J Periodont Res 2008; 43: 162–167

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.01007.x

Comparison of toluidine blue-mediated photodynamic therapy and conventional scaling treatment for periodontitis in rats

Y. L. Qin[1], X. L. Luan[2], L. J. Bi[2], Y. Q. Sheng[1], C. N. Zhou[1], Z. G. Zhang[1]

[1]Department of Physics, Harbin Institute of Technology, Harbin, China and [2]Medical Center of Stomatology, the Forth Affiliated Hospital of Harbin Medical University, Harbin, China

Qin YL, Luan XL, Bi LJ, Sheng YQ, Zhou CN, Zhang ZG. Comparison of toluidine blue-mediated photodynamic therapy and conventional scaling treatment for periodontitis in rats. J Periodont Res 2008; 43: 162–167. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard

Background and Objective: Periodontitis is a disease caused by bacterial infection accompanied with the inflammation of connected tissues and resorption of alveolar bone. The aim of this study was to investigate the *in vivo* photosensitization of periodontal bacteria in rats and to compare its efficacy with that of routine scaling and root planing.

Material and Methods: Periodontitis was developed by submerging ligatures at the subgingival region of maxillary molars in 16 rats. Six weeks later, the infection sites were treated either with 1 mg/mL of toluidine blue plus 12 J/cm^2 red laser irradiation, or by routine scaling and root planing. The therapeutic efficacy was assessed by evaluating the reduction of total bacterial flora and histological changes of periodontal tissues.

Results: Significant reduction of total bacterial flora was achieved by both photodynamic therapy and conventional therapy. The signs of inflammation that accompanied periodontitis, such as redness, increased plaque index and gingival index values, bleeding on probing and inflammatory cell infiltration, were greatly reduced without any obvious detectable injury to host tissues. Both photodynamic therapy and conventional therapy showed similar therapeutic results.

Conclusion: Toluidine blue-mediated photodynamic therapy could effectively treat periodontitis *in vivo* and has high potential in clinical application.

Z. G. Zhang, PO Box 3025, 2 Yikuang Street, Nan-gang District, Science and Technology Park, Harbin Institute of Technology, Harbin 150001, China
Tel: +86 45186402639
Fax: +86 45186412828
e-mail: zhangzhiguo@hit.edu.cn

Key words: periodontitis; photodynamic therapy; scaling and root planing; toluidine blue

Accepted for publication March 6, 2007

Periodontitis is caused by bacterial infection and is accompanied with signs of inflammation, bleeding on probing and pronounced attachment loss (1–3). Consequently, successful periodontal therapy is based on the complete suppression of periodontal pathogenic bacteria and the reduction of inflammatory signs. Conventional treatment for periodontopathy (scaling and root planing in conjunction with or without antibiotics) can greatly reduce the bacterial load and achieve an optimal therapeutic effect in many cases (4–6). However, the efficiency of conventional therapy may not be completely satisfactory in certain cases such as furcations, deep invaginations and concavities (7). Moreover, the increased prevalence of bacteria resistant to antibiotics may further influence

the efficacy of conventional therapy (8,9). To overcome these problems, alternative methods are needed.

With the development of laser technology, the application of laser medicine in the treatment of oral diseases has attracted considerable attention. A series of laser systems, such as CO_2 (10) and neodymium-doped yttrium aluminium garnet (Nd:YAG) (11), have been introduced to treat periodontitis. However, the high output of these infrared lasers may easily lead to negative effects (12). Recently, the erbium-doped yttrium aluminium garnet (Er:YAG) laser (13,14) and the diode laser (15) have been found to to reduce bacteria effectively, causing fewer thermal effects compared with the Nd:YAG and CO_2 lasers. Another potential alternative approach to avoid thermal effects is the use of a low-power laser at the visible region in conjunction with an appropriate photosensitizer (i.e. photodynamic therapy). During photodynamic therapy, reactive oxygen is produced to kill target cells when the photosensitizer is excited by light in the presence of oxygen. This provides photodynamic therapy with many attractive advantages, such as a noninvasive nature, selective targeting, easy repeatability and great safety (16,17). Thus, photodynamic therapy may become a new modality in the management of periodontal disease.

