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Low-dose doxycycline inhibits bone resorption associated with apical periodontitis

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Abstract

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Aim To test the effect of low-dose doxycycline on bone resorption associated with apical periodontitis.

Methodology Apical periodontitis was induced by occlusal pulp exposure in the mandibular first molars of 36 rats. Animals were divided into three groups of 12: group A received doxycycline in drinking water at a dose of 5.85 mg day^{-1}; group B received a dose of 1.48 mg day^{-1} (one-quarter of the original dose); and group C received no medicament and served as the control. A bioassay determined the doxycycline serum levels. After 21 days, the mandibles were removed, radiographed and the radiographs scanned to generate digital images. These images were analysed morphometrically and the total area of the periapical bone resorption of the mesial and distal roots of each tooth was determined and used to compare the groups. Statistical analysis was completed using ANOVA with repeated measures.

Results The mean doxycycline serum level in group A was 0.22(±0.03) μg mL^{-1} and in group B below the detection level of the assay (<0.062 μg mL^{-1}). The mean area of the periapical bone resorption in the control group C was 2.91(±0.61) mm^2. In animals treated with a low-dose doxycycline, the mean size of the bone resorption was significantly smaller at 1.59(±0.59) mm^2 (group A) and 1.72(±0.85) mm^2 (group B) ($P = 0.001$). No significant difference was found in the area of the bone resorption between these two groups A and B.

Conclusions Low-dose doxycycline reduced the area of bone resorption associated with apical periodontitis in the mandibular first molar teeth of rats.

Keywords: apical periodontitis, bone resorption, doxycycline, low-dose, rats.

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Introduction

Apical periodontitis develops in response to bacterial colonization of the root canal. Host response against bacteria emerging through the apical foramen successfully prevents their spread to other sites. Activated macrophages and activated T-lymphocytes serve as essential components of this response. Nevertheless, locally produced inflammatory cytokines, such as $IL1_\beta$ and TNF_β, derived from these cells, trigger periapical bone resorption (Wang & Stashenko 1993).

Periapical bone resorption may be considered a mere undesirable side effect of the essential and successful protective host response at this site of potential bacterial penetration (Metzger 2000). However, it is the major clinical hallmark of apical periodontitis. The presence of bone resorption indicates disease whilst healing is monitored by its reversal and by reduction of the periapical radiolucency. Persistence of the lesion indicates that the balance between bone resorption and healing is still in favour of the former.

For several decades, the main, if not only, intervention in apical periodontitis consisted of effective

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bacterial elimination from the root canal. However, recent advances in understanding the mechanisms involved in apical periodontitis allow consideration of other, additional, means of intervention (Metzger 2000). Agents that may inhibit local bone resorption and thus favourably shift the balance between healing and resorption, once bacteria have been eliminated, may be considered. Enhancement of healing kinetics using a pharmacological intervention presents an attractive option. This may allow earlier decisions regarding the survival and potential prosthodontic use of the affected teeth.

Three main pharmacological targets can be identified: local cytokine production, their effect on their target cells and the bone resorption process itself. Glucocorticoids may be used to inhibit the local production of IL-1 by activated macrophages in the inflamed periapical tissues. This occurs through a post-transcriptional mechanism (Politis *et al.* 1992). Recently, dexamethasone was shown to inhibit the formation of bone resorption associated with apical periodontitis in rats (Metzger *et al.* 2002). The potential of IL-1-receptor-antagonist was also tested in the same model; the inhibition of the effect of IL-1 on its target cells inhibited periapical bone resorption (Stashenko *et al.* 1998).

The present study was designed to test another pharmacological agent that is known to affect the bone resorption process: doxycycline, a member of the tetracycline family. Doxycycline has been widely used both experimentally and clinically to control and inhibit bone resorption (Grevstad 1993, Chang *et al.* 1994, Grevstad & Boe 1995, Cummings & Torabinejad 2000, Bezerra *et al.* 2002, Yaffe *et al.* 2003, Preshaw *et al.* 2004a, Buduneli *et al.* 2005). This effect of tetracyclines is independent of their antimicrobial activity (Golub *et al.* 1998). The inhibition of bone resorption by doxycycline occurs even when a low, sub-antimicrobial dose is administered (Bezerra *et al.* 2002, Buduneli *et al.* 2005). Furthermore, chemical modified tetracyclines (*e.g.*, CMT-1, CMT-3 and CMT-8), which lack any antibacterial effects, are still effective in the inhibition of bone resorption (Sasaki *et al.* 1998, Ramamurthy *et al.* 2002).

