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The effect of spironolactone on experimental periodontitis in rats

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Background: Elevated levels of tumour necrosis factor (TNF) have been found in patients with adult periodontitis. Animal studies have shown that TNF plays an important role in the pathogenesis of periodontitis. New findings suggest that the aldosterone-inhibitor spironolactone possesses an anti-TNF effect. The purpose of the study was to determine the anti-TNF effect of spironolactone in an endotoxic shock rat model and to disclose the effect of oral administration of spironolactone on the development of experimental periodontitis in rats.

Methods: The study was divided in two parts. Part 1: oral administration of spironolactone (100 mg/kg) followed by intravenous lipopolysaccharide (1 mg/kg) infusion 45 min later. Blood samples were taken before and 90 min after lipopolysaccharide infusion to determine the TNF levels in spironolactone treated and non-treated rats. Part 2: oral administration of spironolactone [100 mg/(kg day)] starting 2 days prior to induction of experimental periodontitis established by peridental ligatures. Morphometrical and radiographical registrations of alveolar bone destruction were carried out to determine the effect of spironolactone on the progression of experimental periodontitis.

Results: In part 1 the endotoxic shock model showed a significant reduction in TNF levels in the spironolactone-treated group compared to the non-treated group, suggesting that spironolactone acts as a TNF inhibitor. In part 2 spironolactone-treated rats did not demonstrate significantly less alveolar bone destruction compared to non-treated rats.

Conclusions: The insignificant effect of spironolactone treatment could be explained by the fast metabolism of spironolactone and that spironolactone does not completely inhibit TNF production in rats. Moreover, many other cytokines and mediators involved in alveolar bone destruction may account for the lacking response to spironolactone.

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Periodontitis is thought to be caused by accumulation of bacteria on the tooth surfaces in the periodontal pockets. Bacterial products including lipopolysaccharides cause inflammation, resulting in the production of inflammatory mediators (cytokines), some of which are thought to be key factors in the periodontal tissue destruction (1–3). Due to their tissue destructive potential, proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1 are considered important factors in the pathogenesis of periodontal disease (1, 2). Both cytokines are able to stimulate fibroblasts to produce matrix metalloproteinases and induce bone resorption via osteoclasts (4).

Higher levels of TNF at both protein and mRNA level have been revealed more frequently in biopsies from inflamed gingiva than in biopsies from healthy gingival tissue (5). Even though experiments have suggested that TNF plays an important role in inflammation-related tissue destruction, the exact role of this cytokine in the pathogenesis of periodontal disease is yet to be discovered.

Comparison of sites injected with placebo and sites injected with function-blocking soluble receptors to interleukin-1 and TNF in a primate model of experimental periodontitis showed that the placebo sites had 80% more inflammatory cells in close contact with the bone. Furthermore, the function-blocking soluble receptors to interleukin-1 and TNF reduced the number of osteoclasts by 67%, and the periodontal bone loss was reduced by 60% (6).

Another study in rats showed that simultaneous treatment with recombinant-human-TNF and experimental induction of periodontitis resulted in significantly greater inflammatory response and periodontal breakdown than either treatment alone (7).

It has recently been shown that the aldosterone-inhibitor spironolactone also inhibits production of TNF (8). Consequently, this drug may inhibit the progression of periodontal disease. Spironolactone can be administered orally and has been used for many years in the treatment of hyperaldosteronism (9).

Intravenous lipopolysaccharide injection in rats has been used to examine heart and renal functions during an endotoxic shock. Due to the infectious agents a host response characterized by large amounts of inflammatory mediators are released including cytokines such as TNF (10). This makes the endotoxic shock rat model suitable for testing the anti-TNF effect of spironolactone.

The purpose of this study was twofold: (i) to determine the anti-TNF effect of spironolactone in an endotoxic shock rat model and (ii) to disclose the effect of oral administration of spironolactone on the development of experimental periodontitis in rats.

Materials and methods

The Danish National Experimental Animal Inspectorate approved the experimental protocol.

The study was conducted at the Departments of Pharmacology and Experimental Medicine and Periodontology, the Panum Institute, University of Copenhagen, Denmark. The animals had a minimum of 1 week of acclimatization before the start of the experiment.

The study was divided into two parts.

Part one

Oral administration of spironolactone followed by intravenous lipopolysaccharide injection. Blood samples were taken to determine the anti-TNF effect of spironolactone.

