

A Comparison of Two Models of Experimental Periodontitis in Rats

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Summary

Rats are being used in models of experimental periodontitis because the periodontal anatomy in the molar region bears much resemblance to that of man. Furthermore, rats are available with different genomes and microbial status.

The main purpose of the study was to compare two different methods of inducing experimental periodontitis in rats, by ligature or LPS injection.

Rats were bred and housed on wire-mesh floor with no bedding material and fed a special diet to avoid periodontal destruction caused by impaction of bedding and dietary fibers interdentially. Because no bedding was used it was suggested that in future studies PVC tubes (diameter: 7 cm, length: 12-15 cm) be placed in the cages to improve the environment for the rats. The possible effect of doing so was therefore also examined in this study.

Periodontitis was established either with peridental silk ligatures for 1 or 4 weeks or with gingival injections of lipopolysaccharide (LPS) every other day for a 6-day-period to provoke inflammation. For each experimental group a corresponding control group was established. In all groups the number of rats was 14. In addition 10 rats receiving no treatment were placed in cages with PVC-tubes. Alveolar bone loss was measured by means of morphometrical and radiographical methodologies.

A previously described method for breeding and housing periodontitis-free rats was reproduced. The access to PVC-tubes did not result in differences in alveolar bone destruction when compared to the 4-week control group and therefore, PVC-tubes may be used as an environmental improvement for the rats in future studies.

Compared to the control groups significantly more alveolar bone loss was established in the ligated rats both after 1 and 4 weeks, with the 4-weeks-ligature group having significantly more alveolar bone destruction than the 1-week-ligature group. No effect of LPS injections could be demonstrated and therefore, the study did not confirm earlier findings of significant effect of LPS injection on alveolar bone destruction as compared to saline injection.

Introduction

Periodontitis is one of the most common infectious diseases in the world (Slavkin, 1999). The disease is

characterized by destruction of the tooth supporting tissues, ultimately leading to tooth loss and reduced mastication. The disease is caused by accumulation of bacteria on the tooth surface in the periodontal pockets. Bacterial products containing lipopolysaccharides (LPS) cause inflammation, which includes the production of inflammatory mediators such as cytokines (Page, 1991; Kjeldsen et al., 1993), some of which are thought to be key factors in the periodontal tissue destruction (Page & Kornman, 1997).

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Many animal models have been used to investigate the pathogenesis and treatment of periodontitis. Although similar pathogenic reactions to pathogenic stimuli may differ in different species, studies in animal models may elucidate biological mechanisms, which cannot be studied in humans.

Rats have often been used in models of experimental periodontitis since periodontal anatomy in the molar region bears much resemblance to that in man. Furthermore, rats are easy to handle and can be obtained with different genomes and microbial status. Finally, experimental periodontitis can be induced in a relatively short period of time (Klausen, 1991; Weinberg & Bral, 1999; Björnsson *et al.*, 2003).

It has been revealed that at least 25% of normally bred Sprague-Dawley rats show pronounced periodontal destruction on delivery, mainly due to impaction of diet fibers and bedding materials (Björnsson *et al.* 2003). This phenomenon causes considerable inter-individual variation, which may obscure the effects of the treatment under investigation. It may also be a potential risk factor for systemic health of the rats (Slots 2003). To avoid this problem a model for breeding periodontitis-free rats has been developed, which includes special diet and bedding conditions and a pre-examination of the rats' periodontal health so that rats with existing periodontal destruction are not included in the study (Björnsson *et al.* 2003).

A number of different methods to induce experimental periodontitis in rats have been described, including ligature placement around teeth (Sallay *et al.* 1982), inoculation with bacteria (Heijl *et al.* 1980) or injection of bacterial products (Llavaneras *et al.* 2001, Ramamurthy *et al.* 2002).

The purpose of the present study was to compare two different methods to induce experimental periodontitis in rats: a ligature model, which uses periodontal ligatures to provoke bacterial accumulation leading to tissue destruction (Sallay *et al.*, 1982), and an LPS model that uses gingival injections of LPS to provoke the destructive inflammatory reaction characteristic of periodontitis (Llavaneras *et*

al., 2001, Ramamurthy *et al.*, 2002). The Danish National Experimental Animal Inspectorate approved the protocol but because no bedding material was used, suggested that PVC tubes with a diameter of around 7 cm and length 12-15 cm could be placed in the cages to improve the environment for the rats. To assist future studies the possible effect of such tubes was therefore also examined.