At sub-antimicroboial low doses, doxycycline can be used without the risk of developing resistant bacterial strains (Ciancio & Ashley 1998). With no antibacterial effect, no selective pressure exists on the bacteria and therefore no resistant strains emerge (Greenstein 1995, Pallasch 2003). Low-dose doxycycline has been successfully applied to treat marginal periodontitis with favourable results (Ciancio & Ashley 1998, Lee *et al.* 2004, Preshaw *et al.* 2004a,b). Its effect has been attributed primarily to inhibition of metallo-proteases, essential for the breakdown of connective tissue and bone resorption (Golub *et al.* 1998, Ramamurthy *et al.* 2002). On the other hand, some reports indicate that tetracyclines may also be effective in reducing the amounts of IL-1 released by activated macrophages (Shapira *et al.* 1997).

The present study was designed to test the effect of systemically administrated low-dose doxycycline on the bone resorption associated with induced apical periodontitis in the rat.

Materials and methods

The Animal Care Committee of Tel Aviv University approved the experimental protocol. During the experiment, animals were treated according to the standards set by the Committee. No signs of stress or discomfort were observed in any animal (inspected daily). Individual weight-gain curves served as an additional indicator of the well being of the animals.

Experimental design

Apical periodontitis was induced in rats by the method first described by Kakehashi *et al.* (1965) and allowed to develop for 21 days. Systemic doxycycline was administered and its effect on the size of the bone resorption evaluated. The experiment included three groups of 12 rats each: group A received a low-dose of doxycycline in drinking water throughout the experiment; group B received one-quarter of the former dose; and group C received no doxycycline and served as the control.

Animals

The study used 36 female Wistar rats (Tel Aviv University breeding) which were randomly divided into three groups of 12 animals each. At the start of the experiments, animals were 2 months old and weighed, on average, 229(± 14) g. Animals were kept two per cage and fed pelleted rat diet, *ad libitum*. Rats were weighed every third day. The weight gained in the experimental groups was compared with that of the control group and with normal animals (no procedure). At the end of the experiment, average weight was 272(±12) g. In the experimental groups, weight gain did not differ from the controls or from normal animals (data not presented).

Induction of apical periodontitis

Animals were anaesthetized by an intra peritoneal injection of Ketamine (90 mg kg^{-1}; Ketaset, Barneveld, Fort Iowa, IA, USA) and Xylazine (5 mg kg^{-1}; Kepro, the Netherlands). The occlusal surface of each of the two mandibular first molars was perforated using a new No. 0.5 round bur, at low speed, exposing the pulps. The occlusal penetration was of the size of the active part of the low speed bur used, which was allowed to fully penetrate the pulp chamber. Penetration was verified by insertion of a bent size 20 K-file through the occlusal opening and into the entrance of the distal root canal. The pulps remained exposed for 21 days, as previously determined (Metzger *et al.* 2002).

Doxycycline treatment

Doxycycline (doxycycline hyclate; Sigma, St. Louis, MO, USA) was diluted in distilled water at a concentration of either 40 or 10 mg mL^{-1} and kept frozen in aliquots of 1 mL, in the dark, until used. Each aliquot was then diluted in 200 mL of drinking water, resulting in concentrations of 0.2 mg mL^{-1} (group A) and 0.05 mg mL^{-1} (group B). Aluminium foil, wrapped around the small drinking water bottles, kept the medicament out of the light. Water bottles were replaced every second day and the amount consumed was recorded. Doxycycline treatment started 3 days prior to pulp exposure.

Radiography

At day 21, animals were anaesthetized and blood samples were collected by cardiac puncture. Animals were killed using CO_2 and the mandibles removed and placed in 2% NaOH for 48 h to facilitate thorough soft tissue removal. Mandibles were then stored in buffered 5% formalin. The jaws were radiographed using an EP-21 periapical dental film (Eastman Kodak, Rochester, NY, USA) with 0.32 s exposure from a distance of 20 cm, using a Gendex X-ray machine (Milan, Italy). Jaws placed on their clean buccal side allowed a uniform angulation as previously determined (Metzger *et al.* 2002) (Fig. 1). All radiographs were developed simultaneously.

