# Periodontal disease induced by *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in Wistar rats

# Periodontite induzida pela *Porphyromonas gingivalis* e *Fusobacterium nucleatum* em ratos Wistar

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## ABSTRACT

Prior studies have shown that it is necessary to place ligatures around molars to study periodontal destruction in rats. The present research aims to examine a periodontal disease model in which specific pathogen-free Wistar rats are orally exposed to *Porphyromonas gingivalis* associated with *Fusobacterium nucleatum*. Periodontitis was induced by specific infection with *P. gingivalis* and *F. nucleatum*. Twenty adult male Wistar rats were divided into two groups. The control animals were not infected. The experimental animals were repeatedly infected with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* for one week. For the next three weeks, the animals were repeatedly infected with *P. gingivalis* alone. The distance from the cemento-enamel junction (CEJ) to the alveolar bone crest of the second molar was measured at different sites: buccal-distal (d), buccal-furcation region (f), buccal-mesial (h), and area region. The Mann-Whitney test was applied ($p<0.001$). The results showed that all values obtained were significantly greater in the infected group. Infected group values for the measures d, f, h, and area were 0.41 mm, 0.46 mm, 0.67 mm, and 1.04 mm2, respectively, while in the control group, values for the measures d, f, h, and area were 0.19 mm, 0.26 mm, 0.26 mm, 0.88 $mm^2$, respectively. Our study showed that four weeks following infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* bone loss in Wistar rats could be identified.
**Uniterms**: Rats. Periodontitis. Bone resorption. *Porphyromonas gingivalis*. *Fusobacterium nucleatum*.

## INTRODUCTION

Experimental animal models have been proposed for the study of periodontal disease, such as the muridae, which has been widely used for this purpose. This family of mammals presents some key advantages, including a relatively low cost and a greater ease in handling than monkeys and dogs[1]. In addition, the histological, microbiological, and clinical aspects of periodontal disease in rats are similar to those observed in humans[2,3].

The induction of periodontal disease in rats is greatly facilitated by placing a ligature or elastic ring around the molars in a subgengival position[4,6]. More recently, to produce a model for any treatment modality, *Porphyromonas gingivalis* has been implanted in the ligatures to enhance the induction of periodontitis[3,7]. Evaluation of animal models developed with bacteria other than *Porphyromonas gingivalis* indicates that certain bacteria can induce periodontal disease, while others seem unable to do so[8]. The trigger for the beginning of this disease is the presence of complex microbial biofilms[9]. Different microbial complexes have been associated with the sequence of colonization on the tooth surface as well as with disease severity[10]. Interactions between *Porphyromonas gingivalis* and *Fusobacterium nucleatum* result in specific coaggregation, which enhances the ability of the organism to effectively colonize the subgengival sulcus[11]. Fusobacteria are notable for their specific interactions with several species of oral bacteria and have been identified as a "coaggregation bridge organisms"[12].

Therefore, the purpose of the present study was to evaluate, by morphometric analysis, the effects of oral infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* on the rate of bone loss in the alveolar bone crest in Wistar rats.

## MATERIALS AND METHODS

### Animals

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This research was approved by the Research Ethics Committee from Positivo University. Twenty adult male Wistar rats, with a mean weight of 385 g, were divided into two equal groups. They were kept in temperature-controlled rooms and received water and food *ad libitum*. Standard conditions of light (12 h light and dark cycles) were maintained during the experiment. The animals were monitored daily and were randomly assigned as control uninfected animals (n=10) and experimental infected animals (n=10). The experimental animals were repeatedly infected with *Porphyromonas gingivalis* (*P. gingivalis*) and *Fusobacterium nucleatum* for one week. For the following three weeks, the animals were repeatedly infected with *P. gingivalis* alone.

## The oral infection model

To induce periodontal disease, all rats from the experimental group were orally infected with *P. gingivalis* ATCC 33277*. *P. gingivalis* was grown at 37°C in an anaerobic chamber for 48 hours in a 5% modified sheep blood agar. Cells were removed from the plates by scraping them onto PBS with a bent glass rod, and the bacteria were suspended in 2% carboxymethyl cellulose (CMC). Ten rats from the experimental group received 0.5 ml suspensions (1.0x1012 cells/ml) by oral gavage once a day, 3 days a week, for 4 weeks13, while the control animals received only 2% CMC by gavage.

To assure installation of periodontitis, *P. gingivalis* infection was combined with 0.5 ml (1.0x1012 cells/ml) of *Fusobacterium nucleatum* ATCC 10953 infection*. No damage to the soft tissues, such as ligatures, was caused before the microorganisms were introduced. Eight weeks after the first infection, all of the rats from group 1 and 2 were sacrificed by $CO_2$ inhalation.

