ORIGINAL ARTICLE

The optimization of ligature/bone defect-induced periodontitis model in rats

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Received: 6 September 2021 / Accepted: 10 March 2022 / Published online: 3 June 2022
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Abstract
The destruction of alveolar bone is a crucial manifestation of severe chronic periodontitis, which stem cell-based bioengineered therapies are expected to cure. Therefore, a cost-effective, reproducible, quantifiability and easier to administrate animal model that mimics human periodontitis is of great importance for further endeavor. In this study, we created periodontitis rat models in silk ligation group, bone defect group and bone defect/silk ligation group, respectively. Obvious periodontal inflammation but slight alveolar bone resorption was observed in the ligation group, while surgical trauma was not robust enough to continually worsen the constructed bone defect area in the bone defect group. In the bone defect/ligature group, significant and stable periodontal inflammation was the most enduring with similar evolving pathological patterns of human periodontitis. It also exhibited enhanced clinical similarity and confirmed its superiority in quantitativeness. The present rat model is the first study to reproduce a pathological process similar to human periodontitis with reliable stability and repeatability, manifesting a priority to previous methods. Day 9–12 is the best time for reproducing severe periodontitis syndromes with vertical bone resorption in this model.

Keywords Periodontitis · Animal model · Bone defect · Ligature · Rat

Introduction

Periodontitis is one of the most prevalent microbial dysbiosis-initiated oral diseases, triggered by the formation of the subgingival-colonized polymicroorganisms biofilm and chronically developed by its mutual interactions with dysregulated host immune responses [1–3]. According to current pathogenesis studies, periodontitis-associated microbiome synergistically aggregate in a matrix of extracellular substances, secreting multifarious virulence factors that can stimulate susceptible auto-immunopathological mechanisms and provoke proinflammatory cascades [4–6]. The reciprocally reinforced interplay between the dysbiotic oral microbiota and the disturbed homeostasis eventually result in the destruction of the periodontal tissue including gingiva, alveolar bone, cementum, periodontal ligament and even serious tooth loss [7, 8]. Periodontitis initiation and progression are related to multiple risk factors [9] and worldwide epidemiological data show that a fraction of around 10% of those over 40–50 years in all populations exhibiting severe periodontitis, which later become the prevalent cause of tooth loss in 90% of adults today [10]. It is also associated with systemic diseases such as heart disease, diabetes, Alzheimer's disease and pregnancy complications [11]. Evidence-based strategies toward periodontitis rely heavily on the removal of plaque and calculus by mechanical scaling and root planning [5]. But for severe cases that cannot be fully controlled non-surgically, open-flap debridement clears up contaminated alveolar bone and reduces periodontal pocket [12]. Mechanical and surgical procedures eradicate active inflammatory components to slow down progression, but do not always regenerate the ravaged periodontal tissues [8, 13].
Thus, the discovery and development of stem cells and bio-engineering is making headway and points out the direction for the clinical treatment of periodontitis and periodontal tissue regeneration [14]. While the more promising bio-engineering treatment is still in the research stage, it is necessary to evaluate the effect of periodontal tissue regeneration through in vivo animal experiments to test the efficacy of prevention and treatment of the periodontal disease.

Animal experiments are the indispensable pathways to evaluate any new treatments. At present, animal models used for the study of periodontal tissue regeneration are often created by bone defect modeling method, which establishes
The acute bone defect by surgically removing part of the alveolar bone, periodontal ligament and cementum [15]. Surgical creation of bone defects allows for rapid, stable, and quantifiable access to periodontal bone tissue for study [16]. However, due to the lack of inflammatory microenvironment caused by accumulation of microbial plaque, the model obtained by acute bone defect is not satisfactory in etiology, development and prognosis of periodontitis [17,18]. The acute bone defects caused by surgery lack inflammatory induction process. Therefore, it is not widely used in periodontitis-related research [19]. Instead, this method is more suitable for mechanical traumatic etiology [20,21].