Bioassay for doxycycline in serum

Individual serum samples collected at the end of the experiment were assayed for doxycycline concentration

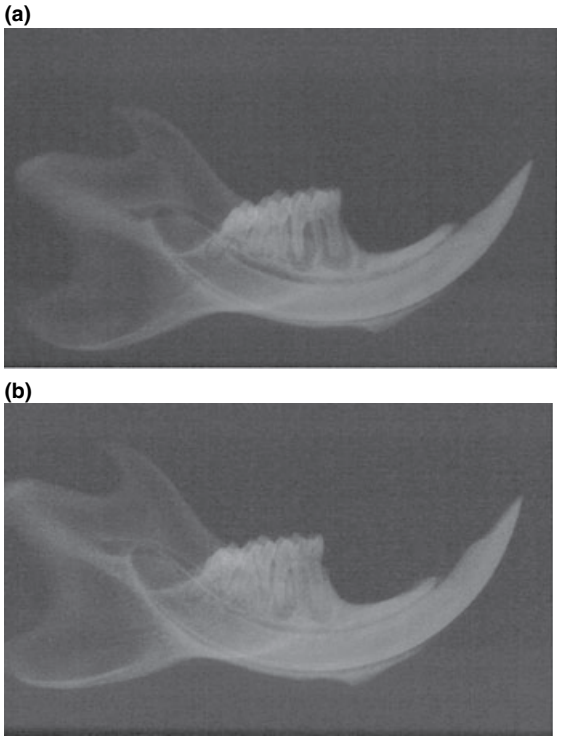

Figure 1 Periapical bone resorption area in first mandibular molar of rats. (a) Larger resorption in the control group; (b) smaller resorption in animals treated with doxycycline. Apical periodontitis was induced by occlusal pulp exposure, open for 21 days.

using the method described by Bennet *et al.* (1966). *Bacillus cereus* (ATCC 11778), which is highly sensitive to tetracycline, was grown overnight in Brain–Heart Infusion broth and 200 μL of the culture, containing 2×10^8 colony forming units, were evenly spread on the surface of Brain–Heart Infusion agar plates. Four full-thickness holes, 6 mm in diameter, were prepared in the agar of each plate and served as wells for the tested samples (Bennet *et al.* 1966). Serum samples, 60 μL, were placed in each well and plates incubated aerobically at 37 °C for 24 h. A standard curve was generated in a similar manner using predetermined doxycycline concentrations (3.0–0.031 μg mL^{-1}) either in PBS or in PBS containing 50% normal rat serum (Bennet *et al.* 1966). Circular inhibition zones developed around wells containing doxycycline. Two inhibition zone diameters, perpendicular to each other, were measured and the mean diameter used as an inhibition parameter. Each serum sample and each point of the standard curve were assayed in quadruplicate. The mean inhibition zone diameter of each

serum sample was calculated and its individual doxycycline concentration determined using the standard curve.

Morphometric image analysis

Radiographs were scanned using an HP Photosmart film scanner (Hewlett Packard, Singapore), and their digital images analysed using SIGMA SCAN software (SPSS Science Software, San Raffael, CA, USA). The borders of the radiographic image of the periapical bone resorption of the mesial and distal roots of each tooth were traced and their area calculated. The area of the root tip was excluded. Tracing was performed manually, on the screen, on an enlarged image, with the operator blinded as to the animal and treatment group from which the sample came. Measurements, after several initial practicing sessions, were with intra-operator reproducibility of ±8%. A total of 24 teeth with 48 periapical areas of bone resorption were measured in each group.

Statistics

ANOVA with-repeated-measures was used to analyse the weight gain of the animals. The areas of bone resorption were first tested for normal distribution, using the Kolmogorov–Smirnov test (SPSS, version 14.0). Once this was verified (see Results), ANOVA with-repeated-measures with Tukey's *post hoc* test was used to analyse the size of periapical bone resorption. Analysis was performed with within-subject-factors: area of the individual periapical bone resorption on left and right and the total of areas of the resorption of the mesial and distal roots of each given tooth for left and right sides and with between-subject-factor: treatment.

Results

Doxycycline dose in drinking water

Preliminary measurements were taken to determine the amount of drinking water consumed daily by normal rats, similar in age and weight. Mean consumption was 27.8(±2.6) mL day^{-1}. On this basis, the doxycycline concentration in the drinking water of group A was set at 0.2 mg mL^{-1} (calculated daily dose of 5.4 mg per animal), and of group B at 0.05 mg mL^{-1} (calculated daily dose of 1.35 mg per animal).