It has been demonstrated that photodynamic therapy is effective in killing periodontal pathogens such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Streptococcus sanguis* (18–21). Recently, a study suggested that photodynamic therapy is also effective in suppressing a community of microorganisms in a biofilm (22). However, these studies were carried out on pure cultures of bacteria. In fact, the causative agents of periodontitis *in vivo* are present as an ecosystem, which includes hundreds of species of bacteria. Our previous *in vitro* study (23) showed that it is possible to achieve lethal photosensitization of multibacterial species from periodontal patients, but the therapeutic effect may be different from that obtained from cultured single-species bacteria. Before clinical application, it is necessary to evaluate further whether the *in vivo* photosensitization of bacterial flora in periodontitis is as effective as conventional therapy, and to ascertain that photodynamic therapy is not harmful to the host oral tissues. To our knowledge, few studies have been reported on this issue. In order to explore the effectiveness of photodynamic therapy in destroying periodontal bacteria *in vivo*, to compare its efficacy with that of routine scaling and root planing procedures, as well as to evaluate whether such photodynamic therapy treatment does not exert harmful effects on the host oral tissues, we carried out the present toluidine blue/photodynamic therapy experiment in rat periodontitis models. Toluidine blue was chosen as the photosensitizer in this study, because it has been shown to be potentially one of the safest photosensitizers for treating periodontal disease (18,24–27).

Material and methods

Animals

Sixteen healthy male Wistar rats (8–10 wk old; 200–230 g; The Animal Facilities, Forth Affiliated Hospital of Harbin Medical University, China) were used in this study. No inflammatory changes and/or plaque accumulation were observed in any rats before the experiment. During the experimental period, all the rats were housed in accordance with the regulations of the Home Office of the Chinese Government.

Infection procedures

The rats were anesthetized with inhalation of fluothane (FCI Pharmaceuticals, Cheshire, UK) and were placed in a supine position. For each rat, to gain easy access, the mouth was retracted using a mouth retractor to hold away the cheeks and the tongue. Ligatures (orthodontic elastics) were immediately submerged in the subgingival region around the first maxillary molar on each side of the rat mouth to induce periodontal inflammation through plaque accumulation. The clinical alterations were evaluated weekly until a steady state of infection was manifested at the end of week 6. The ligatures were removed and the rats were prepared for photodynamic therapy (photodynamic therapy group) or scaling and root planing (conventional treatment group).

Photosensitizer and laser illumination

Toluidine blue powder (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China) was dissolved in sterile saline, filtered though a 0.22-μm pore-size membrane and subsequently kept in the dark. The light source used was a diode laser (Sanyo Electric Co., Tokyo, Japan) with a maximum output of 0.26 W and a wavelength of 635 nm. The light was distributed by a fiberoptic applicator with a 0.8-mm cylindrical diffusing tip. Before laser irradiation, the output power from the fiber tip was adjusted to 61 mW and the irradiation area was adjusted to a diameter of 7 mm to obtain a light dose of 159 mW/cm^2. The laser output energy was carefully calibrated with a power meter (PS10; Coherent Inc., Santa Clara, CA, USA), and the size of the irradiation area was adjusted by changing the distance between the fiber tip and the irradiation surface.

Treatment

The 16 rats were randomly allocated into either the photodynamic therapy group or the conventional treatment group (eight rats in each group). In the photodynamic therapy group, the infection sites on one side of the mouth were treated by toluidine blue/photodynamic therapy. Before photodynamic therapy, the rats were anesthetized and the mouth retracted, as described above. The infection sites of each rat around the first maxillary molar region were soaked with 50 μL of toluidine blue (1 mg/mL) using a micropipette and left in the dark for 10 min. Then, the optical fiber was immediately placed in the midline of the palate and the area was exposed to

12 J/cm^2 of red light at 159 mW/cm^2 for 75 s. The untreated infection sites on the opposite side of the mouth were used as a control.