To overcome the shortcomings of bone defect modeling, some studies have combined the bone defect method with periodontitis silk ligation and successfully established periodontitis models of large animals (such as in miniature pigs and beagle dogs) [22,23]. Intraosseous defect is created on the alveolar bone and directly exposed to the oral environment [24], then the ligated silk threads are placed around the cervical region of the teeth to ensure long-term plaque deposition and accelerate the natural process of inflammation [25]. This method not only ensures a more standardized morphology of the surgically created defect but also allows for reliable reproducibility of the study according to any given scheme, combining the rapid creation of periodontal bone tissue defects with the inflammatory microenvironment maintained by silk thread ligation method. It is a more appropriate model for the occurrence, development and prognosis of periodontal diseases in an inflammatory environment.

At present, there has been no report on the establishment of regenerative periodontitis model in rats by bone defect combined with silk ligation. In this regard, we proposed a novel surgical procedure to create bone defects in the mandibular molar region of rats by removing alveolar bone and tying the teeth with silk ligature. We observed and recorded the clinical manifestations of periodontitis. Micro-computed tomographic, histological and histomorphometric were also performed to analyze the inflammation and bone remodeling degrees. Our protocol have overcome the surgical operational obstacles of narrow oral region, small teeth of rats [26] and administrated reproducible and standardized bone defects in a rapider and greater manner. Afterwards, silk ligature was sutured around the cervical portion for the accumulation of plaque and calculus, providing an ideal habitat for the endogenous oral microbiota to cause periodontitis by dysregulating host immune system in rats. These optimizations are expected to better mimic the pathological process of periodontitis in both acute and chronic inflammation. We believe the present solution can facilitate the use of periodontitis models in periodontal regeneration research and shed light for the future study in the clinical effect of periodontal tissue regeneration therapy.

**Materials and methods**

**Surgical induction of experimental animal model**

Forty female SPF Wistar Rats, aging 8–12 weeks and weighing 220–240 g [27], were obtained from the Animal Science Center of Southern Medical University (Guangzhou, China). Animals were acclimated for 1 week before periodontitis induction and they were housed under conventional condition with free access to water and food after periodontitis induction. This study was reviewed and approved by the animal care and use committees of Southern Medical University. Rats were randomly divided into sham surgery group (only gingival incision and suture), ligature group (ligation of bilateral mandibular first molars), bone defect group (operation of buccal alveolar bone defect of bilateral mandibular molars) and bone defect/ligature group (operation of bilateral mandibular molars buccal alveolar bone defect and ligation of bilateral mandibular first molars). All rats were fasted for 24 h. Weights were recorded and the anesthetic dosage were calculated according to body weight with 1% pentobarbital sodium (5 ml/kg) injected intraperitoneally. The rats were fixed on the designed bed for rat dental surgery (previously Computer-aided designed and 3D printed in polylactic acid Fig. 1A-1), and the tongue was pulled by the surgical silk thread to fully expose the visual field of the operation (Fig. 1A2–3). The mucoperiosteal flap was raised and the alveolar bone was removed using surgical bur to create experimental periodontal defect between the mandibular first molar and second molar (Fig. 1B1–4). The created
alveolar bone defects were 1 mm wide, 1 mm long and 1 mm deep (Fig. 1A4–6). Subsequently, silk suture of the ligament was ligated around the cervical portion of the first molar and gingiva was intermittently sutured (Fig. 1B5–6). Ligation and periodontal condition were checked every day. Rats were respectively sacrificed on Days 3, 6, 9, 12, 15 and 18 after modeling (Fig. 1C). The body weight and vital signs such as heart rates and blood pressure were recorded before each rat was sacrificed.