The actual drinking water consumption was monitored during the experiment. In group A, animals consumed an average of 29.3(±3.5) mL day^{-1} (actual daily dose of 5.85(±0.69) mg per animal), and in group B, the mean consumption was 29.52(±2.77) mL day^{-1} (actual daily doxycycline dose of 1.48(±0.14) mg per animal).

Doxycycline serum levels

Doxycycline treatment resulted in a mean serum level of 0.22(±0.03) μg mL^{-1} in group A, with a range from 0.17 to 0.30 μg mL^{-1}, whilst in group B serum levels were below the lowest detection level of the bioassay (<0.062 μg mL^{-1}). No Doxycycline was detected in the control group. No difference was found between standard curves generated with and without normal rat serum (data not presented).

Statistical consideration

The areas of the periapical bone resorption were first tested for normal distribution, using the Kolmogorov–Smirnov test (SPSS, version 14.0). When each root was tested alone (mesial right, distal right, mesial left, distal left), distribution in each group did not differ from normal distribution ($P = 0.637$, 0.990, 0.290 and 0.272, respectively). Nevertheless, when the total area of both mesial and distal bone resorption of a given tooth was used as the parameter, distribution was closer to normal ($P = 0.945$ and 0.661 for left mesial + distal roots and right mesial + distal roots, respectively). Results of analysis for distal roots alone or mesial roots alone gave similar results with the same conclusions (data not presented) Therefore, the total of periapical areas per tooth was used as the parameter for analysis and presentation.

Effect of doxycycline on periapical bone resorption

The area of periapical bone resorption that developed in the control group measured an average of 2.91(±0.61) mm^2 (per tooth) (Table 1). In group A, doxycycline treatment of 5.85(±0.69) mg day^{-1} resulted in a significant reduction in the size of periapical bone resorption to a mean of 1.59(±0.59) mm^2 ($P = 0.001$). The lower dose of doxycycline in group B (1.48(±0.14) mg day^{-1}) also resulted in a significant reduction in the size of periapical bone resorption to a mean of 1.72(±0.85) mm^2 ($P = 0.001$). No significant difference in the size of bone resorption was found between the two doxycycline-treated groups.

Table 1 Effect of low-dose doxycycline on the size of periapical bone resorption area in rats[a]

	Group A	Group B	Group C
Doxycycline dose (mg day^{-1})	5.85 ± 0.69	1.48 ± 0.14	0
Size of bone resorption area (mm^2)	1.59 ± 0.59	1.72 ± 0.85	2.91 ± 0.61

[a]Mean total area of the periapical bone resorption area of the mesial and distal roots, as measured from a digitized radiograph.

Discussion

This preliminary feasibility study tested for the potential of doxycycline to modulate periapical bone resorption. The total area of periapical bone resorption surrounding the mesial and distal roots was used to give a 'tooth' value. This was performed as this parameter had a more normal distribution than the area of either the distal or mesial roots alone. As this differs from other studies carried out with a similar model (Stashenko *et al.* 1998, Tjäderhane *et al.* 2007), statistical analysis was also carried out on the distal and mesial bone resorption areas alone, with similar results.

Systemic low-dose doxycycline treatment inhibited the formation of periapical bone resorption by 40–45%. It could be possibly attributed to either its antibacterial activity or to its inhibitory effects on bone resorption, or both. Neither the higher dose (5.85 mg day^{-1}) nor the lower dose (1.48 mg day^{-1}) resulted in serum levels that may be considered as clinically antimicrobial in rats. A therapeutic antimicrobial doxycycline dose in rats should result in serum levels ≥1 μg mL^{-1} (Slots & Rams 1990, Chang *et al.* 1994, Ramamurthy *et al.* 2002). In the present study, the higher doxycycline dose resulted in a serum level of only 0.22(±0.03) μg mL^{-1}, approximately one-fifth of that of an effective therapeutic dose. Serum samples from group A partially inhibited bacterial growth in the bioassay. Therefore they could, apparently, have some antimicrobial effect *in vivo* which could have contributed to the inhibited development of the periapical bone resorption. Nevertheless, the lower doxycycline dose in group B resulted in such a low serum concentration that it failed to inhibit the growth of even the extremely sensitive bacterium (*Bacillus cereus* ATCC 11778). As this dose was as effective in inhibiting periapical bone resorption as the dose that had a partial inhibitory effect in the bioassay, it may indicate that the inhibitory effect of the doxycycline was most probably through its effect on bone resorption and not its antibacterial properties.

