

Periodontal disease induced in Wistar rats - experimental study

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Abstract: Objectives: The aim of the present research was to develop a reproducible experimental model for the induction of periodontal disease in Wistar rats, using ligatures in the lower frontal group. Material and Methods: Ten male adult Wistar rats obtained from Laboratory animal facility - Centre for Experimental Medicine, "Iuliu Hațieganu" University of Medicine and Pharmacy, with average weight 180-200 g, were included in this study. Ligatures in "8" with 4/0 nonresorbable sterile silk thread were placed in the inferior frontal group under general anesthesia. After 14 days the animals were euthanized and samples representing the cephalic extremity were stored in formalin and prepared for histological processing. Results: Periodontal disease induction by ligature placement caused a significant inflammation of periodontal tissue and alveolar bone loss, observed at 14th day. Histopathological analysis showed a progressive mononuclear cell infiltration and an increase in the osteoclast numbers were evident. Conclusions: In our study we demonstrated by clinical and histopathological analysis that this modified "ligature" model of periodontitis in rats has several advantages: short-term of induction of disease-14 days, pronounced clinical inflammation of periodontal tissues and advanced resorption of the alveolar bone, simplifying the intervention of inducing periodontal pathology.

Key Words: periodontitis, animal model, ligature, inflammation, bone loss.

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Introduction

Animal models have an important role in the generation of new knowledge in medical sciences, including periodontology. These experimental models have distinct advantages because they can reproduce in vivo cellular characteristics and reactions that occur in humans. Animal models in periodontal disease are particularly important in the development of the scientific basis for understanding the pathological processes (Graves et al 2012). Periodontitis is a highly prevalent, chronic immune-inflammatory disease of the periodontium that results in progressive loss of gingival tissue, the periodontal ligament and adjacent supporting alveolar bone with significant impact on human health (Pihlstrom et al 2005).

Periodontitis was associated with systemic diseases such as diabetes, autoimmune diseases and cardiovascular complications (Desvarieux et al 2005; Cullinan & Seymour 2013; Gulati et al 2013; Gurav 2014).

To study the phenomenon of periodontal inflammation and the effect of periodontal treatment, several animal models have been adopted (Do et al 2013; Eggert et al 1980; Schou et al 1993). Periodontal disease can be divided into different phases and each one may be studied separately depending upon the animal

model. These phases involve the development of a bacterial biofilm-colonization, invasion across the epithelium into connective tissue by bacteria and their products, induction of a destructive host response in connective tissue and bone resorption, limitation of a repair process that follows tissue damage. By selecting the appropriate animal model each of these phases can be analyzed individually, whereas in human studies it is difficult to isolate a specific step and in vitro studies lack the complexity to examine specific phases (Graves et al 2012).

Rodents and rats in particular, are relevant models for experimental periodontal research (Struillou et al 2013). The structure of the dental gingival area is similar to that observed in humans with a shallow gingival sulcus and attachment of the junctional epithelium to the tooth surface (Yamasaki et al 1979). However, there are some differences like the keratinisation of the crevicular epithelium in rats. Another difference relates to the relationship between the gingival and junctional epithelium with desmosomal contact between the most superficial cells of the gingival epithelium and the non keratinized cells of the junctional epithelium (Listgarten 1975).

The junctional epithelium is a pathway for foreign substances, bacterial endotoxins and for inflammatory cell exudations, similar to what occurs in humans.



Figure 1 - Macroscopic aspect after placing the ligature in the lower frontal group - day 0 (a) and day 14 th (b)

The occurrence of periodontal diseases in rats is less frequent than in human and the pathology can be induced by inoculating bacteria, giving a carbohydrate - rich diet and fixing ligatures around the teeth. The most common experimental model of periodontitis is a “ligature” model.

Studies on rodents have obtained periodontal disease by placing of ligatures in the gingival sulcus around the molar teeth and increasing biofilm accumulation, as well as disrupting the gingival epithelium, exacerbating osteoclastogenesis and bone loss (Oz & Puleo 2011).

