHRs and WTK rats

In the peripheral plasma from SHRs and WTK rats, Fig. 4 illustrates that in SHRs, even without periodontal disease, the levels of CCL20 and CXCL5 chemokines were increased. The CINC-2α level was analyzed in all samples, but no detection was observed. The standard curve of the assay was good, but all samples did not have any amount of CINC-2α in the period observed.

Expression of *iNOS* mRNA in gingival tissue from SHRs and WTK rats

Expression of iNOS mRNA was observed in gingival tissue from WTK rats and SHRs with experimentally induced periodontal disease; however, SHRs appeared to express higher levels of *iNOS* mRNA compared with WKT rats with periodontal disease (Fig. 5).

CRP levels in gingival tissue of SHRs and WTK rats

The level of CRP was elevated in rats with experimentally induced periodontal disease; however, the systemic concentration of protein was increased

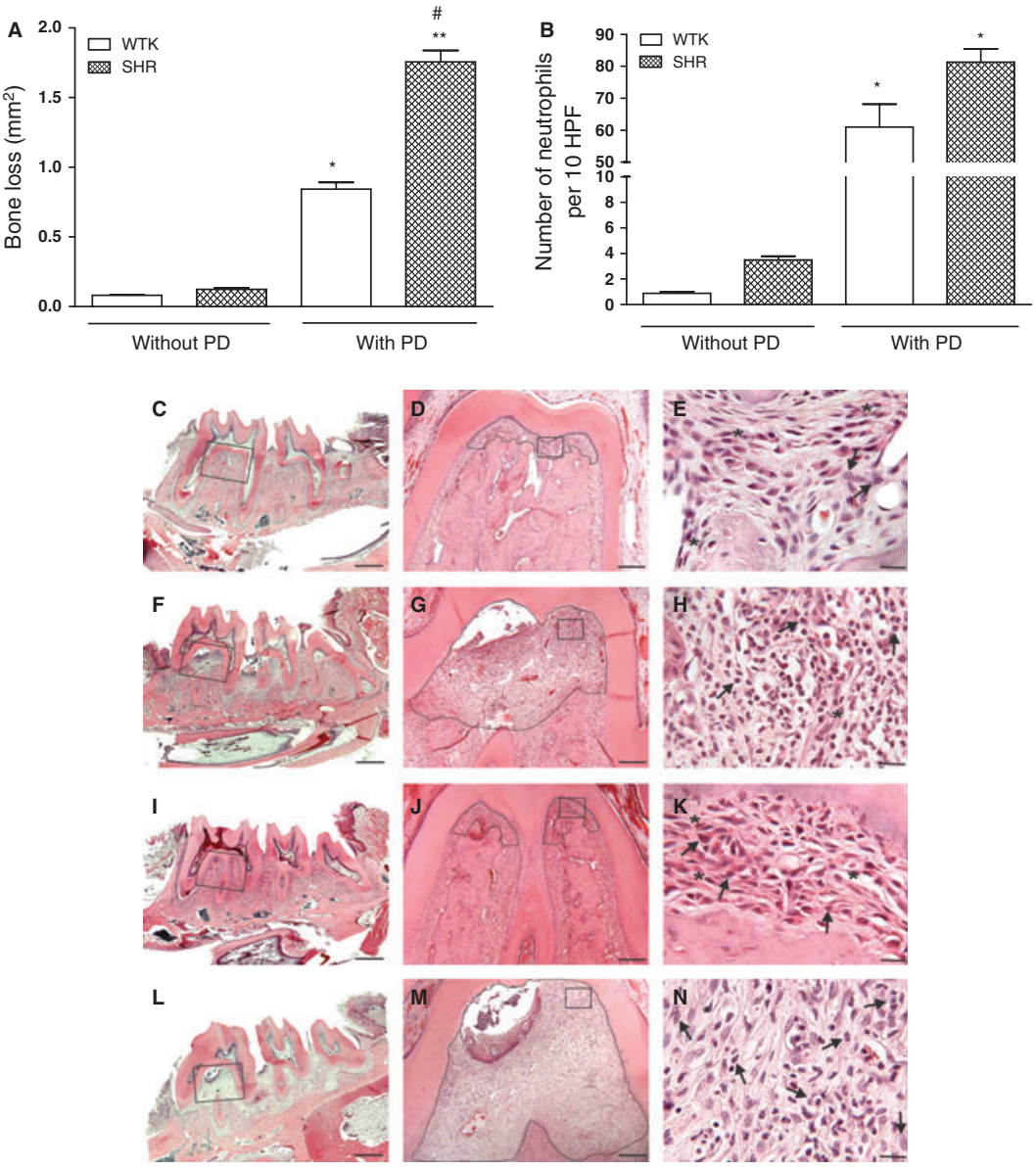

Fig. 1. Bone-loss level and neutrophil migration in spontaneously hypertensive rats (SHRs) and in normotensive (WTK) rats, with and without periodontal disease. (A) Periodontal ligament area in hypertensive and normotensive rats with and without periodontal disease. The area (mm^2) of periodontal ligament for unligated teeth and bone loss for ligated teeth in the furcation region was determined histometrically as the area between the inter-radicular bone crest and the furcation roof. The results are expressed as mean ± standard error of the mean of the area between the inter-radicular bone crest and the furcation roof. *$p < 0.001$ (WTK rats without periodontal disease vs. WTK rats with periodontal disease), **$p < 0.001$ (SHRs without periodontal disease vs. SHRs with periodontal disease) and #$p < 0.05$ (WTK rats with periodontal disease vs. SHRs with periodontal disease) (results obtained by analysis of variance with Bonferroni correction). (B) Neutrophil recruitment into the inter-radicular bone crest and into furcation roof tissue was counted in 10 high-power fields (HPF; × 1000 magnification). The results are expressed as mean ± standard error of the mean of a group of 15. *$p < 0.001$ (WKT rats without periodontal disease vs. WKT rats with periodontal disease) and **$p < 0.001$ (SHRs with periodontal disease vs. SHRs without periodontal disease; and WKT rats with periodontal disease vs. SHRs with periodontal disease) (analysis of variance with Bonferroni correction). (C–N) Photomicrographs with hematoxylin and eosin staining of histological aspects, furcation sections and cellular components in SHRs and WTK rats with or without periodontal disease. Mandibular sections of (C) WTK rats without periodontal disease, (F) WTK rats with periodontal disease, (I) SHRs without periodontal disease and (L) SHRs with periodontal disease (original magnification, 16×; scale bar = 1 mm). (D, G, J, M) Area between the inter-radicular bone crest and the furcation roof. The boxed area represents the measure used to establish the histometric analysis (original magnification, 40×; scale bar = 200 μm). (E, K) Note the high number of fibroblasts and the small number of neutrophils in nonligated groups compared with ligated groups (H, N) (original magnification 1000×, scale bar = 20 μm; asterisk indicates fibroblasts; arrows indicate neutrophils). PD, periodontal disease.

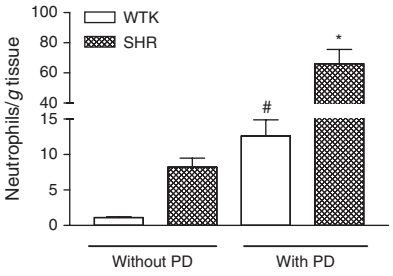

Fig. 2. Myeloperoxidase level in spontaneously hypertensive rats (SHRs) and in normotensive (WTK) rats with and without periodontal disease. Gingival tissue of SHRs and WTK rats was collected, homogenized and the myeloperoxidase level was measured. The results are expressed as mean ± standard error of the mean of a group of six animals in three independent experiments, with similar results obtained each time. *$p < 0.001$ (SHRs with periodontal disease vs. SHRs without periodontal disease) and $p < 0.001$ (WTK rats with periodontal disease vs. WTK rats without periodontal disease) (analysis of variance with Bonferroni correction). PD, periodontal disease.

in SHRs with periodontitis compared with WTK rats (Fig. 6).

Discussion

Hypertension and periodontitis are inflammatory diseases that stimulate specific and nonspecific defense mechanisms, with synthesis of various pro-inflammatory mediators (8, 22, 23 24). Thus, the present study focused on the impact of hypertension and/or periodontitis on alveolar bone and the inflammatory process in the gingival tissue of rats.