In the conventional treatment group, the rats were also anesthetized and the mouth retracted, as described above. The infection sites on one side of the mouth were treated by scaling and root planing, whereas the lesions on the opposite side of the mouth received neither photodynamic therapy nor conventional treatment and served as a control. Scaling and root planing were accomplished by using sickles to scrape the supragingival plaque and curettes to remove the subgingival plaque. Then, the inflammatory gingivae were cleaned by topical application of 0.5% chlorhexidine (Shanxi Dasheng Chemical Tech. Co. Ltd, Xi'an, China).

Microbial samples

One month after treatment, bacterial samples were taken from the tooth plaque around the infectious gingivae using a sickle and were immediately placed in 3 mL of sterile saline. Two-hundred microlitres of bacterial suspension was cultured on Luria–Bertani nutrient agar at 37°C for 24 h. The bacterial colonies growing on the agar were photographed and analyzed automatically with IMAGE PRO-PLUS 5.0 software (Media Cybernetics Inc., Silver Spring, MD, USA). All experiments were performed in triplicate and all measurements were performed in duplicate. Data were collected and the difference between the two groups was analyzed statistically using the *t*-test; *p*-values of < 0.05 were considered statistically significant.

Clinical and histological evaluation

After collection of the microbial samples, the following clinical parameters for the treated infection and control sites were measured by the same experienced operator (X. L. Luan): plaque index; gingival index; and bleeding on probing. Both plaque index and gingival index were evaluated according to the Löe and Silness method (28). Then, the rats were killed by cervical dislocation under inhalation anesthesia. The maxilla was removed and the gingivae around the first molar were photographed using a digital camera to record the changes of gross appearance (redness and edema).

Following clinical observation, the tissues around the first molar were stripped off and fixed immediately in 10% formalin for 72 h. The bone specimens were flushed in running water and then decalcified with nitric acid for 36–48 h. The specimens were embedded in paraffin, cut into sections and stained with hematoxylin and eosin for histological evaluation to examine inflammatory changes.

Results

Comparison of suppression of periodontal bacterial flora by photodynamic therapy and conventional treatment

Both photodynamic therapy and conventional treatment showed a pronounced bactericidal effect, and the survival of bacteria after treatment was 4% and 4.3%, respectively, compared with control sites ($p < 0.001$). There was no significant difference between the therapeutic effects of the two treatment modalities ($p > 0.05$) (Fig. 1).

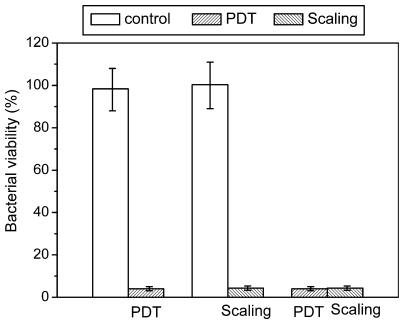

Fig. 1. Effect of photodynamic therapy and scaling treatment. The photodynamic therapy group received 1 mg/mL of toluidine blue and 12 J/cm^2 laser irradiation. Data represent mean values ($n = 8$) and error bars represent standard deviations of bacterial viability in individual rats ($n = 8$). PDT, photodynamic therapy.

Evaluation of the periodontal gross lesions

Comparison of the gross appearance of treated and untreated infected gingivae is shown in Fig. 2. Inflammatory signs, remarkable redness and edema were found in the control rats (Fig. 2A,C) and disappeared 1 mo later, after photodynamic therapy (Fig. 2B) and conventional treatment (Fig. 2D). The plaque index and gingival index values for the control groups were 3 and 2, respectively. Compared with the control groups, both therapy groups exhibited lower plaque index and gingival index values, of 1 and 1, respectively. For bleeding on probing, lower positive values were found for the therapy groups than for the corresponding control groups. These data indicated that a similar therapeutic result was obtained by photodynamic therapy and conventional scaling.