Animals — 10 male MOL:SPRD (358 ± 10 g) were housed in standard type III cages and maintained on a standard rodent diet and had free access to water.

The animals were anaesthetized with isofluran-nitrous oxide and implanted with permanent medical grade Tygon catheters into the abdominal aorta and the inferior caval vein, respectively, via a femoral artery and vein (11, 12).

After instrumentation the animals were housed individually for 7–8 days until the day of treatment.

Treatment — The rats were randomly divided into two groups with five rats in each.

1 Spironolactone group (SPIR1): treatment with spironolactone given orally and intravenous lipopolysaccharide.
2 Control group (CON1): intravenous lipopolysaccharide.

Before the experiment all rats were adapted to the restraining cages used for the experiment by training them for 90 min. Furthermore, the SPIR1 group was trained in oral gavage twice.

The SPIR1 group received 100 mg/kg spironolactone (minimum 97%, S3378, Sigma-Aldrich, Vallensbaek Strand, Denmark) by oral gavage using a baby feeding tube. Forty-five minutes after spironolactone administration to the SPIR1 group, the rats in both groups were placed in restraining cages and blood samples for determination of TNF and spironolactone were taken from the arterial catheter. Immediately hereafter 1 mg/kg lipopolysaccharide (*Escherichia coli*, serotype 055:B5; Sigma-Aldrich) was infused via the venous catheter with an infusion rate of 0.1 ml/min.

Ninety minutes after lipopolysaccharide infusion, blood samples were taken again and the animals killed (Mebumal i.v.).

Spironolactone was suspended in 3 ml of tap water and vortexed for 10 s. To ensure complete administration of spironolactone, the syringe and tube were re-flushed with 1 ml of water.

Lipopolysaccharide was diluted in isotonic saline to a final concentration of 1 mg/ml.

Part two

The effect of spironolactone after oral administration on the progression of experimental periodontitis in rats using a ligature model previously described (13, 14).

Animals — Forty male MOL:SPRD rats bred as described by Björnsson (14) were housed on wire mesh floors and fed a finely milled pellet diet (Altromin 1314 fortified, Altromin, Lage, Germany) and tap water ad libitum. The weight of the rats at the start of the experiment was 362 ± 60 g.

Treatment — A pre-experimental examination was performed under general anaesthesia (hypnorm/midazolam) to exclude rats with periodontal probing depths exceeding 0.5 mm.

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

Spironolactone was given three times daily (at 07.00, 15.00 and 23.00 h) for 9 days to the SPIR2 group. Treatment started 2 days prior to induction of experimental periodontitis.

The daily dose of spironolactone was 100 mg/kg split in three portions. Each portion consisted of 1/3 of the daily dose suspended in 2 ml of tap

Table 1. Group distribution, number of animals (N) in each group at the beginning of the experiment, treatment of the animals in each group, and duration of the experiment within each group

Group	*N*	Treatment	Duration
SPIR2	12	Ligature-induced experimental periodontitis and orally administered spironolactone	9 days
LIG2	12	Ligature-induced experimental periodontitis. Served as control group for SPIR2	1 week
CON2	14	No treatment. Served as control group for SPIR2 and LIG2	1 week

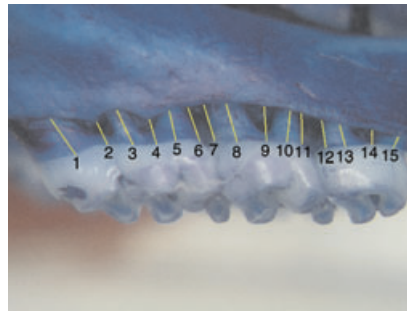

Fig. 1. Digital photograph of the left vestibular maxilla. The lines indicate the 15 measuring sites.

water. The suspension was delivered as described above.

Experimental periodontitis was induced by placing 4/0 silk ligatures (Perma-Hand® Seide, Ethicon GmbH, Norderstedt, Germany) around the cervix of the second maxillary molar in each side under general anaesthesia with hypnorm-midazolam (14).

On the fourth day after induction of experimental periodontitis, the rats were anaesthetized and loose or lost ligatures replaced. Forty-five minutes after oral administration of spironolactone, blood samples were taken from the orbital plexus on the fourth day and by cardiac puncture on the seventh day. Blood samples were taken under anaesthesia. The rats were killed and decapitated immediately after cardiac puncture.

All blood samples were refrigerated for 10 min followed by 10-min centrifugation at 1300 *g*, sera pipetted and stored at −20°C.