Experimental studies have demonstrated that ligation-induced periodontitis results in degeneration of collagen and causes an increase in the numbers of neutrophils and osteoclasts in the alveolar process (25), which results in bone resorption. This occurs in both normotensive and hypertensive animals, but few studies have claimed that the inflammatory response and bone loss, which characterize periodontal disease, are exacerbated in SHRs (7,18). In the present study, it was found that SHRs had higher bone loss in the presence of periodontal disease than did WTK rats. It is worth reporting that in the presence of periodontal disease, a higher rate of bone loss was found in SHRs (7). Cardiovascular disease and osteoporosis are major public health problems that pose increased morbidity and mortality. Although traditionally viewed as separate diseases, with an increased incidence with aging, evidence indicates similar pathophysiological mechanisms underlying these diseases.

Myeloperoxidase is stored in neutrophils and macrophages and is released into the extracellular fluid on establishment of the inflammatory process. It is involved in oxidative stress and has been considered as one of the possible markers of atherosclerotic plaque instability. In the experiments conducted in the present study, the presence of neutrophils was

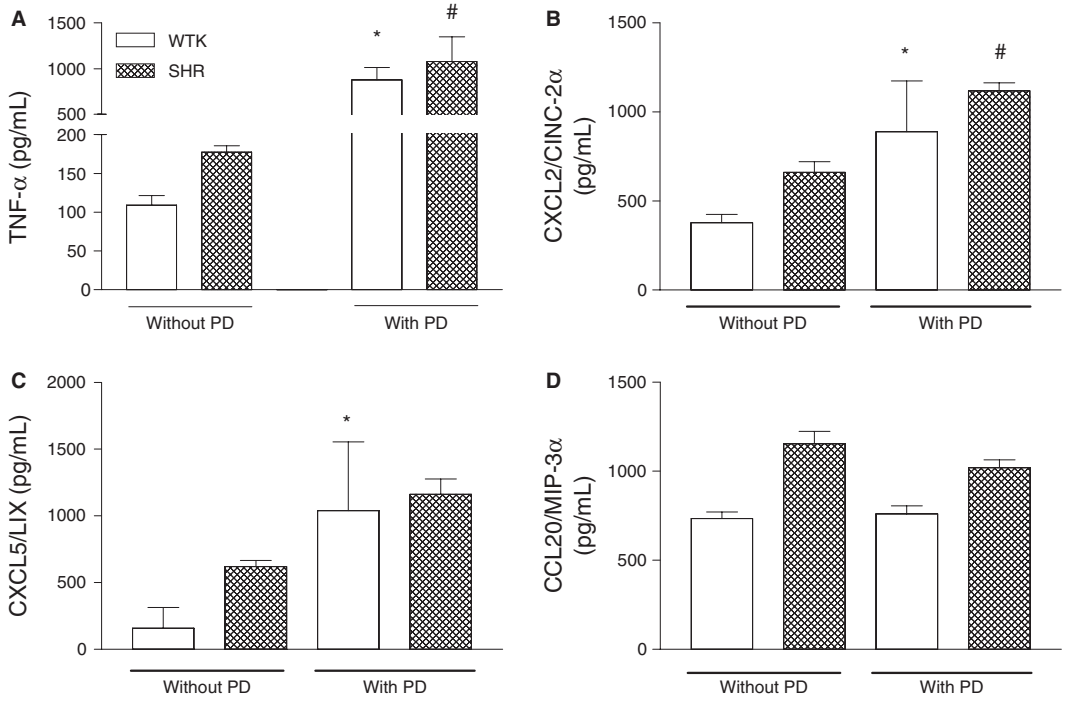

Fig. 3. Levels of tumor necrosis factor-α (TNF-α), CXCL2/CINC-2α, CXCL5/LIX and CCL20/MIP-3α in gingival tissue from spontaneously hypertensive rats (SHRs) and from normotensive (WTK) rats, with and without periodontal disease. Gingival tissue of SHRs and WTK rats was collected, homogenized and the TNF-α (A), CXCL2/CINC-2α (B), CXCL5/LIX (C) and CCL20/MIP-3α (D) levels were measured by ELISA. The results, of a group of six animals in three independent experiments (with similar results obtained each time), are expressed as mean ± standard error of the mean. *$p < 0.001$ (WTK rats with periodontal disease vs. WTK rats without periodontal disease) and $p < 0.05$ (SHRs with periodontal disease vs. SHRs without periodontal disease) (analysis of variance with Bonferroni correction). PD, periodontal disease.