**Observation of clinical manifestations**

The degree of periodontitis, the probing depth, edema, probing bleeding and looseness of the gingiva of the mandibular first molars of rats were measured with a Williams periodontal probe (002-0531, Shanghai Kangqiao Dental Device Company, China) at each time point. Any inflammatory changes such as loss of gingival papilla, dislocation of gingival position or contour, redness, swelling and ulcer would be recorded. The periodontal pocket depths (PPD) in the buccal, mesial, distal and lingual sites of the mandibular first molars were respectively recorded with average values calculated. The Gingival Bleeding Index (GBI) and the range of tooth mobility (TM) of mandibular first molars are scored as previously described.

**Micro-computed tomography (micro-CT) analysis**

To observe alveolar bone loss at different time points and evaluate the effect of inflammation on bone tissue, the mandibles were fixed with paraformaldehyde. Micro-CT (Scano-Medical, Viva CTμ80, Switzerland) was used for computerized tomography and three-dimensional reconstruction. To assess alveolar bone loss, distances from the Cemento-Enamel Junction (CEJ) to the alveolar bone crest (ABC) were measured at four sites of first molars (mesio-buccal, disto-buccal, mesio-palatal, and disto-palatal) in three-dimensional images viewed from buccal and palatal sides, with the assistance of the image analysis system RadiAnt Dicom Viewer (Medixant, Poland). Using the function of multi-plane reconstruction, the buccal-lingual cross section was set to the long axis of the distal root of the mandibular first molar, then the periodontal ligament widths were measured at apical 1/3, mid-root 1/3, and cervical 1/3. Images from different specimens were evaluated in a random sequence. The measurements were repeated two times per site.

**Histomorphometric analysis**

To observe periodontal tissue inflammation and bone remodeling in rats, mandible was fixed with 10% paraformaldehyde, decalcified and embedded in paraffin. The tissue blocks were made into 5 μm thick tissue sections through buccal and lingual direction, which were stained by hematoxylin–eosin (HE) and observed under a light microscope. To evaluate the degree of inflammatory cell aggregation and the integrity of alveolar bone and cementum, HE staining was visualized with confocal microscope (LSM 700, Carl Zeiss, Oberkochen, Germany). To observe the attachment loss, the Leica image analysis system was used to measure the distance from the cementum-enamel junction (CEJ) to the root of the junctional epithelium (50X) [19]. The surgical area between the first and second molars was analyzed with 0–3 double-blind scoring system under light microscope [20]. The sections of different specimens were evaluated according to random sequence and the measurements were repeated twice.

**Statistical analysis**

Statistical analysis was performed using Statistical Package for the Social Sciences ver. 13 software (SPSS, Chicago, IL) and Graphpad Prism software (Graphpad, US). Data were representative of three or more independent experiments and all results were expressed as mean values ± standard deviation (SD), All data are subjected to Kolmogorov–Smirnov normality distribution testing and passed. Ranking data for GI and TM were evaluated Wilcoxon Rank sum test. Results were considered significant for \( P < 0.05 \). Quantitative data were evaluated by one sample \( t \)-test, one-way ANOVA and two-way ANOVA analysis, the \( P < 0.05 \) was considered statistically significant.

**Results**

**Clinical manifestations**

Weigh change in rats after periodontitis induction surgery is an important indicator for experimental safety and growth evaluation [29]. Generally, all rats presented increase in their weights during the analysis period of 18 days (Fig. 2A). Differences of weight gain between four groups were not found to be statistically significant (\( P > 0.05 \)). Rats of the bone defect plus ligation group lost about 2% of body weight on postoperative Day 3, which may be due to the acute trauma and loss of blood, but they regained their initial body weight at Day 6, confirming the ability to eat normally after surgery treatment. Statistical analysis indicated that no abnormalities were found both in blood pressure (Fig. 2B) and heart rate (data not shown) of all rats.