Analytical methods

TNF-α determination — TNF-α concentration was determined in duplicate by enzyme-linked immunosorbent assay (rat TNF-α kit, KRC3012, range: 15.6–1000 pg/ml, sensitivity < 4 pg/ml, interassay CV: ≤ 4.3%, intra-assay CV: ≤ 2.7%, Biosource, Nivelles, Belgium).

Serum concentrations of spironolactone — The spironolactone concentrations in sera were examined using high pressure liquid chromatography (HPLC) previously described by Kaukononen *et al.* (15, 16).

HPLC was performed using a Waters 515 HPLC pump and a Waters 486 tunable absorbance detector. System and data management was controlled by a Millennium[32] version 3.05.01 software. A Waters Spherisorb cartridge column (5 µm packing) ODS-2 was used with an integrated precolumn at ambient temperature. The mobile-phase (67% methanol in water) flow-rate was 1.0 ml/min; detection was performed at 238 nm. Stock solutions and dilutions of standards were prepared in pure acetonitrile. Testosteron 2 µg/ml was used as internal standard. Samples were prepared by adding 200 µl serum to 200 µl of acetonitrile containing internal standard and were vortex-mixed for 10 s before centrifugation at 10600 *g* for 15 min. The supernatant was pipetted and placed in Waters 717 plus auto sampler vials and 80-µl samples were injected.

Morphometrical registration of bone destruction — The rat heads were boiled for 10 min and defleshed manually. They were left for 24 h in 3% H_2O_2 and stained for 1 min in methylene blue (1 g/100 ml) to delineate the cemento-enamel junction (17). A previously described method for quantifying periodontal bone destruction in rats (14, 18) was used with slight modifications. Periodontal bone loss was evaluated morphometrically by measuring the distance between the cemento-enamel junction and the buccal alveolar bone crest at 15 sites in each upper jaw. All measurements were made along the long axis of the roots (14) (Fig. 1). The measurements were performed electronically (DP-Soft version 3.2, Olympus Europa GMBH, Hamburg, Germany) on 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 right side molars in the maxilla was 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 periodontal bone loss in each animal. All registrations were carried out blinded.

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 slab 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 (14).

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

All measurements on the X-rays were performed with DP-Soft version 3.2. Measurements were performed on the mesial and distal aspect of the second molar in each side. The apex (A) of the mesial or distal root and the corresponding mesial or distal cusp (C) tip were identified and the distance between A and C was traced and measured in mm. A line was traced from the deepest bone defect interproximally intersecting AC at a right angel. Finally, the intersection of the

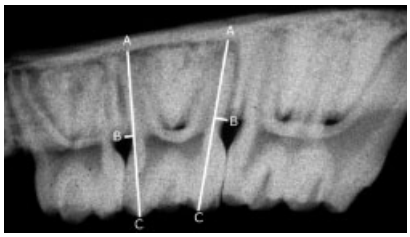

Fig. 2. Digitized X-ray of the left maxilla. AB indicates the distance from apex (A) to intersection (B) and AC the distance from apex (A) to cusp tip (C).

two lines (B) was located and the distance from apex to the intersection (AB) was measured in mm. Periodontal bone support was calculated according to the formula periodontal bone support = AB/AC × 100% (14, 17) (Fig. 2). All registrations were carried out blinded.

Statistical analysis

All calculations and statistical analyses were performed using the statistical analysis system (SAS) version 8.0 (SAS Institute Inc., Cary, NC, 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

Part one

As shown in Fig. 3, all rats had TNF concentrations below detection range (15.6 pg/ml) before the lipopolysaccharide administration.

Ninety minutes after lipopolysaccharide administration, the TNF concentrations had increased significantly in both groups ($p < 0.01$). Comparison of CON1 and SPIR1 groups showed that there was significantly higher TNF concentration in the CON1 group than in the SPIR1 group ($p \sim 0.0335$, two sided).

The HPLC chromatograms showed small amounts of spironolactone. However, due to the small quantities the spironolactone concentration could not be determined. To ensure that the spironolactone peak located on the chromatogram indeed was caused by spironolactone, additional samples of sera were spiked with spironolactone. This resulted in overlapping peaks.