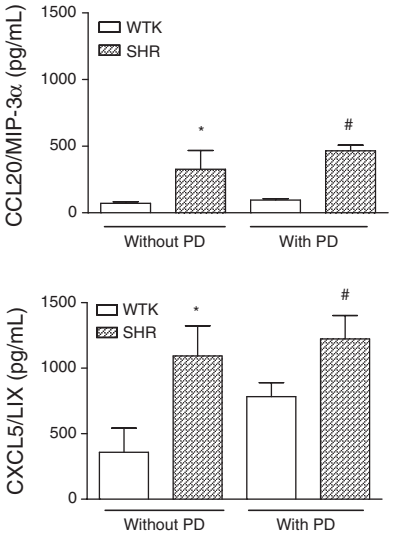

Fig. 4. Levels of CCL20/MIP-3α and CXCL5/LIX in peripheral blood plasma from spontaneously hypertensive rats (SHRs) and from normotensive (WTK) rats, with and without periodontal disease. Peripheral blood plasma was collected from SHRs and from WTK rats and the CCL20/MIP-3α and CXCL5/LIX levels were measured by ELISA. The results, of a group of six animals in three independent experiments (with similar results obtained each time), are expressed as mean ± standard error of the mean. $*p < 0.001$ (SHRs without periodontal disease vs. WTK rats without periodontal disease) and $p < 0.001$ (SHRs with periodontal disease vs. WTK rats with periodontal disease) (analysis of variance with Bonferroni correction). PD, periodontal disease.

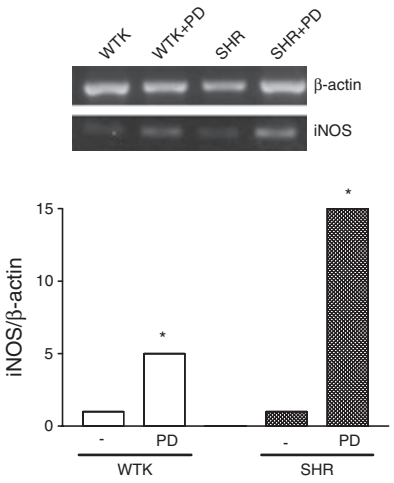

Fig. 5. Expression of inducible nitric oxide synthase (iNOS) mRNA in gingival tissue from spontaneously hypertensive rats (SHRs) and from normotensive (WTK) rats, with and without periodontal disease. Gingival tissue was collected from SHRs and from WTK rats 15 d after experimental induction of periodontal disease, homogenized and analyzed by RT-PCR. Two micrograms of total RNA purified from gingival fibroblasts was used in RT-PCR analysis (1.8% agarose gel), and the products of the PCR were used for iNOS and β-actin detection. The iNOS/β-actin ratio was determined using densitometric analysis. The results represent the mean ± standard error of the mean of three independent experiments, with similar results obtained each time (analysis of variance with Bonferroni's Multiple Comparison Test). $*p < 0.001$ (statistically significant for comparison of WTK rats vs. WTK rats + periodontal disease and for SHRs vs. SHRs + periodontal disease). PD, periodontal disease.

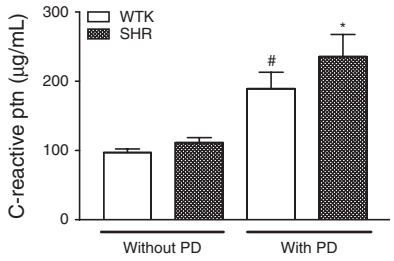

Fig. 6. C-reactive protein level in peripheral blood plasma from spontaneously hypertensive rats (SHRs) and normotensive (WTK) rats, with and without periodontal disease. Peripheral blood plasma of SHRs and WTK rats was collected and the C-reactive protein level was measured by ELISA. The results are expressed as mean ± standard error of the mean of a group of six animals in three independent experiments, with similar results obtained each time. $*p < 0.001$ (SHRs with periodontal disease vs. SHRs without periodontal disease) and $p < 0.05$ (WTK rats with periodontal disease vs. WTK rats without periodontal disease) (analysis of variance with Bonferroni correction). PD, periodontal disease.