## Morphological analysis of periodontal bone level

The mandibles of the sacrificed rats were washed, skinned, and soaked in 1% hypochlorite to remove the remaining soft tissue debris. To measure alveolar bone loss, the dissected lower jaws were stained with 1% Methylene blue to delineate the CEJ more clearly. Each hemimandible was photographed under magnification (x20) with light and position standardized in a stereoscopic magnifying glass (Olympus America Inc. Model SZX9, Center Valley, Pennsylvania, USA) with conventional slide films. The obtained slides were digitalized with a scanner[14]. Using image analysis software (ImageLab®-Softium informatica, São Paulo), the bone loss of the lower second molar was determined by two methods of measurements: distance and area[15]. One independent and one blind examiner, previously calibrated (intra-class correlation coefficient: 0.91), performed the measurements of each sample.

1) *Distance method* – the distance from CEJ to the alveolar bone crest (ABC) of the second molar was measured by a blinded examiner at 7 sites: palatal-distal (c), buccal-distal (d), mesio-buccal of the distal root (e), buccal-furcation region (f), mesio-buccal- of the mesial root (g), buccal-mesial (h), and palatal-mesial (i) in each animal.

2) *Area method* – the area of the stained buccal root surface of the second lower molar was also measured. The alveolar bone surface area of the second molar was obtained from the outlines of the linear measurements of ABC, CEJ, the distal-palatine surface of the second molar, and the mesial-palatine surface of the second molar (Figure 1).

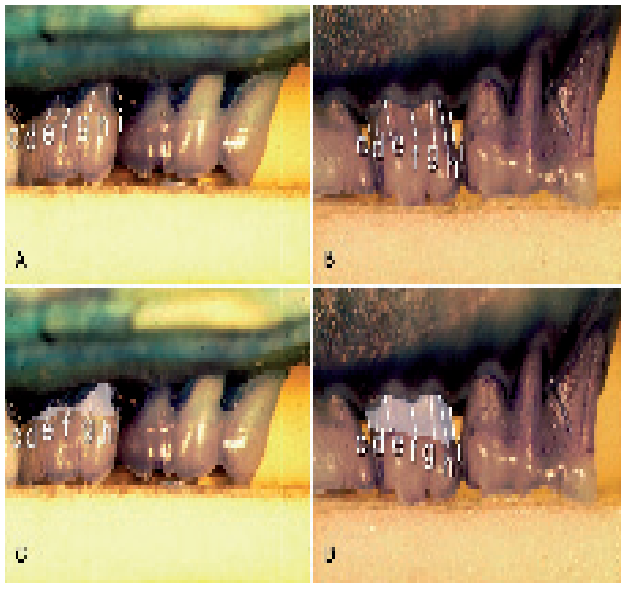

**Figure 1** - The photographs on the left (A,C) identify the control group, while the photographs on the right (B,D) identify the experimental group. Photographs C and D represent the distance from CEJ to the alveolar bone crest of the second molar at seven sites (c, d, e, f, g, h, i). Illustration (C,D) of the landmark used to define the area of alveolar bone. Buccal aspect.

## Statistical Analysis

The nonparametric Mann-Whitney test was applied between the uninfected control group and the experimental infected group. To characterize the studied sample, the data are presented in Table 1: means, 95% confidence interval (95% CI), and median values to summarize the data and the standard error; standard deviation and minimum and

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maximum values to show the variability of the data obtained for measurements d, f, h, and area. Statistical analysis was only performed on the buccal-distal (d), buccal-furcation region (f), and buccal-mesial (h) measurements and the area.

## RESULTS

Evident bone resorption was observed 4 weeks after inoculation with *P. gingivalis* and *F. nucleatum* (Table 1).

**Table 1 -** Descriptive statistics of the measurements from the CEJ to the alveolar bone crest at d, f, h, and the Area in both groups, together with p values

| STATISTICS | d | | f | | h | | area | |
|---|---|---|---|---|---|---|---|---|
| | control | infected | control | infected | control | infected | control | infected |
| **Mean** | 0.196 | 0.461 | 0.233 | 0.429 | 0.257 | 0.679 | 0.862 | 1.062 |
| **Median** | 0.195 | 0.410 | 0.260 | 0.46 | 0.265 | 0.675 | 0.889 | 1.041 |
| **Standard error of Mean** | 0.016 | 0.047 | 0.019 | 0.024 | 0.024 | 0.034 | 0.023 | 0.033 |
| **Standard deviation** | 0.049 | 0.147 | 0.061 | 0.075 | 0.075 | 0.108 | 0.073 | 0.105 |
| **Minimum** | 0.120 | 0.310 | 0.130 | 0.310 | 0.130 | 0.510 | 0.752 | 0.943 |
| **Maximum** | 0.270 | 0.780 | 0.290 | 0.520 | 0.370 | 0.860 | 0.945 | 1.240 |
| **p value\|*** | <0.0001 | | <0.0001 | | <0.0001 | | <0.0001 | |

*Mann-Whitney test

Analysis of the results showed that obtained mean values were significantly greater in the infected group. For example, the d measurement presented a median value of 0.41 in the infected group, while in the control group this value was 0.195. This reveals a greater bone loss in this region. Similar results occurred for the f and h measurements and the area. Thus, the rats that received *P. gingivalis* and *F. nucleatum* inoculation presented greater bone resorption than did those from the control group.