Due to the complexity in performing experiments on rats, modification of existing model is proposed, which differs by fixture of cotton ligature around the central incisor and not around the second molar. The aim of the present research was to develop a reproducible experimental model for the induction of periodontal disease in Wistar rats, using ligatures in the lower frontal group .

Material and methods

Ten male adult Wistar rats obtained from Laboratory animal facility - Centre for Experimental Medicine, “Iuliu Hatieganu” University of Medicine and Pharmacy, with average weight of 180-200 g, were included in this study.

The animals were housed for acclimatization, one week before the start of the experiment. Five rats were housed in each wire cage in a temperature and humidity controlled room ($23 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ relative humidity), under 12-hour light/dark cycle, with access to standard rat chow pellets and water available ad libitum.

General anesthesia was achieved through intramuscular injection with a solution of Ketamine 10% and Xylazine 2% (2:1), 0.12 ml /100 g body weight.

Anaesthesia was installed in 4-5 min after administration. Body weight was determined for each subject. The animals were placed on a proper operating table, wich allowed open-mouth maintenance of the rats to facilitate acces to the teeth. The surgical procedure was performed by two oral surgeons with an experience of ten years.

Ligatures in “8” with 4/0 nonresorbable sterile silk thread were placed in the inferior frontal group. This ligature acted as a

gingival irritant for 14 days and promoted the accumulation of plaque and subsequently development of periodontal disease. After placing ligatures the animals were kept in the same conditions 5 subjects in each cage. Subjects were observed for 14 days. Daily we performed ligatures control and we checked the animals in terms of proper nutrition and body weight.

On completion of the experiment, after 14 days the animals were anesthetized with eter and euthanized by cervical dislocation. Samples representing the cephalic extremity were stored in formalin and prepared for histological processing.

Experimental protocol was performed in accordance with present laws regarding animal welfare and ethics in animal experiments (Directive 86/609 EEC/1986; Romanian Law 205/2004; Romanian Law 206/2004; Romanian Law 471/2002; Romanian Law 9/2008; Romanian Order 143/400).

Analytical method

Histological evaluation

After euthanasia the heads were cleaned of skin, muscle and connective tissue and fixed for 72h in 10 % neutral buffered formalin. After complete fixation the samples were decalcified in an 1/1 mixture of 8% formic acid and 8% clorhidric acid for 3 weeks. When decalcification was completed the oral tissue was trimmed longitudinally and dehydrated through successive baths of Isopropyl alcohol (70%, 90%, 95%, and 100%), clarified in xylene, and embedded in paraffin wax (Prophet et al 1992).

Multiple tissue sections were cut from each paraffin block at 4 μm thickness with a rotary microtome (Leica RM2135). Afterwards, tissue sections were stained with hematoxylin and eosin (H&E) and examined for descriptive histology under an Olympus BX41 microscope. The bright field microscopic images were taken with an Olympus UC30 camera and processed using Olympus Stream Basic image analysis software. The specimens were examined by two experts in histology.

Results

The surgical procedure that we presented is a reproducible one. As shown in Figure 1a, the ligatures placed induced 14 days later macroscopically detectable inflammatory modifications as in Figure 1b.