observed at the site of injury, by determining the level of myeloperoxidase dosage, which is considered a marker of activated neutrophils (26). Correlating with the myeloperoxidase level, we observed neutrophil recruitment into the gingival tissue in hypertensive and normotensive rats with periodontal disease. However, SHRs showed an increased recruitment of neutrophils to the site of injury compared with WTK rats after experimental induction of periodontitis and even in its absence. These data strengthen the idea that the accumulation of neutrophils may be higher in hypertensive subjects owing to the presence of the primary disease, and that the second source of inflammation – periodontitis – results in the recruitment of an even larger number of inflammatory cells. Neutrophil accumulation in tissues is a characteristic of acute inflammatory conditions, including host defence against bacterial infection (27,28) and also in the initiation and development of atherosclerosis (29,30). In our study, the majority of recruited cells observed in the inflammatory gingival tissue were neutrophils, with few lymphocytes, macrophages or eosinophils detected. Studies have shown that neutrophil infiltration and activation in inflamed tissue is mediated by cytokines, such as TNF-α, and mainly by CXC chemokines (31,32). Activated neutrophils are able to produce these mediators, leading to an amplification of the loop of polymorphonuclear accumulation and activation (33,34). In our model we observed an increased level of TNF-α already in the SHRs, which was enhanced after induction of periodontitis.

TNF-α is a mediator of immune and inflammatory responses (35). TNF-α, together with other molecules, is known to modify heart function through different mechanisms (36). Data from the literature show that patients with myocardial infarction have higher levels of TNF-α (37). As described previously, we observed that normotensive and hypertensive rats with periodontal disease had elevated levels of TNF-α; however, the hypertensive rats without periodontal disease had a slight tendency for increased TNF-α compared with normotensive rats. Possibly, high blood pressure and the presence of atherosclerosis in SHRs are pre-existing factors that increase the concentrations of TNF-α and chemokines in gingival tissue, even without the induction of periodontal disease. Data from the literature have

suggested that systemic diseases co-induce the inflammatory process as both disorders involve functional interference, comprising the amplification of innate immunity and an adaptive response evoked to increase the production of cytokines/chemokines (38–40). This may also be related to the slight tendency for increased CXCL2, CXCL5 and CCL20 concentrations, and even the myeloperoxidase level, in SHRs with periodontitis and could be potentiated in animals with periodontitis, favoring the accumulation of neutrophils in tissues. TNF-α is a proinflammatory cytokine with neutrophilotactic activity, which may act as a trigger to induce chemokine production (19,31).

Chemokines belong to a family of specialized chemotactic cytokines that function as potent mediators of inflammation and have the ability to recruit and activate specific subpopulations of leukocytes (41). The chemokines CCL2/MCP-1, CCL3/MIP-1α, CXCL10/IP-10, CCL5/RANTES and CCL11/eotaxin modulate the functions of fibroblasts, and endothelial cells play an important role in the pathogenesis of myocardial infarction, thrombosis, ischemia reperfusion injury and the healing process after infarction (41,42). In the present study, it was found that gingival tissue from SHRs tended to produce CXCL2/CINC-2α and this was potentiated after periodontitis induction, whereas the WTK rats were stimulated to produce CXCL2/CINC-2α when periodontal disease was experimentally induced. Data from the literature have presented results demonstrating the expression of CXCL2/CINC-2α in gingival tissue of rats with periodontal disease. According to these results, CXCL2/CINC-2α expression may be responsible for recruiting neutrophils to combat the invading pathogen in periodontal disease (43). In the model used in the present study, the recruitment of neutrophils was observed in hypertensive rats, and this migration was enhanced in the presence of periodontal disease. It may be suggested that CXCL2 and TNF-α work in a coordinated manner to induce neutrophil migration to the inflammatory focus.

Studies have shown that CXCL5/LIX, a CXC chemokine, is also a chemoattractant for neutrophils and a potent angiogenic factor (44,45). This chemokine acts at the same receptor as CXCL2/CINC-2α (CXCR2) (46) and is produced concurrently in response to interleukin-1β and TNF-α in lung epithelial cells (A459) (47). The expression and production of CXCL5/LIX was observed in monocytes and dendritic cells stimulated by lipopolysaccharide from *Escherichia coli* (48). However, there are no studies to date demonstrating the production of CXCL5/LIX in periodontal tissue, as shown by the results of the present study. The induction of this chemokine in normotensive rats may be important for collaborating in the recruitment of neutrophils and tissue repair in periodontal tissue. However, data from the literature show that CXCL5/LIX is expressed by resident myocardial cells during ischemia/reperfusion and is induced by oxidative stress or by TNF-α in cultured cardiomyocytes (49). If the results of the present study are carefully analyzed, it will be noted that CXCL5/LIX production was increased in SHRs, both systemically and in the gingival tissue. This suggests that TNF-α may be released by stimulating CXCL5/LIX production by muscle tissue and entering the systemic circulation as well as the inflammatory site of the secondary lesion. In WTK rats, CXCL5/LIX was observed only during the induction of periodontal disease.