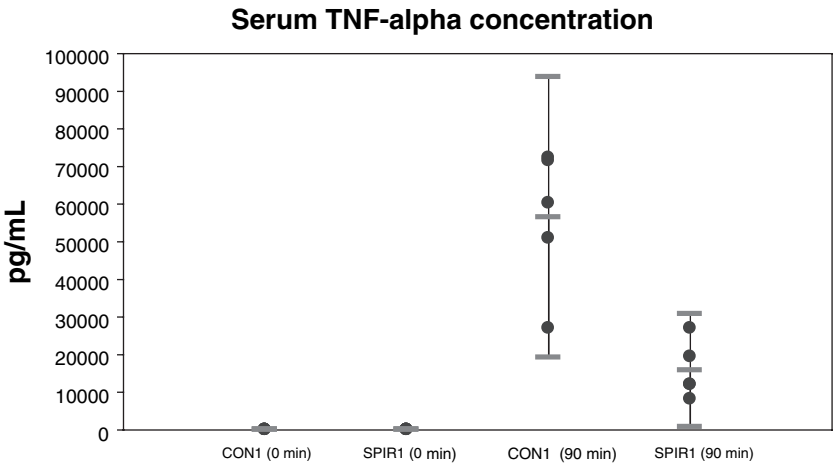

Fig. 3. Tumour necrosis factor-α (TNF-α) concentration for the individual rat within each treatment group (dots) at baseline (0 min) showing levels below detection range (15.6 pg/ml) and 90 min after lipopolysaccharide administration. The mean ± 2 × standard deviation of each treatment group is indicated by horizontal lines.

Part two

Four rats were excluded from the study after pre-experimental examination due to 0.5-mm periodontal probing depths. Three rats were lost during anaesthesia, two from the SPIR2 group and one from the LIG2 group.

Three rats from the LIG2 group and one from the SPIR2 group had loose ligatures when checked on the fourth day and none had loose or lost ligatures on the seventh day.

Periodontal bone loss — The periodontal bone loss for the individual rat within each treatment group is illustrated in Fig. 4. No differences are seen between the groups SPIR2 and LIG2, whereas CON2 differs significantly from the aforementioned groups ($p < 0.05$).

Periodontal bone support — Figure 5 shows the periodontal bone support results for the individual rats. Periodontal bone loss describes the amount of lost periodontal bone, whereas periodontal bone support is an assessment

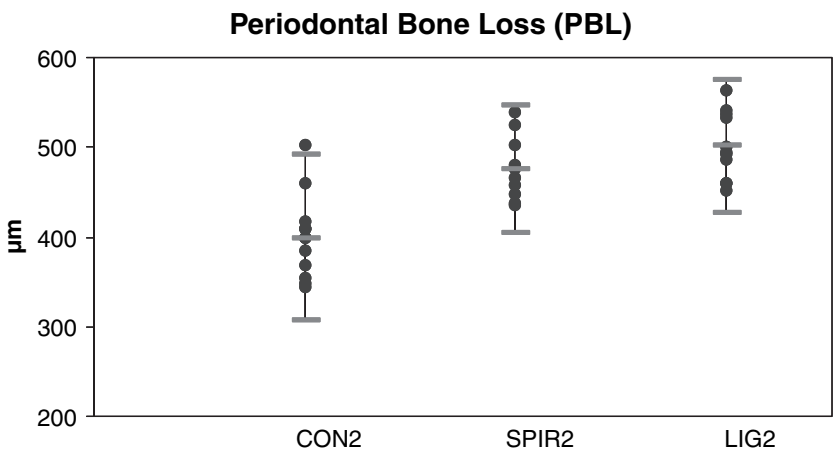

Fig. 4. Periodontal bone loss for individual rats in each group (dots). The mean ± 2 × standard deviation of each group is indicated by horizontal lines.

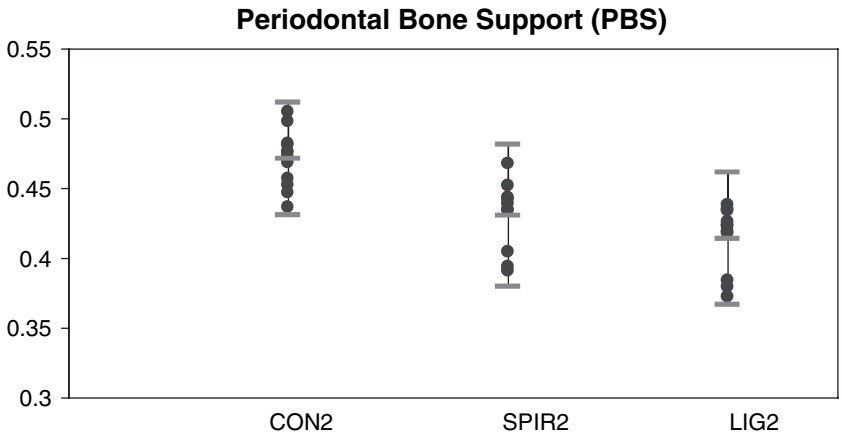

Fig. 5. Periodontal bone support for individual rats in each group (dots). The mean ± 2 × standard deviation of each group is indicated by horizontal lines.

of remaining periodontal bone. The expected inverse relation can be seen by comparing Figs 4 and 5.