Histological evaluation of the inflammatory changes

In the periodontal gingiva, no serious destructive changes, such as hemorrhage and necrosis, were found in rats of either the control or treatment groups. The gingival epithelium was intact. Also, no damaging changes were observed in the maxillary bone structures in rats of control or treatment groups. The major pathological alteration observed in the rats of the control groups was widespread and extensive infiltration of inflammatory cells, mainly lymphocytes and plasma cells in the lamina propria of mucosa and subgingival connective tissue (Fig. 3A). Infiltration of inflammatory cells was greatly reduced after photodynamic therapy. The lamina propria of the mucosa was clear with very few infiltrating lymphocytes (Fig. 3B). A considerable reduction of inflammatory cell infiltration was also found after scaling and root planing treatment (Fig. 3C). Overall, the histological changes found in rats after photodynamic therapy and conventional treatment were similar, with no distinct differences observed.

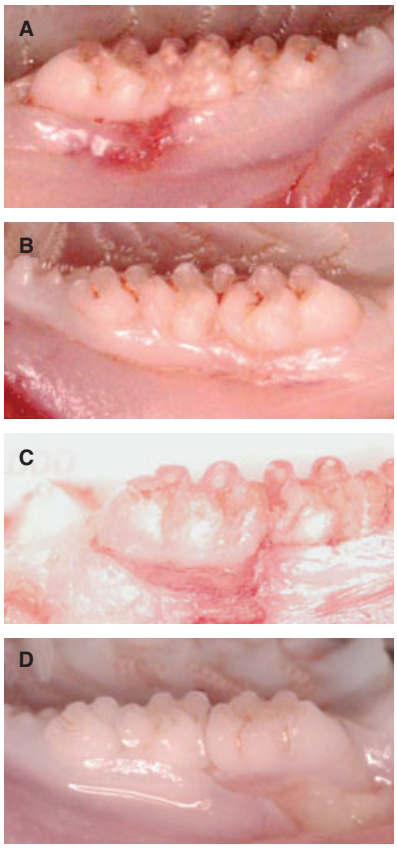

Fig. 2. Photographs of infected rat gingivae. (A) Control in the photodynamic therapy group; (B) 1 mo after photodynamic therapy; (C) control in the scaling treatment group; and (D) 1 mo after scaling treatment.

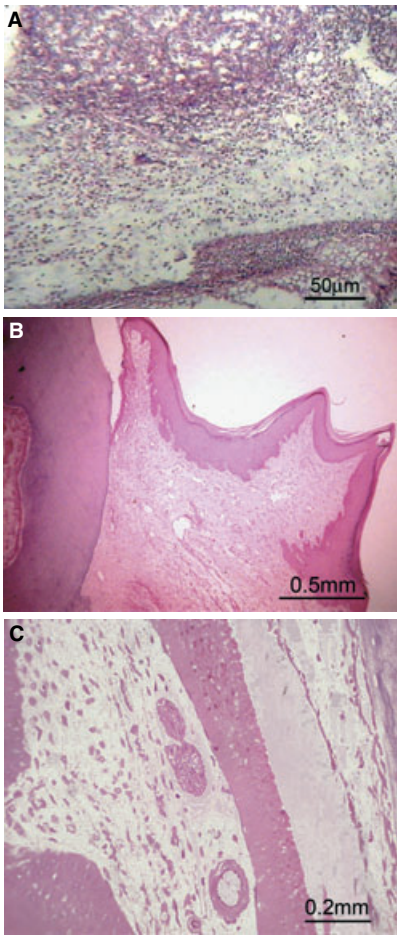

Fig. 3. Histological sections of periodontal structures of rats (A) without any treatment, (B) treated by photodynamic therapy and (C) treated by scaling and root planing.