General status of the periodontal tissue was assessed by commonly used clinical indexes including Gingival Bleeding Index (GBI) (Fig. 2C) and Tooth Mobility (TM) (Fig. 2D). In terms of clinical indicators of periodontitis,
the mean GBI from 0 to 18 days postoperatively in the bone defect plus silk ligation group showed significant differences from the remaining three groups (Fig. 2C). Gingival bleeding by probing was more frequent in either the bone defect group or silk ligation group at the first from Day 3 to 6. While gingival bleeding was more severe in the bone defect plus silk ligation group than in the rest of the groups from Day 3 onwards, it was most severe and persistent at Day 12-15 (Supplemental Material 2).

For tooth mobility (TM), the bone defect plus silk ligation group had a significantly higher mean value of tooth loosening from 0 to 18 days postoperatively compared to either the bone defect or the silk ligation group (one sample t-test, $P < 0.0001$ for the bone defect group, $P < 0.0005$ for the wire group, and $P < 0.0001$ for the sham surgery group). Tooth mobility was highest in the bone defect group on Day 6, and reached its peak on Day 9-12 in both the silk ligation group and the bone defect/silk ligation group (Supplemental Material 2).
Morphometry of micro-CT images

Micro-CT is a very sensitive technique for displaying hard tissue conditions and providing three-dimensional images. Correlation between reconstructions by three-dimensional micro-CT images and histomorphological metrics of periodontitis models has been demonstrated [19]. Therefore, to evaluate periodontal condition, we used micro-CT to measure periodontal ligament width and alveolar bone loss.

Our results manifested a smooth alveolar bone cortex in the sham surgery group during 6–18 days and only mild horizontal resorption of alveolar bone was observed (Supplemental material 2). There was no significant widening of the periodontal ligament in the sham group during the 18 days (Fig. 3A4–6, mean = 0.307, SD = 0.024).

As for the ligation group, during the first 9 days, the alveolar bones cortex was partially dissolved. From day nine onwards, a loss of continuity of the alveolar cortex on the alveolar bone surface and sparse cancellous bone trabecular structures were observed. Mild horizontal resorption with progressive and irregular bone loss pattern was observed on Day 12–18 (Fig. 3A8–9). Periodontal ligament width changed mildly, peaking at 9 days and later with a slight decrease are observed in the silk ligation group on Day 18 (Fig. 3A10–12).

In the bone defect group, the surgical alveolar bone defect between the area of the first and second molar bone was clearly detected (Fig. 3A13). The depth of the bone defect area slightly increased and restorative tissue was formed at the edge of the area from 12 days onwards (Fig. 3A14). The bone defect was partially restored after 18 days (Fig. 3A15). Slight increase of periodontal ligament in the bone defect group was witnessed from 6 days postoperatively (Fig. 3A16) and a gradual decrease from 12 to 18 days (Fig. 3A17–18).

In the bone defect/ligation group, rough surface of the alveolar bone, typical horizontal and vertical resorption were obvious 6 days after surgery (Fig. 3A19). Meanwhile, the surgical defect area remains a relatively identifiable contour. A rapid increase of CEJ-ABC distance in the bone defect/ligation group occurred from Day 3 to 6 (Fig. 3A19), peaking at 9–12 days (Fig. 3A20) and followed by a gradual decrease from Day 15 to 18 (Fig. 3A21). In bone defect/ligation group, PDL width increased rapidly in 6 days onwards (Fig. 3A22), reaching at highest in the Day 9 and decreased gradually afterwards (Fig. 3A23–24). Still, it remains significantly higher than that in the rest groups during the whole period of 6–18 days (Fig. 3C). Overall, the amount of alveolar bone resorption established by bone defect combined with silk ligation was significantly higher than the remaining three groups (Fig. 3B, P < 0.0001), with a most active inflammation of periodontitis maintaining the longest time period (Fig. 3D).