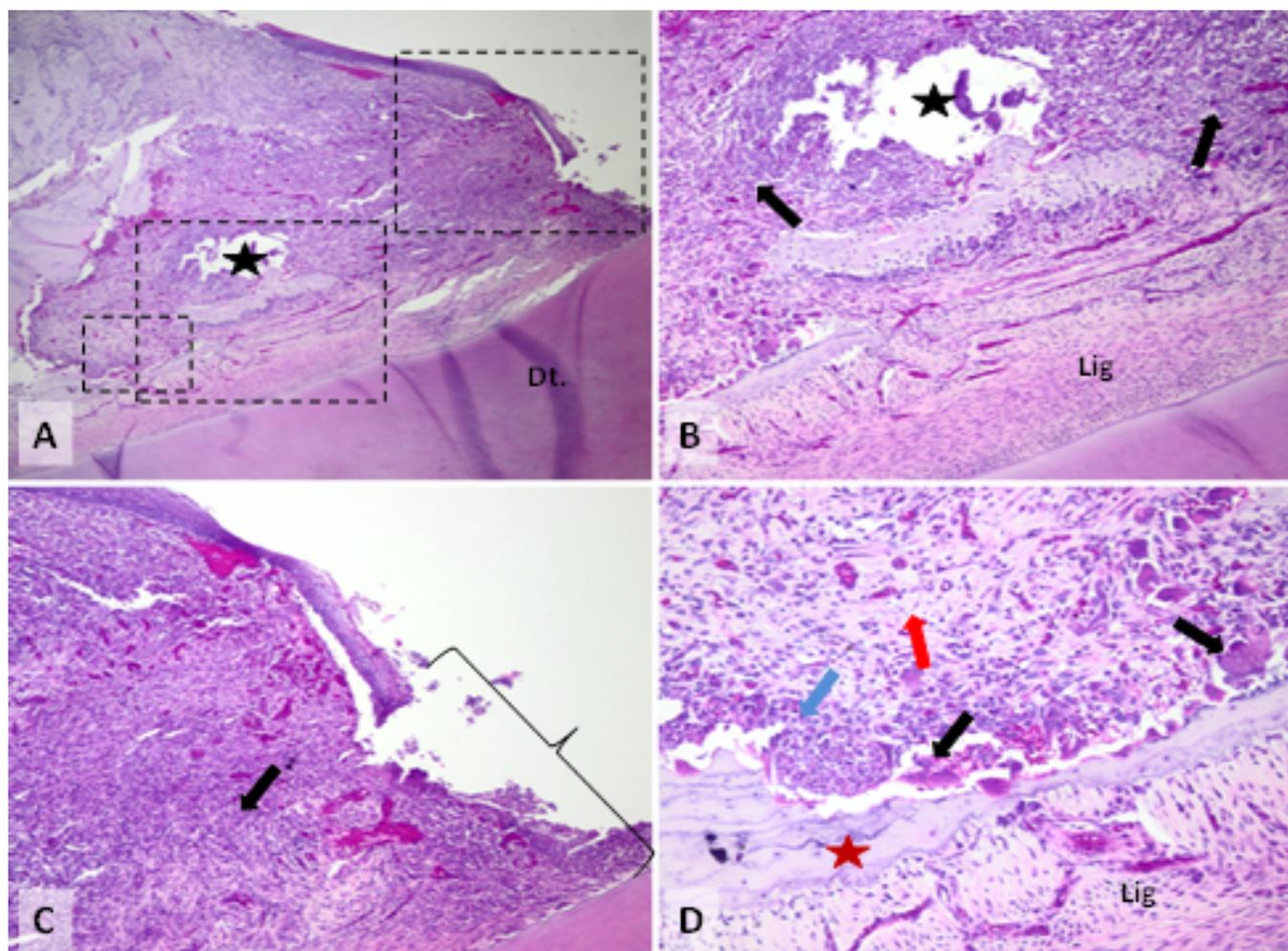


Figure 2 - Histopathological aspects of periodontal tissue of rats, which has been experimentally induced periodontal disease-14 days after the placement of ligature

First changes occurred three days after applying the ligature when gingival tissue began to lose its normal aspect and structure. The gingival colour changed from pink to intense red. Plaque accumulation was detected around the ligated silk thread including the dentogingival junction. The changes observed were accentuated since third day until fourteenth day, when subjects were sacrificed. Therefore, inflammatory modifications initially identified were associated with an increased tooth mobility and the gingival tissue bleeding on probing .

Descriptive histology

The histological evaluation was carried out using descriptive histology.

The main histological aspects of periodontal tissue of rats sacrificed at 14 days revealed an intense inflammatory response to irritation caused by ligature thread (marked by star) that includes gingival tissue, periodontal ligament and alveolar bone (Figure 2A). This acute inflammatory reaction is marked by a mixed inflammatory infiltrate - neutrophil and mononuclear and by a massive fibroplasia (Figure 2B). The histopathological aspect of the superficial gingival tissue revealed thinning and ulceration appeared consecutively of the ligature. The neutrophilic exudates prevails at the periphery of the gingival tissue defect and a dense granulation tissue is present in the subjacent region (Figure 2C). As seen in Figure 2D a pronounced alveolar osteolysis was detected with the presence of the border of

osteoclasts involved in bone matrix resorption (black arrow). The polymorphonuclear exudates (blue arrow), numerous cellular debris secondary of inflammatory process and the granulation tissue containing few inflammatory cells (red arrow) can all be observed.