The two models were compared by morphometrical and radiographic examination of the alveolar bone loss.

Materials and Methods

Study design

100 male MOL:SPRD (337±60g) rats bred as described by Björnsson (Björnsson *et al.*, 2003) were housed in type III cages on wire mesh floors, two rats in each cage and fed a finely milled pellets diet (Altromin, 1314 fortified, Germany) and tap water ad libitum. 10 of these rats had PVC-tubes in their cages for the purpose of environmental improvement. The rats were kept at 21-23 °C and with a relative humidity of 50-70% with 12-14 air changes per hour and a 12 hours light/dark cycle.

A pre-experimental periodontal examination was performed under general anaesthesia (hypnorm/midazolam) to exclude rats with periodontal destruction, defined as probing pocket depths exceeding 0.5 mm (Björnsson *et al.*, 2003). The weights of the rats were recorded at regular intervals during the experiment.

The experiment was conducted at the Departments of Experimental Medicine, Pharmacology and Periodontology, the Panum Institute, University of Copenhagen. The animals had one week of acclimatisation before the beginning of the experiment.

Treatment

LPS model

LPS (Escherichia coli serotype 055:B5, Difco, Franklin Lakes, USA) was diluted in isotonic saline to a final concentration of 1 mg/ml. 10 µl LPS or saline were injected into the buccal and palatal interdental papillae between the first and second

maxillary molar under general anaesthesia (hypnorm/midazolam) as previously described (Llavaneras *et al*, 2001; Ramamurthy *et al*, 2002). The administration of LPS and saline was performed using a Hamilton 10µl syringe (1701 RN) with a custom-made needle (gauge 31, length 25mm, point style 4).

Ligature model

4/0 silk ligatures (Perma-Hand[®] Seide, Ethicon GmbH, Norderstedt, Germany) were placed around the cervix of the 2nd maxillary molar in each side under general anaesthesia (hypnorm-midazolam) (Björnsson *et al*, 2003).

The rats were randomly divided into seven treatment groups (Table 1).

The rats in groups CON1, CON4 and TUB received no treatment.

LPS and saline were administered to the LPS and SAL groups, respectively, every other day for six days as described above, beginning with day 1. The rats in these groups were euthanised on the 6th day (Llavaneras *et al*, 2001, Ramamurthy *et al*, 2002).

Experimental periodontitis was induced with periodental ligature in groups LIG1 and LIG 4 as described. The rats in group LIG 1 were anaesthetised on day 4 and loose and lost ligatures replaced, while the rats in group LIG4 were con-

trolled once a week (Björnsson *et al*, 2003).

The rats in the LIG1 and CON1 groups were euthanised one week after induction of experimental periodontitis while the rats in groups LIG4, CON4 and TUB were euthanised 4 weeks after the beginning of the experiment.

Morphometrical registration of bone destruction

The heads from the eutanised rat were boiled in water for 10 min. and defleshed manually. They were left for 24 hours in 3 % H₂O₂ and stained for 1 min. in methylene blue (1g/100mL) to delineate the cemento-enamel junction (CEJ) (Klausen *et al*, 1989). A method for quantifying periodontal bone destruction in rats previously described (Chang *et al*, 1994; Björnsson *et al*, 2003) was used with slight modifications. Periodontal bone loss (PBL) was evaluated morphometrically by measuring the distance between the CEJ and the buccal alveolar bone crest at 15 sites in each upper jaw. All measurements were made along the long axis of the roots (Björnsson *et al*, 2003)(Figure 1). The measurements were performed electronically (DP-soft ver. 3.2 for Windows, Olympus Europa GmbH, Germany) with digital stereomicroscope photography (5050zoom, Olympus digital camera). To standardize the measuring procedure, the jaw specimen was placed so that the occlusal plane of the left and

Table 1. Treatment groups, number of animals in each group at beginning of experiment, treatment of the animals in each group and the duration of experiment for each group.