Histological analysis

Histopathologic assessment is the golden criterion for periodontal healing and regeneration in animal models of periodontitis. The evaluation of the central portion of the surgical site of the bone defect yields representative histometric data [23]. The depth of the periodontal pockets was measured on the HE sections (Fig. 4A and 3E). The level of attachment loss was determined by measuring the distance from the CEJ to the apical extent of the attached epithelium (Fig. 4A). In the present study, we also set up a double-blind scoring system based on the degree of the infiltration of inflammatory cells in succular epithelium, gingival connective tissues, alveolar bone loss, periodontal ligaments continuity, Sharpey’s fibers completeness in the position of the distal root of the mandibular first molar (Fig. 3D).

The primary clinical change observed in the rats in the bone defect/ligation group from Day 3 onwards was the appearance of reddish hyperemia and shiny acute edema. Pathologically, the proliferated capillaries and capillary loops can be witnessed near the succular epithelium with emerged PMNs and destructed collagen. As expected, the periodontal pocket depth in the bone defect group maintained at a low level during the first 6 days after surgery (Fig. 3E). No significant difference of gingival soft tissue inflammation was found compared to the sham surgery group (Fig. 3D), indicating that the bone defect surgery alone does not cause persistent irritation of the periodontal ligament. There was a distinct mechanical defect area in the buccal alveolar ridge in bone defect group with blood clot and osteoblasts observed on the Day 6 due to the initiation of bone healing process.

In the silk ligation group, histological ravage of normal color and contour of the gingival tissue was observed from Day 9 onwards (Fig. S1). Loose gingival tissue and disconnected apical periodontal ligament could be witnessed due to the continuously plaque accumulation but the inflammation infiltration was not severe and the resorption in cortical bone was uneven. The ligation group did not produce deep periodontal pockets until 9 days after surgery (Fig. 3E). In bone defect group, initial osteogenesis of the surgical area was observed with fibrous new bone formation. Meanwhile, in the bone defect/ligation group during Day 9–12 a large number of inflammatory cells infiltrated and ulcerated in the periodontal pockets as the lesion progressed (Fig. 4B13). Polymorphonuclear leukocytes appeared under the crevicular epithelium and penetrated the junctional epithelium into the gingival sulcus. Periodontal pockets deepened when the epithelial attachments moved along the root surface apically leading to loss of attachment. Macrophages appeared under the affected epithelium and the junctional epithelium was detached from the tooth surface, at which point the inflammation reached its peak (Fig. 4B14).
Fig. 3 Micro-CT images and histomorphometry of the periapical first molar in rats. A CT images of rat periodontalitis models. Left (3D reconstructed images): Particularly significant horizontal and vertical bone loss, rough alveolar bone surfaces were observed in the bone defect/ligature group of 6–12 days (A19–20). Smooth surfaces of alveolar bones were witnessed in the sham surgery group (A-1–3). The surgical defect areas were clear in the bone defect group (A13). Unevenly loss and rough surface of alveolar bones had been observed in the ligature group (A8–9). Right (Two-dimensional micro-CT sections of periodontal ligament width): No significant widening of the periodontal ligament during the 18 days in sham surgery group and bone defect group (A4–6). In ligation group, the periodontium was slightly widened in the early period of 6 days (A10), and gradually returned to the original level afterwards (A11–12). B Bone defect depth measurements of four groups. C Measurements of the periodontal ligament width. D Histomorphometric measurements of PDD of first molar in HE staining sections. E Depth of PDD of first molar by Micro-CT panel sections.
On the 15–18 days after the surgery, infiltration of inflammatory cells was no longer found in the bone defect group. The repaired alveolar bone was still cortically spongy but the defected triangle-shaped alveolar crest was already flattened.