CCL20/MIP-3α is a CC chemokine produced in normotensive and hypertensive animals without disease, which means that its concentration did not change in the presence of periodontitis. There was a tendency towards an increase in the concentration of CCL20/MIP-3α in SHRs, but this was not statistically significant when compared with WTK rats. It is known that CXCL2/CINC-2α and CCL20/MIP-3α are important in the recruitment of neutrophils and T lymphocytes, by interacting with endothelial cells at the lesion site (50,51). The authors suggest that, as a result of resident cells stimulation with TNF-α, the presence of CXCL2/CINC-2α and/or CCL20/MIP-3α and/or CXCL5/LIX released by them may facilitate the accumulation of neutrophils at the site of injury. Moreover, it is known that in hypertension, accumulation of neutrophils occurs, both systemically and at the lesion site, and that these neutrophils are capable of producing mainly chemokines and TNF-α (52). TNF-α is able to mediate the influx of neutrophils induced by CXCL1/KC and CXCL5/LIX (31). These findings strengthen the possibility that TNF-α induces CINC-2α and LIX, which mediate neutrophil recruitment to the inflammation site, as observed in the model in the present study. Furthermore, it was observed that the systemic production of CCL20/MIP-3α was increased in SHRs but not in WTK rats, suggesting that in SHRs the high blood pressure itself enhances this chemokine, possibly by facilitating the recruitment of neutrophils.

Data from the literature indicate that the SHR model is not only a model for hypertension research but also a model for abnormal lipid metabolism as the levels of triglycerides and free fatty-acids are enhanced when compared with WKT rats. Furthermore, neutrophils from SHRs are able to produce nitric oxide and iNOS, whereas neutrophils from WKT rats are not. Together, these data suggest that SHRs are a strong candidate for development of atherosclerosis with neutrophil accumulation in the arteries (53,54).

The CRP is an acute-phase protein that is released during inflammation. Its name arises from the ability to precipitate C-polysaccharides from *Streptococcus pneumoniae*, present in the development of periodontal disease and other illnesses such as pneumonia, meningitis and endocarditis (55). There is laboratory evidence that CRP is a sensitive marker of inflammation and/or metabolic processes associated with atherothrombotic and cardiovascular events, suggesting that CRP may contribute to their pathogenesis (56,57). Data in the literature have shown that CRP is present in patients with hypertension and atherosclerosis, suggesting that this protein is released by the stimulus for endothelial injury

(58,59). The results of the present study demonstrate that both SHRs and WTK rats have high levels of CRP following experimental induction of periodontal disease. However, there was no statistically significant difference between the groups with periodontitis, although there was a tendency towards increased protein concentration in the group of SHRs with periodontal disease.

The enzyme responsible for nitric oxide production – nitric oxide synthase – was identified in various cell types, suggesting that it has a physiological and pathophysiological role, mainly through its direct action on inflammed tissue, bone and immunopathology. In hypertensive individuals the level of nitric oxide is increased to provide greater inhibition of norepinephrine and cause vasodilation in order to decrease blood pressure (60). In periodontal disease the production of nitric oxide is increased, suggesting that it is an inflammatory marker of periodontitis (61). According to the results of the present study, the expression of *iNOS* mRNA was increased in gingival tissue from rats with experimentally induced periodontal disease. In addition, this expression was higher in SHRs with periodontitis. Corroborating the results of the present study, other authors have shown that SHR animals stimulated with NaCl are able to express iNOS with higher intensity than are Wistar rats, and that the expression was increased upon stimulation with atrial natriuretic peptide (62). Taken together, the hypertensive state of the genetic SHR seems to favor the inflammatory process, measured using markers such as CXCL2, CXCL5, CCL20, TNF-α, iNOS and CRP, with greater intensity than in normotensive rats. Nevertheless, the periodontitis potentiated the inflammatory process in SHRs.

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