Group	N	Treatment	Duration
SAL	14	Saline injections. Served as control for LPS	6 days
LPS	14	LPS injections	6 days
CON1	14	No treatment. Served as control for LIG1 and SAL	1 week
LIG1	14	Periodental ligature	1 week
CON4	14	No treatment. Served as control for LIG4 and TUB	4 weeks
LIG4	14	Periodental ligature	4 weeks
TUB	10	No treatment. Rats with PVC-tubes in cages	4 weeks

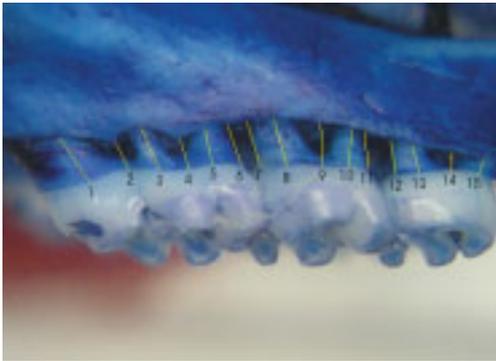


Figure 1. Digital photograph of the left vestibular maxilla the lines indicate the 15 measuring sites.

right side molars in the maxilla were aligned when observed in the microscope. The mean of the 15 measurements from the left and right side of the maxilla was used as a measure of PBL in each animal. All measurements were carried out blind.

Radiographical registration of periodontal destruction

The defleshed alveolar process with the three molars was dissected from each side of the maxilla. Each specimen was attached to a plastic slip on top of an x-ray film (Kodak x-ray, OMAT, MA). To obtain a sufficient reproducibility of the alignment of the molars on the film, two criteria had to be fulfilled: the teeth should not overlap each other interproximally and the buccal root of each molar should be superimposed on the corresponding palatal root (Björnsson *et al*, 2003).

The radiographs were scanned and digitized (Sprint Scan 4000 model cs-4000, Polaroid corporation, Cambridge, Mass) with a resolution of 4000 dots/inch.

All measurements on the x-rays were performed with DP-soft ver. 3.2 (Olympus Europa GmbH, Germany). Measurements were performed on the mesial and distal aspect of the 2nd molar in each side. The apex (A) of the mesial or distal root and the corresponding mesial or distal cusp tip (C) were identified, and the distance between A and C was

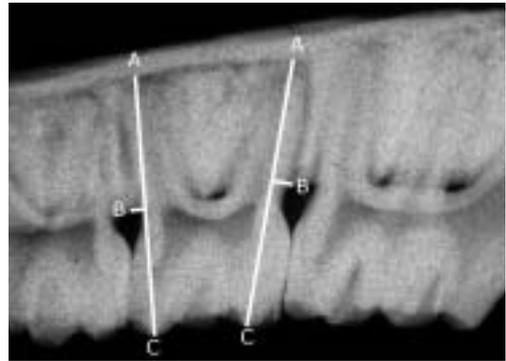


Figure 2. Digitized x-ray of the left maxilla. AB indicates the distance from apex (A) to intersection (B) and AC the distance from apex (A) to cusp tip (C).

traced and measured in mm (AC). A line was traced from the deepest bone defect interproximally intersecting AC at a right angle. Finally the intersection of the two lines (B) was located and the distance from apex (A) to the intersection (B) was measured in mm (AB). Periodontal Bone Support (PBS) was calculated according to the formula $PBS = AB/AC \times 100\%$. (Klausen *et al*, 1989; Björnsson *et al*, 2003) (Figure 2). All measurements were carried out blind.

Statistical analysis

All calculations and statistical analysis were performed using the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc., SAS Campus Drive, Cary, NC 27513, USA). One-way analysis of variance was used to demonstrate differences between the treatment groups. The analysis was supplemented with a Duncan multiple range test for further description of the differences. Level of significance, $p=0.05$.

Results

Five rats were excluded from the study after pre-experimental examination due to 0.5 mm periodontal probing depths. Seven rats were lost during anesthesia: two rats under the pre-experimental examination, four from the LPS group and one from the LIG1 group.

Table 2. Mean body weight (gram) of the groups when weighed during the experiment and total weight gain at the end of the experiment. SAL = Saline injections; LPS = LPS injections; CON1 = No treatment (1 week); LIG1 = Periodental ligature (1 week); CON4 = No treatment (4 weeks); LIG4 = Periodental ligature (4 weeks); TUB = No treatment, PVC-tubes in cages.