As found in part one, the HPLC chromatograms showed detectable but immeasurable amounts of spironolactone because of the small quantities.

Discussion

In the present study, the effects of spironolactone on TNF and experimental periodontitis in rats were investigated.

Part one of the study showed that serum TNF concentrations were significantly reduced in the spironolactone-treated group 90 min after lipopolysaccharide infusion compared to a control group. As the injected lipopolysaccharide dose and the TNF levels before lipopolysaccharide administration were similar in the two groups, the finding indicates that spironolactone when present in the blood, shown by the HPLC analysis, acts as a TNF-inhibitor. This is in compliance with earlier *in vitro* studies using human whole-blood and purified blood mononuclear cells in which an anti-TNF effect of spironolactone was revealed (8).

The higher TNF levels, especially in the control group, of the present study than reported in earlier studies could be due to the fact that the lipopolysaccharide infusion was performed more rapidly and with a larger amount of endotoxin than previously described (12, 19).

In part two we reproduced a previously described model of experimental periodontitis in rats, demonstrating that placement of a silk ligature around the cervix of the second maxillary molar produces significant alveolar bone destruction in 1 week (13).

TNF has been found to be an important proinflammatory mediator of bone destruction in experimental periodontitis on rats (7). Elevated expression of TNF and its p55 receptor has been found in sites with adult periodontitis, suggesting that TNF plays a role in the tissue destruction of periodontitis (20). Therefore, spironolactone, which was found in part one to be a TNF-inhibitor in rats, could perhaps be suitable for the prevention of tissue destruction associated with periodontitis. Moreover, spironolactone has been found to inhibit the production of other pro-inflammatory cytokines in humans, such as interleukin-6, oncostatin M, interferon γ and osteopontin, all of which play a significant role in the pathogenesis of periodontitis (8).

The possible effect of spironolactone on the progression of experimental periodontitis in rats was investigated in part two. The study showed no significant reduction of bone destruction in the spironolactone-treated group.

As the anti-TNF effect of spironolactone was already established in part one of the present study, we did not examine TNF levels in part two. However, the lack of effect could be due to the fast metabolism of spironolactone in the rat (half-life 0.72 ± 00.17 hours after i.v. administration) (16), which may result in periods with no or insignificant amounts of spironolactone in the blood. This assumption was supported by the HPLC analyses, revealing only small amounts of spironolactone. Administration via ALZET osmotic pumps would have been preferable, as this could assure a higher and more constant spironolactone concentration. Unfortunately, it was not possible to deliver the high daily dose of spironolactone required in this study using ALZET osmotic pumps because of the hydrophobic character of spironolactone, which prevented us from making a suitable solution.

The insignificant effect of spironolactone treatment on the progression of experimental periodontitis in rats could also be explained by the fact that spironolactone does not completely inhibit TNF production, as seen in part one, and also that spironolactone does not appear to inhibit several other proinflammatory mediators involved in alveolar bone destruction (21).

Furthermore, the limited number of observations should be taken into account, and although statistically insignificant, a lower mean, maximum and minimum value of bone destruction was found in the spironolactone group as compared to the non-treated ligated group.

The possible effect of spironolactone treatment in humans with periodontal disease is yet to be investigated. Results other than the present ones may be achieved, because the metabolism of spironolactone in humans is slower [post steady state elimination half-life 1.4 h (22)] and unchanged spironolactone has been found up to 8 h after a single oral dose of 200 mg spironolactone (23).

Anti-TNF antibody treatment is being used in the treatment of many chronic inflammatory diseases, such as rheumatoid arthritis and morbus Crohn. These treatments are often expensive and require parenteral administration (24). Spironolactone is,

on the other hand, inexpensive and can be administered orally.

Future studies may show whether spironolactone or derivatives are suitable for the treatment of human inflammatory conditions including periodontitis.

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