## DISCUSSION

The present findings suggest that oral infection with *Porphyromonas gingivalis* ATCC 33277 is associated with *Fusobacterium nucleatum* ATCC 10953 induced periodontal disease in male Wistar rats. Placement of a ligature around the molars or associated with *P. gingivalis* has been widely used to induce periodontitis in animal models[3,16]; however, a subgengival ligature may induce apical migration of the junction epithelium by trauma without the induction of marginal periodontitis.

Placement of a cotton ligature around the molars to facilitate plaque accumulation and the development of periodontitis has been well studied[2-5]. This procedure requires general anesthesia for test animals and the replacement of the ligature each time that it is lost. This results in added stress and life risk to the animals. Plaque accumulation over a long period of time, achieved by placing an elastic ring around the molars in a submarginal position, could also promote root surface caries.

Previously established by Crawford *et al*.[8], specific types of bacteria vary in their ability to cause periodontal disease in a rat models in such a way that subtle differences in bone loss patterns could be observed, depending on the particular organisms used for infection. Also, in the aborementioned study, animals were infected by oral inoculation with colonies of *Actinomycetemcomitans naeslundii, viscosus*, and *Streptococcus mutans*, whereas, in the present study, only periodontal pathogens were used to inoculate Wistar rats. Moreover, Crawford *et al*.[8] did not observe bone loss at the histological level.

*Shoji et al*.[4] promoted bone loss by placing an elastic ring around the cervix of the right mandibular first molar. Importantly, in the present study, promotion of bone loss in the inter-radicular area of the second mandibular molar and the interdental alveolar bone in Wistar rats was achieved by four weeks of oral infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* with no need for further clinical intervention. Therefore, the experimental rat model described in this communication, by morphometric analysis, rapidly reproduces periodontal disease following an inoculation with *P. gingivalis* and *F. nucleatum* and may well represent an alternative to developing periodontal disease in a rat model.

## CONCLUSION

The periodontal disease model generated by *P. gingivalis* and *F. nucleatum* inoculation yields reproducible and predictable changes that are similar to those seen in adult human periodontitis.

## RESUMO

Tem sido demonstrado na literatura, em modelo experimental com animais, que seria necessária a colocação de amarrias ao redor dos molares para se obter com êxito a destruição das estruturas periodontais. Este estudo teve o objetivo de induzir doença periodontal pela inoculação oral da *Porphyromonas gingivalis* associada à *Fusobacterium nucleatum* em ratos da linhagem Wistar. Vinte ratos machos foram divididos em dois grupos. O grupo controle não foi infectado pelas bactérias. Os animais do grupo experimental foram infectados oralmente por *Porphyromonas gingivalis* associada à *Fusobacterium nucleatum* durante uma semana. Após esse período, foi inoculado apenas P gingivalis por mais 3 semanas. Depois da periodontite estabelecida, a perda óssea foi determinada pela análise morfométrica. Um examinador independente, cegado e previamente calibrado realizou as medidas. A distância da junção esmalte cemento até a crista óssea alveolar (JEC-COA) do segundo molar inferior foi medida em diferentes faces: disto-vestibular (d), região da furca vestibular (f), vestíbulo-mesial (h) e área. Foi aplicado o teste estatístico de Mann-Whitney ($p<0.001$). Os resultados mostraram que os valores das medidas foram significativamente maiores no grupo que foi a inoculado pelas bactérias. Os valores para as medidas d, f, h e área do grupo infectado foram 0,41mm, 0,46mm, 0,67mm, 1,04 $mm^2$, respectivamente, enquanto que os valores das medidas d, f, h e área do grupo controle foram 0,19mm, 0,26mm, 0,26mm, 0,88 $mm^2$, respectivamente. O presente estudo demonstrou que 4 semanas de infecção pelas bactérias *Porphyromonas gingivalis* e *Fusobacterium nucleatum* promoveu perda óssea em ratos da linhagem Wistar.

**Descritores**: Ratos. Periodontite. Reabsorção óssea. *Porphyromonas gingivalis*. *Fusobacterium nucleatum*.

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