Groups	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 14	Day 21	Day 28	Total weight gain
SAL	353	-	356	-	356	364	-	-	-	-	11
LPS	370	-	352	-	349	361	-	-	-	-	-9
CON1	346	-	-	356	-	-	364	-	-	-	19
LIG1	363	-	-	369	-	-	376	-	-	-	13
CON4	348	-	-	-	-	-	368	387	401	412	63
LIG4	340	-	-	-	-	-	360	376	387	397	57
TUB	340	-	-	-	-	-	350	367	380	395	55

Body weights

The rats were weighed throughout the experiment. (Table 2)

It can be observed that the administration of LPS obviously led to a weight loss compared to the weight after saline injection, whereas only minor differences were seen within the 1-week groups and within the 4-week groups

Loose or lost ligatures

The rats were checked for loose or lost ligatures at certain days during the experiment (Table 3).

Only in one case was the ligature completely lost, but loose ligatures were a common finding.

Periodontal Bone loss (PBL)

PBL for the individual rat within each group is illustrated in Fig 3. No significant difference was

seen between the SAL, LPS, CON1 and CON4 groups whereas LIG1 and LIG4 had significantly more PBL than the aforementioned groups ($p < 0.05$). Further, it was found that LIG4 had significantly more PBL than LIG1. The amount of bone loss in the group supplied with the environmentally improving PVC tube did not differ significantly when compared to the CON4 group.

Figure 4 shows PBL excluding sites other than the four closest to the injection area (site 5,6,7 and 8, see Figure 1). This was done particularly to investigate the local effect of the LPS injections and the possible traumatic effect of the injection. Almost the same relationships between the groups are displayed in Figure 4 as in Figure 3. Comparison of the LPS SAL CON1 and CON4 groups with the Duncan multiple range test showed a significantly higher PBL value in the LPS group compared to the

Table 3. Loose/lost ligatures when controlled during the experiment. LIG1 = Periodental ligature (1 week); LIG4 = Periodental ligature (4 weeks);

Groups	4 th day	One week	Two weeks	Three weeks	Four weeks
LIG4	-	6/1	10/0	5/0	6/0
LIG1	3/0	2/0	-	-	-

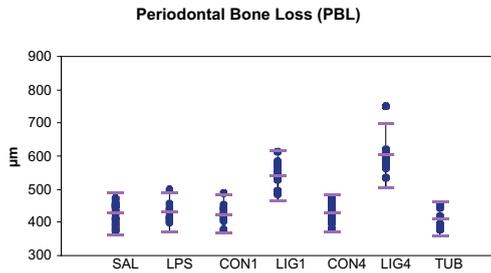


Figure 3. PBL for individual rats in each treatment group (dots); the mean $\pm 2 \times$ standard deviation of each group indicated by horizontal lines. SAL = Saline injections; LPS = LPS injections; CON1 = No treatment (1 week); LIG1 = Peridental ligature (1 week); CON4 = No treatment (4 weeks); LIG4 = Peridental ligature (4 weeks); TUB = No treatment, PVC-tubes in cages.

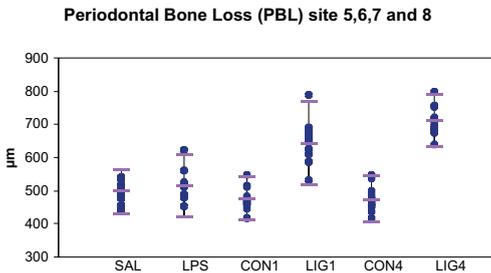


Figure 4. PBL for individual rats in each treatment group at site 5,6,7 and 8 (dots); the mean $\pm 2 \times$ standard deviation of each group (lines). SAL = Saline injections; LPS = LPS injections; CON1 = No treatment (1 week); LIG1 = peridental ligature (1 week); CON4 = No treatment (4 weeks); LIG4 = Peridental ligature (4 weeks).

untreated control groups. However, PBL in the SAL group had a PBL value, which did not differ significantly from either the LPS or CON1 and CON4 groups. Again, it was found that the amount of PBL was significantly higher in the LIG4 group compared to the LIG1 group ($p < 0,05$).