The space between the rat incisor and the socket is veiled with the presence of a dense inflammatory exudate at this level (Figure 3A). The necrotic material present here is composed of cellular debris, bacteria and neutrophilic exudates (red arrow). Also can be noted the presence of the subjacent granulation tissue (blue arrow) (Figure 3B). Periodontal ligament, partially degenerate can be seen in the bottom of Figure 3C. The phenomenon of the alveolar bone resorption also displays at this point with an important border of osteoclasts (black arrow - Figure 3D).

Discussions

Periodontitis is one of the most prevalent diseases in humans, so many studies have used experimental animals to investigate its pathogenesis. Ligature placement in the teeth has been proposed to obtain an experimental periodontitis condition more quickly than periodontitis naturally occurs (Do et al 2013).

In this study, we have shown that placement of a silk thread around the cervical region of the lower incisors induced gingival inflammation and the first symptoms of periodontitis from the third day of experiment. Significant alveolar bone loss was

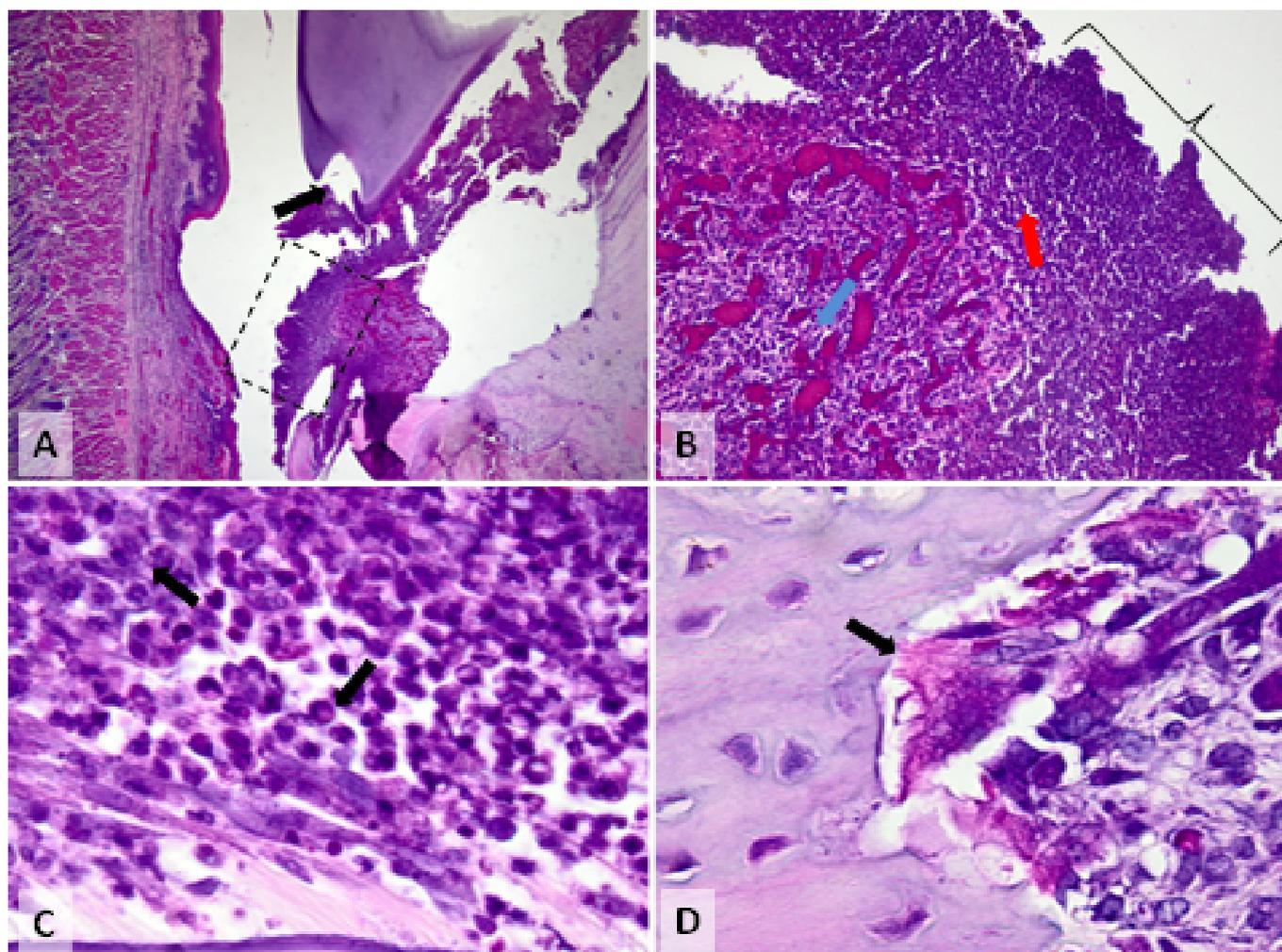


Figure 3 - Histopathological aspects of periodontal tissue and the apical region of rats incisor - 14 days after the placement of ligature

proven by histopathological analysis after 14 days, the data obtained being in accordance with results of previous studies in the literature (Liu *et al* 2000; Chumakova *et al* 2014; Terrizzi *et al* 2013). Some studies demonstrated that loss of attachment and bone occurs in a 7 day period (Bezerra *et al* 2002; Bezerra *et al* 2000). Other investigators have conducted experiments over much longer periods of time (Kuhr *et al* 2004; Nociti *et al* 2001). Alveolar bone loss in the ligature model at rats is dependent upon bacteria similar to human periodontitis. Therefore in gnotobiotic rats placement of ligatures does not induce significant gingival inflammation or periodontal bone loss but we cannot exclude the possibility for mechanical trauma by the ligatures, which could thus contribute to bone loss (Graves *et al* 2008). The experimental protocol proposed provided the expected results, the silk thread acting as a bacterial plaque retentive factor that contributes to periodontitis. It has been proposed in previous studies that bacterial stimulation induces a host response that leads