Discussion

A wide range of bacterial species that could cause periodontal disease have been reported to be killed by toluidine blue and laser irradiation (18,25). However, most previous studies focused on the bactericidal effects of photodynamic therapy on pure cultures of bacteria (18–22). In fact, in the human oral cavity, there are hundreds of species of bacteria, which comprise a complex ecosystem. Thus, the response to photodynamic therapy of an entire *in vivo* bacterial community may differ greatly from that of their *in vitro* cultured isolates in many aspects, such as growth rate, metabolic activity and gene expression (29,30). To investigate the *in vivo* bactericidal effect of toluidine blue-mediated photodynamic therapy on the whole bacterial flora, we established a periodontal disease model in rats caused by natural infection rather than by a single specific species of bacteria, and carried out treatment under *in vivo* conditions, simulating clinical situations as closely as possible.

The results obtained in this study demonstrated that toluidine blue-mediated photodynamic therapy could cause lethal photosensitization of the whole bacterial flora *in vivo*. Meanwhile, clinical observations and histological examinations revealed a remarkable reduction of inflammatory reactions in the periodontal gingiva following toluidine blue-mediated photodynamic therapy, and no adverse effects were observed in adjacent tissues. These results further support the fact that toluidine blue-mediated photodynamic therapy may successfully suppress multispecies bacteria and preserve normal adjacent tissues without causing any adverse effects. Our findings are in accordance with those of Kömerik *et al.* (18), who reported that toluidine blue-mediated photodynamic therapy could kill *P. gingivalis* without destroying normal connective tissues. However, the study of Kömerik *et al.* was limited to pure cultures of bacteria. Furthermore, in their study, inflammation was not induced in the rat gingival tissues, which were used only for monitoring the effect of saliva and serum. As described above, the experimental models of the present study were used to analyze the therapeutic effect on multiple bacterial species, reflecting more closely the clinical situation, and provided useful new evidence on the effectiveness of photodynamic therapy for treating periodontal disease.

It should be noted that much higher doses of photosensitizer and light (1 mg/mL of toluidine blue combined with 12 J/cm^2 laser irradiation at 635 nm) were used in this study than in a previous study on pure cultures of bacteria (25 μg/mL of toluidine blue and 2.2 J/cm^2 laser) (26), because our preliminary *in vitro* study (23) showed that much higher doses of photosensitizer and light were required to obtain an optimum bactericidal effect when the whole periodontal bacterial flora taken from human dental plaques were treated, and that the best therapeutic effect was observed using 1 mg/mL of toluidine blue combined with 12 J/cm^2 laser irradiation at 635 nm. Consequently, those parameters were adopted in the present study to investigate the *in vivo* bactericidal effect on the whole bacterial community. It is encouraging to find that a prominent therapeutic effect was still obtained in rats by using higher doses of photosensitizer and light.

Recently, clinical applications of different lasers have been well documented. The Er:YAG laser (31) and the diode laser (32) have been reported to cause a significant bacteriocidal effect in addition to scaling. In a more recent study, Hayek *et al.* (33) demonstrated that compared with conventional

therapy, azulene-photodynamic therapy could successfully suppress *Prevotella* sp., *Fusobacterium* sp., and *S. beta-haemolyticus* in peri-implantitis. Similarly to the studies mentioned above, our study further showed that toluidine blue-mediated photodynamic therapy could be a potential adjuvant to conventional scaling and root planing.

In the present study, photodynamic therapy showed a therapeutic effect similar to that of scaling and root planing. In clinical situations, photodynamic therapy is expected to have more benefits than conventional therapy. For example, some sites, such as furcations, deep invaginations and concavities in the periodontal area, are difficult to access with hand instruments (7). The use of photodynamic therapy, however, is not affected by this problem, as it is based on photosensitizer and light irradiation and thus it can easily irradiate those inaccessible places. Another problem with conventional therapy is the increase of bacterial resistance to antibiotics, whereas photodynamic therapy, using reactive oxygen species to kill bacteria in a short time, is highly unlikely to cause bacterial resistance, such as that to antibiotics (34–37).