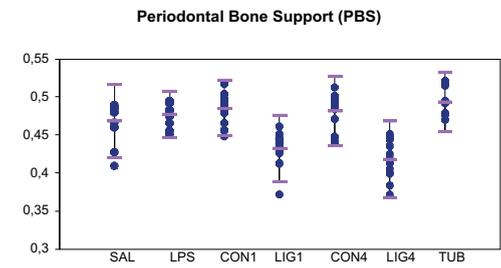


Figure 5. PBS for individual rats in each treatment group (dots); the mean $\pm 2 \times$ standard deviation of each group indicated by horizontal lines. SAL = Saline injections; LPS = LPS injections; CON1 = No treatment (1 week); LIG1 = Peridental ligature (1 week); CON4 = No treatment (4 weeks); LIG4 = Peridental ligature (4 weeks); TUB = No treatment, PVC-tubes in cages.

Periodontal Bone Support (PBS)

PBL describes the amount of lost periodontal bone whereas PBS is an assessment of remaining periodontal bone. The expected inverse relation can be seen by comparing Figures 3 and 5.

Figure 5 shows the results for the individual rats. The same pattern in significant differences as seen in PBL was found for PBS, but in contrast the difference between LIG1 and LIG4 was insignificant. Again, the amount of bone loss in the TUB group supplied with the environmentally improving PVC tube did not differ significantly from the CON4 group.

Discussion

In the present study we have reproduced a previously described method for breeding and housing periodontitis-free rats (Björnsson *et al*, 2003). The use of PVC-tubes in the bedding-free cages did not influence alveolar bone destruction and can therefore be used to improve the environment in the cages for the rats in future studies.

Surprisingly, this study did not confirm earlier findings that gingival LPS injection could induce experimental periodontal disease (Llavaneras *et al*, 2001,

Ramamurthy et al, 2002) since there was no significant difference between gingival injection of LPS and saline. The localised effect of LPS was limited. At sites 5,6,7 and 8, which were adjacent to the injected area, there was a significant difference between the LPS treated and the untreated control groups but the LPS treated group was not significantly different from the saline group. This suggests that the periodontal breakdown found in those groups was due to the trauma from the injection regime and only to a minor degree due to the substances administered.

The traumatic influence of the needle injection may itself initiate inflammation and bone destruction. However, this effect appears to be limited since there was no significant difference in periodontal bone destruction between saline injected rats and the corresponding control group. The insignificant effect of LPS injections on alveolar bone loss could be due to technical difficulties when performing the injections. However, the LPS group was the only group with a marked body weight loss (table 2), and the group had a much higher morbidity than any other group indicating a systemic effect of LPS. Another possibility is that the type of LPS used in this study (*Escherichia coli* serotype 055:B5, Difco, Franklin Lakes, USA) may cause less alveolar bone destruction than the type of LPS used earlier. This hypothesis could not be verified since information about the serotype of the *E coli* used in earlier studies was not available (*Llavaneras et al, 2001; Ramamurthy et al, 2002*).

According to the Danish legislation the experimental model that results in fewer animals being used

while still obtaining the same amount of information should be used. This is in accordance with the principle of the Three R's (reduction, refinement and replacement) as defined by William Russel and Rex Burch in 1959 in their book *The Principles of Humane Experimental Techniques*.

The demand for fewer animals and the eventually small differences between treatment groups, e.g. small differences in periodontal destruction between the treated and untreated groups, make the pre-examination indispensable. Performing no pre-examination even when a few animals have pre-experimental periodontal destruction may blur the outcome of a study or demand a larger sample to reveal a significant difference between treatment groups due to greater variation within groups.

The study verified that significant bone destruction could be achieved by placing a silk ligature around the cervix of the 2nd maxillary molar for four weeks as previously described by Björnsson (*Björnsson et al, 2003*). Furthermore, we found that it is possible to induce significant experimental periodontitis in rats in only one week using a silk ligature tied around the 2nd maxillary molar.

Since four weeks of ligation resulted in more periodontal bone loss (PBL) than did one week ligation, four week ligation may be advantageous in studies where disease progression is assumed to be slow. On the other hand one-week ligation has the advantage of lower cost, shorter duration and less manipulation of the animals.

Finally, in our hands, the previously reported LPS method proved to be unreliable.

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