Due to the gradual immune adaption of bacteria, the ligature group manifested a tendency of rapid decreasing of acute inflammation after Day 15. The subsided inflammation at the gingival margin and relieved shallowing of the PD were
Infiltration of inflammatory cells in the api-
chronic periodontal ligament, showing similarity
plaque accumulation yet. This stage can be regarded as the
the clinical manifestations and pathological progress [35].
the clinical manifestations of the established stage in
Significant horizontal and vertical bone resorption was
by alveolar bone removal surgery in association with ligature
periodontitis method, the model we induced manifest a better clinical similarity,
significantly higher intensity and a more standardized bone
area of experimental periodontitis during the same period [34].
In present study, we obtained various methods to evalu-
ate the model outcomes of periodontitis at different time
points, including the micro-CT radiological analysis and
HE stained histopathological sections, all reporting obvious
time-pattern changes and specificity. The present rat models
we established, induced by acute alveolar bone defect and
chronic silk ligature, is the first to successfully mimic the
pathological changes in periodontal tissue and stages divi-
sions in human periodontitis (Table 1). At Day 3, experimen-
tal region between the mandibular first and second molars
of rats showed clear-cut triangular bone defect area (Fig. S1).
Mild hyperemia of blood vessels was observed in the
gingival tissues with low clinical indexes of GBI and TM
(Fig. 2C, D), indicating that ligation had not led to obvious
plaque accumulation yet. This stage can be regarded as the
initial lesions of human periodontitis, showing similarity
in clinical manifestations and pathological progress [35].
In 6–9 days, the activity of periodontitis increased due to
plaque accumulation, initiating inflammatory cell infiltr-
ation. As more of the gingiva becomes affected, bleeding may
be spontaneous as the clinical manifestations of marginal
gingivitis [36] (Fig. S1). Rough surface of alveolar bone
was captured both by micro-CT and histopathologic sec-
tions, corresponding to the early lesions of human periodon-
titis (Fig. 3A 19). With the progress of the experiment, the
destruction of periodontal tissue exacerbated (Figs. 4A and
S1) in the Day 9 to 12 (Fig. 4B 13). The connection between
the junctional epithelium and the dentin surface was greatly
loose with polymorphonuclear leukocytes (PMNs) infiltr-
ation. The connective tissue showed significant RBC extrava-
sation and collagen fibers disappearance (Fig. 4B 14). In our
study, bone loss peaked at 9 days postoperatively (Fig. 3B).
Significant horizontal and vertical bone resorption was
formed causing the height of alveolar crest decreased, and
the loss of buccal bone reached more than 1/2 (Fig. 3A20
and 23). By far, the expression of periodontal tissue is close
to the clinical manifestations of the established stage in
human periodontitis. From 15 to 18 days, the acute gingival
inflammation was slightly alleviated (Fig. S1). This may be
a result of the conversion of the innate responses of the rat
immune system to adaptive responses to produce protection
of periodontal tissue, but at this point, the reduced alveolar
bone height is not significantly recovered and chronic peri-
odontal destruction will persist (Fig. 3A21).

The breeding and housing costs of rodent animals are
relatively low, making it possible to carry out studies with
sufficient mass for statistical analysis [37]. Rat modeling

**Discussion**

Periodontitis is a chronic inflammatory response that results
from the interaction between the host immune system and
oral pathogens [20]. It is generally believed that periodon-
titis animal models should represent the obvious processes
of plaque attachment, gingival inflammation, attachment
loss and alveolar bone loss observed in human disease [30].
There are different administrations of inducing naturally
occurring periodontitis using plaque accumulation methods,
such as feeding inducive-diet [31, 32] or placing ligature
[25]. By far, no single satisfying model similar to the patho-
logic process of human periodontitis has been proposed [32].
Invasive surgical alveolar bone defect was proved to increase
the maximum tense of inflammatory, without altering the
mechanism of periodontitis development or the peak time-
point of acute periodontitis compared to ligation method
[33]. For these reasons, we choose to induce animal model

witnessed (Fig. S1). Acute inflammation in the bone defect/ligature group also decreased slowly after Day 15 but still
remained significantly higher from the other groups till Day
18 (Fig. 3D). The PDL width gradually decreased in the
later stage in bone defect/ligation group (Fig. 3C), while the
height of the defect alveolar bone was less regenerated than
that in the bone defect group (Fig. 3B).
### Table 1 Differences of stages of gingivitis and periodontitis between humans and rats