We conclude that toluidine blue-mediated photodynamic therapy is effective in the treatment of periodontitis *in vivo*. Compared with scaling and root planing, photodynamic therapy could cause lethal photoinactivation of periodontal pathogenic bacteria and complete reduction of inflammatory reactions in the periodontal gingivae with no detectable damage. These encouraging results suggest that further clinical investigations are worth undertaking.

Acknowledgements

This research work was jointly supported by the SIDA Asian-Swedish Research Partnership Program and the Important Science and Technology Plan Sponsored by Harbin Municipal Government. We are grateful to Mrs Chengyi Hu, the Forth Affiliated Hospital of Harbin Medical University, for her invaluable assistance in preparing the histological sections.

References

1. Slots J. Subgingival microflora and periodontal disease. *J Clin Periodontol* 1979;**6:**351–356.
2. Harper-Owen R, Dymock D, Booth V, Weightman AJ, Wade WG. Detection of unculturable bacteria in periodontal health and disease by PCR. *J Clin Microbiol* 1999;**37:**1469–1473.
3. Lino Y, Hopps RM. The bone-resorbing activities in tissue culture of lipopolysaccharides from the bacteria Actinobacillus actinomycetemcomitans, Bacteroides gingivalis and Capnocytophaga ochracea isolated from human mouths. *Arch Oral Biol* 1984;**29:**59–63.
4. Badersten A, Niveus R, Egelberg J. 4-year observations of basic periodontal therapy. *J Clin Periodontol* 1987;**14:**438–444.
5. Berkstein S, Reiff RL, McKinney JF, Killoy WJ. Supragingival root surface removal during maintenance procedures utilizing an air-powder abrasive system or hand scaling. An *in vitro* study. *J Periodontol* 1987;**58:**327–330.
6. Killoy WJ. Chemical treatment of periodontitis: local delivery of antimicrobials. *Int Dent J* 1998;**48:**305–315.
7. Wasserman B, Hirschfeld I. The relationship of initial clinical parameters to the long-term response in 112 cases of periodontal disease. *J Clin Periodontol* 1998; **15:**38–42.
8. Slots J, Rams TE. Antibiotics in periodontal therapy: advantages and disadvantages. *J Clin Periodontol* 1990;**17:**479–493.
9. Walker CB. The acquisition of antibiotic resistance in the periodontal microflora. *Periodontol 2000* 1996;**10:**79–88.
10. Tucker D, Cobb CM, Rapley JW, Killoy WJ. Morphologic changes following in vitro CO_2 laser treatment of calculus-ladened root surfaces. *Lasers Surg Med* 1996;**18:**150–156.
11. Romanos GE. Clinical applications of the Nd:YAG laser in oral soft tissue surgery and periodontology. *J Clin Laser Med Surg* 1994;**12:**103–108.
12. Israel M, Cobb CM, Rossmann JA, Spencer P. The effects of CO_2, Nd:YAG and Er:YAG lasers with and without surface coolant on tooth root surfaces. An in vitro study. *J Clin Periodontol* 1997; **24:**595–602.
13. Ando Y, Aoki A, Watanabe H, Ishikawa I. Bactericidal effect of erbium:YAG laser on periodontopathic bacteria. *Lasers Surg Med* 1996;**19:**190–200.
14. Aoki A, Ando Y, Watanabe H, Ishikawa I. *In vitro* studies on laser scaling of subgingival calculus with an erbium:YAG laser. *J Periodontol* 1994;**65:**1097–1106.
15. Moritz A, Gutknecht N, Doertbudak O *et al*. Bacterial reduction in periodontal pockets through irradiation with a diode laser: a pilot study. *J Clin Laser Med Surg* 1997;**15:**33–37.
16. Dougherty TJ, Gomer CJ, Henderson BW *et al*. Photodynamic therapy. *J Natl Cancer Inst* 1998;**90:**889–905.
17. Huang Z. A review of progress in clinical photodynamic therapy. *Technol Cancer Res Treat* 2005;**4:**283–293.
18. Kömerik N, Nakanishi H, MacRobert AJ, Henderson B, Speight P, Wilson M. *In vivo* killing of *Porphyromonas gingivalis* by toluidine blue-mediated photosensitization in an animal model. *Antimicrob Agents Chemother* 2003;**47:**932–940.
19. Chan Y, Lai CH. Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy. *Lasers Med Sci* 2003;**18:**51–55.
20. Wilson M, Burns T, Pratten J, Pearson GJ. Bacteria in supragingival plaque samples can be killed by low-power laser light in the presence of a photosensitizer. *J Appl Bacteriol* 1995;**78:**569–574.
21. Sarkar S, Wilson M. Lethal photosensitization of bacteria in subgingival plaque from patients with chronic periodontitis. *J Periodont Res* 1993;**28:**204–210.
22. Zanin IC, Goncalves RB, Junior AB, Hope CK, Pratten J. Susceptibility of Streptococcus mutans biofilms to photodynamic therapy: an *in vitro* study. *J Antimicrob Chemother* 2005;**56:**324–330.
23. Qin YL, Luan XL, Bi LJ *et al*. Toluidine blue-mediated photoinactivation of periodontal pathogens from supragingival plaques. *Lasers Med Sci* 2007;**87:**88–94.
24. Williams JA, Pearson GJ, Colles MJ, Wilson M. The effect of variable energy input from a novel light source on the photoactivated bactericidal action of toluidine blue O on Streptococcus mutans. *Caries Res* 2003;**37:**190–193.
25. Matevski D, Weersink R, Tenenbaum HC, Wilson B, Ellen RP, Lepine G. Lethal photosensitization of periodontal pathogens by a red-filtered Xenon lamp *in vitro*. *J Periodont Res* 2003;**38:**428–435.
26. Bhatti M, MacRobert A, Meghji S, Henderson B, Wilson M. Effect of dosimetric and physiological factors on the lethal photosensitization of *Porphyromonas gingivalis in vitro*. *Photochem Photobiol* 1997;**65:**1026–1031.
27. Bhatti M, MacRobert A, Meghji S, Henderson B, Wilson M. A study of the uptake of toluidine blue O by *Porphyromonas gingivalis and* the mechanism of lethal photosensitisation. *Photochem Photobiol* 1998;**68:**370–376.
28. Lőe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontol Scand* 1963;**21:**533–551.
29. Paster BJ, Boches SK, Galvin JL *et al*. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;**183:**3770–3783.

30. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999;**284:** 1318–1322.
31. Ishikawa I, Aoki A, Takasaki AA. Potential applications of Erbium:YAG laser in periodontics. *J Periodont Res* 2004;**39:**275–285.
32. Moritz A, Schoop U, Goharkhay K *et al*. Treatment of periodontal pockets with a diode laser. *Lasers Surg Med* 1998;**22:**302–311.
33. Hayek RRA, Araujo NS, Gioso MA *et al*. Comparative study between the effects of photodynamic therapy and conventional therapy on microbial reduction in ligature-induced peri-implantitis in dogs. *J Periodontol* 2005;**76:**1275–1281.
34. Wilson M. Bactericidal effect of laser light and its potential use in the treatment of plaque-related diseases. *Int Dent J* 1994; **44:**181–189.
35. Wilson M. Lethal photosensitisation of oral bacteria and its potential application in the photodynamic therapy of oral infections. *Photochem Photobiol Sci* 2004;**3:**412–418.
36. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *J Antimicrob Chemother* 1998;**42:**13–28.
37. Lauro FM, Pretto P, Covolo L, Jori G, Bertoloni G. Photoinactivation of bacterial strains involved in periodontal diseases sensitized by porphycene-polylysine conjugates. *Photochem Photobiol Sci* 2002; **1:**468–470.