The present results agree with Chang *et al.* (1994) who found that *Porphyromonas gingivalis*-induced alveolar bone loss could also be inhibited by administering 5 mg day^{-1} of doxycycline, a dose similar to that used in this study. Similar results with low-dose doxycycline in rats have also been reported using either nylon thread ligature (Bezerra *et al.* 2002) or endotoxin (Buduneli *et al.* 2005) to induce alveolar bone loss.

It is well documented that doxycycline inhibits periodontal bone resorption in rats. Grevstad *et al.* demonstrated that surgically induced alveolar bone loss was prevented by doxycycline administered systemically (Grevstad 1993, Grevstad & Boe 1995). Similar results have been reported on inhibition of surgery-associated crestal bone loss by doxycycline, administered either locally (Yaffe *et al.* 2003) or systemically (Cummings & Torabinejad 2000).

The results of the present study are in apparent conflict with those reported recently by Tjäderhane *et al.* (2007). That study addressed a similar issue, using the same model and tested the effect of systemically administered chemically modified tetracycline (CMT-3), which was administered by oral gavage. This treatment was expected to inhibit periapical bone resorption by its inhibitory effect on matrix metalloproteinases. Their results indicate an enhancement of periapical bone resorption in animals treated with the CMT-3 rather than an inhibition, as found in the present study. These results are in apparent conflict with most reports on the effect of tetracyclines or CMT-3 in rats, in most of which bone resorption associated with marginal periodontitis was the model. Therefore, it will be of interest to carry out a study in which doxycycline will be compared with CMT-3, as well as to other CMT, in the same model of apical periodontitis.

Even with a clear effect, widespread use of tetracyclines has its potential drawbacks as resistant strains may emerge through selective pressure on oral bacteria (Greenstein 1995, Pallasch 2003). Golub *et al.* (1998) have clearly demonstrated that the effect of tetracyclines on bone resorption is independent of their antimicrobial properties. Furthermore, the inhibition of matrix metallo-proteinases, essential for connective tissue breakdown, occurs at levels well below those required for the antimicrobial effects (Vernillo & Rifkin 1998). Consequently, low, sub-antimicrobial doses of doxycycline have been tested clinically for treatment of periodontal disease, with favourable results (Golub *et al.* 1990, 1992, Greenstein 2004).

The commonly used method for oral administration of doxycycline in rat studies is oral gavage, using a canula inserted into the esophagus. It has the benefit of assuring an accurate dose as well as bypassing the oral cavity. A previous study using the same model concluded that dexamethasone inhibited the development of periapical bone resorption (Metzger *et al.* 2002). As the gavage procedure may present a recurrent stressful event for the animals, which may result in elevation of endogenous cortico-steroids, this procedure was avoided and drinking water was used as a substitute. This may have a drawback, as a potential local effect of the doxycycline cannot be precluded (Toth *et al.* 1986). To investigate this point a direct comparison between the two methods, using the same model, may be required.

The present study does not preclude the possibility that the doxycycline treatment affected periapical bone resorption also through down regulation of cytokine production. To test for this hypothesis, a study in larger animals may be required, in which intra-canal exudates may be quantitatively tested for presence of IL 1_{β} and TNF_{β} (Matsuo *et al.* 1994, Kuo *et al.* 1998a,b).

The results indicated that modulation of bone resorption in apical periodontitis with doxycycline may represent a potential option; however, further studies will be required to explore this possibility. Systemic administration was used in the present study as it was designed as a preliminary feasibility trial, conducted in a simple, small animal, model. As for potential clinical implication, one should keep in mind that the relatively closed environment of the root canal and periapical tissue may lend itself rather easily to *local* pharmacological intervention with low-dose doxycycline, as well as other potential modulators. Nevertheless, further studies, using larger animals in which intra-canal manipulation is possible, will be required before such clinical application may be considered.

Conclusions

1. Low-dose doxycycline inhibited development of bone resorption associated with apical periodontitis in rats.
2. This inhibition was most probably unrelated to doxycycline's antimicrobial activity.
3. Low-dose doxycycline may be potentially considered for pharmacological modulation of periapical bone resorption; further studies will be required to explore this possibility.

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