| Stage                  | Humans       | Clinical Findings                                                                 | Underlying microscopical features                  | Rats          | Clinical Findings                              | Underlying microscopical features                  |
|------------------------|--------------|----------------------------------------------------------------------------------|----------------------------------------------------|--------------|------------------------------------------------|----------------------------------------------------|
| I. Initial lesion      | 2–4          | Gingival fluid flow                                                              | Vascular dilation                                    | 0–3          | NOT clinically evident                          | Gingival vasculature                               |
|                        |              |                                                                                  | Infiltration by PMNs                                 |              | Subclinical gingivitis                          | PMNs exudation                                     |
|                        |              |                                                                                  | Perivascular collagen loss                           |              |                                                | Fibrin deposition                                  |
| II. Early lesion       | 6–8          | Erythema Bleeding on probing                                                      | Vascular proliferation                               | 3–6          | erythema of the gingival margin                 | PMNs into pocket area                              |
|                        |              |                                                                                  | Lymphocytes infiltration                            |              | “marginal gingivitis”                           | Collagen destruction                              |
|                        |              |                                                                                  | Increased collagen loss around infiltrate            |              |                                                | Proliferated capillaries and capillary loops       |
| III. Established lesion| 14–21        | Changes in color, size, texture, and so on                                       | Vascular proliferation and blood stasis             | 6–9          | bluish tinge may become superimposed on the reddened gingiva (anoxemia) | Plasma cells increase                              |
|                        |              |                                                                                  | Plasma cells infiltration                            |              |                                                | Gingival edema                                     |
|                        |              |                                                                                  | Continued loss of collagen                           |              |                                                | Widened intracellular spaces with PMNs             |
|                        |              |                                                                                  |                                                    |              |                                                | Congested vessels                                  |
| IV. Advanced lesion    | > 28         | consistent bleeding (gingival index = 2)                                        | Fibrosis of the gingiva                              | 9–12         | Probing bleeding                                | RBCs extravasate                                   |
|                        |              |                                                                                  | Widespread tissue damage                             |              | Pocket form                                     | Slight alveolar bone loss                          |
|                        |              |                                                                                  | Plasma cells and neutrophils dominating epithelium |              | Attachment loss                                 |                                                    |
| V. Periodontitis       | For years    | Plaque and calculus                                                              | Degeneration and inflammatory exudate               | 15–18 onwards| Oral inflammatory changes (erythema, edema, hemorrhage) intensify | alveolar bone is lost via osteoclastic activity    |
|                        |              |                                                                                  | Gingival swelling, redness                           |              |                                                |                                                    |
|                        |              |                                                                                  | Edema                                              |              |                                                |                                                    |
|                        |              |                                                                                  | Deep periodontal pocket                              |              |                                                |                                                    |
|                        |              |                                                                                  | Bleeding on probing                                  |              |                                                |                                                    |
|                        |              |                                                                                  | Attachment loss                                     |              |                                                |                                                    |
|                        |              |                                                                                  | Bone loss (angular/ vertical or horizontal)         |              |                                                |                                                    |
|                        |              |                                                                                  | Increased tooth mobility                             |              |                                                |                                                    |
was therefore faster, easier and more cost-effective to study the underlying mechanism of systemic inflammation and its effect on periodontal healing [38]. We successfully reduced the obstacles of fixing rat’s body position and exposing the surgical region in the narrow oral cavity by designing a customized dental surgery bed (Fig. 1A1–3). Overall, the application of our process can effectively improve the efficiency of periodontitis modeling in rats (the average successful rate is 82.6%, data not shown).