to inflammatory cell infiltration, osteoclast formation, bone loss and the loss of tooth attachment (Wahl *et al* 1993). The histopathological analysis performed in the present study demonstrated the presence of neutrophils and mononuclear cell infiltration, an increased number of osteoclasts, in turn leading to cementum and alveolar bone resorption. These inflammatory alterations could be explained by a local release of inflammatory mediators. In subsequent research we

aim to assess the systemic implications of these inflammatory mediators, researching the correlation between periodontal disease, periodontal treatment and general health status.

The continuous growth of rodent teeth can put difficulties in understanding and interpreting this animal model, but the problem seems to have been overcome in the past decade making available new directions for research in periodontology. Therefore, the rodent incisor is a unique model for the study of dental pathological processes, because it continues to grow and differentiate throughout life and the odontogenic tissues remain functional during the lifetime. The rodent incisor is also a sensitive recorder of altered mineral metabolism (Kuijpers *et al* 1996). Our study has some limitations, in that the sample size is small and a model of experimental periodontitis with silk ligature placed around the lower incisors was not yet adopted, requiring further research. Within the limits of this study, we were able to achieve periodontal inflammation and bone resorption at 14 days after ligature placement. This could be a possible candidate for a reproducible experimental periodontitis model. The advantage of this experimental model is the simplification of surgical intervention with reducing the operative trauma of the subjects. Also, it is easier to follow the integrity of silk thread and daily changes occurring in dental and gingival tissues without additional trauma to animals and without requiring general anesthesia.

Conclusions

In our study we demonstrated by clinical and histopathological analysis that this modified “ligature” model of periodontitis in rats has several advantages: short-term of induction of disease-14 days, pronounced clinical inflammation of periodontal tissues and advanced resorption of the alveolar bone, simplifying the intervention of inducing periodontal pathology.

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