Meanwhile, previous study found that ligature alone did not induce stable and lasting periodontal bone loss in rats [25, 39], which is also proved as the regression of inflammation and the healing of alveolar bone were observed in the ligature group of our study from Day 12 to Day 18 (Fig. 3A8–9). The decrease of CEJ-ABC was also rather random in individual rat, making it difficult to achieve standardized measurement for comparable data analysis. Therefore, we also optimized the surgical bone removal protocol in rats according to pre-described anatomical landmarks (Fig. 1A4–6). The 3D micro-CT reconstructed images of the mandibular first and second molars showed similar triangular area of bone defect after operation, which proved that the operation location was reliably repeatable (Fig. 3A13 and 19). The method proposed in this study not only produces a standardized morphological defect area, but also ensures reliable data for repetitive comparison research according to any given scheme. For an instance, any amount of regenerative osseous tissue provoked by certain regenerative periodontal treatment (manifesting as a blurring margin and the shallowing of the triangle surgical defect, Fig. 1D) might be easily identified, measured and quantitated.

In summary, having integrated the advantages of both acute bone defect and chronic silk ligature methods, our rat model better represent the evolving process of human periodontitis, showing a great similarity between rats and humans in the divisions of clinical syndromes and pathological changes [35]. It can be fully applied to the study during the period of Day 9–12 when reaches the most active peak. Present protocol proves to establish a suitable experimental model for the regenerative research of periodontitis, as the stability and reproducibility of alveolar bone resorption triumphs over the rest of the methods as demonstrated above. The optimization of this model is anticipated to contribute to the application of periodontitis animal model in the future research, especially in the evaluation of clinical efficacy as well as the underlying mechanism of periodontal regeneration therapy.

Limitations

1. Even though we designed a novel surgical rat beds via 3D-printing and proposed the protocol with clear anatomical marks, the surgical skill of fabricating bone defect requires skilled operators to perform. Rats’ mouth has very narrow view and difficult to put surgical instruments in. There are certain technical barriers and practices are needed and the failure of death culture and loss of ligation are estimated to be about 20%. Another limitation for the model of rodents is the minimal amount of gingival tissue available and therefore a large number of animals is required to achieve statistically meaningful results.

2. As the induction of bone defects is an essential method for evaluating alveolar bone regeneration and the current mainstream animal models of periodontitis mainly apply wire ligation, our optimized model only compared with bone defect and ligation group. Yet, there are many other administrations could be used in establishing periodontal animal models, for example, LPS injections, high-glucose diet or Pg. inoculated periodontitis models. To achieve a general applicability, we will add more control groups for future studies.

3. Murine dental structures are not totally identical to human dental structures. Although murine molars have cementoenamel junctions that enable attachment loss measurements, the structure of the mice periodontal apparatus and host susceptibility is not exactly the same as that of humans. The ligature aids in facilitating bacterial adherence and colonization, while some human oral microorganisms may not be able to colonize the murine oral cavity. Furthermore, their resistance to bacterial challenge differs from that of humans and these should always be considered before planning the study and during interpretation of the results.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10266-022-00715-7.

**Acknowledgements** This work was supported by the National Natural Science Foundation of China [81803710], Foundation of President of Nanfang Hospital [grant no. 2018B014] and GuangDong Basic and Applied Basic Research Foundation [grant No. 2021A1515011503]. We thank Dr. Lina Yu from the Department of Pathology, Nanfang Hospital, Southern Medical University for her guidance on the production of histological analysis. We thank Ms. Xujia Lai, for her technical support for the statistical analysis in this paper.

**Author contributions** All authors have made substantial contributions to conception and design, establishment of animal model, analysis or interpretation of data in this study. JG carried out histomorphometric analysis, observation of clinical index and drafted the manuscript. SC carried out micro-CT analysis and participated in observation of clinical index, all data analysis and manuscript drafting. MO participated in the study design and the data analysis, helped to perform the tissue sample preparation and revise the manuscript. DL perform the tissue sample preparation, participated in the data analysis and advised on the study design. ZW advised on the data analysis and helped to revise the manuscript. XZ advised on the data analysis and clinical index.
Conflict of interests
The authors declare no competing interests.

Declarations

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