

Antioxidant and pro-oxidant properties of chlorhexidine and its interaction with calcium hydroxide solutions

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Abstract

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Aim To evaluate the antioxidant and pro-oxidant properties of chlorhexidine (CHX).

Methodology The scavenging and generation of reactive oxygen species (ROS) by CHX in the presence or absence of saturated Ca(OH)₂ solutions was evaluated. The reaction emitted chemiluminescence in the presence of lucigenin thus was determined by a luminometer to evaluate the levels of ROS production. Changes in DNA conformation were analysed by agarose gel electrophoresis. Paired Student's *t*-test was used to compare the difference between groups.

Results Chlorhexidine (0.00002–0.02%) effectively scavenged 56–88% of the superoxide radicals generated by the xanthine/xanthine oxidase reaction. Through analysis of PUC18 DNA conformation chan-

ges, CHX was shown to be a mild scavenger of hydroxyl radicals generated by H₂O₂ plus FeCl₂. However, CHX (>0.083%) decreased the mobility of PUC18 plasmid DNA with potential production of DNA–DNA cross-link and severe DNA breaks (presence of DNA smear) at further higher concentrations. Furthermore, CHX induced ROS production including H₂O₂ and superoxide radicals in 0.1N NaOH (pH = 12.76) or Ca(OH)₂ (pH = 12.5) solutions.

Conclusion Chlorhexidine exhibited both antioxidant and pro-oxidant properties under different conditions. These events are possibly involved in the killing of root canal and periodontal microorganisms when CHX and Ca(OH)₂ were used in combination or separately. Potential genotoxicity and tissue damage when extruded into the periradicular tissue and at higher concentrations should be considered during periodontal and endodontic practice.

Keywords: Ca(OH)₂, chlorhexidine, DNA breakage, H₂O₂, reactive oxygen species.

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Introduction

Apical periodontitis is an ubiquitous disease mainly caused by root-canal infection. Without adequate endodontic treatment, infection of the root canal and periradicular tissue may result in loss of supporting

apical periodontium and increases the possibility of tooth extraction. Root-canal infection, which shows similar microbial pathogens to periodontal infection, is also seen linked to increased risk of coronary heart disease (Caplan *et al.* 2006, Joshipura *et al.* 2006), highlighting the important role of infection control in the oral cavity to promote systemic health.

Chlorhexidine (CHX, N,N1-Bis(4-chlorophenyl)-3,12-diimino-2,4,11,13, tetraaza tetradecadiimide) has been widely used as an antiseptic agent for

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routine dental plaque control and the irrigation of root canals as well as a medicament during root-canal treatment. CHX is an effective antimicrobial agent, which exhibits potent antimicrobial and anti-inflammatory effects. Rinsing twice daily with 0.2% CHX inhibits dental plaque formation (Loe & Schiott 1970). One epidemiological study has found that mouth rinsing with 0.12% CHX combined with perborate solution (which generates H₂O₂) resulted in more effective short-term plaque reduction than a rinse with CHX alone (Dona *et al.* 1998). An *ex vivo* study further supports the synergistic antibacterial effects of CHX and H₂O₂ against *Streptococci* species (Steinberg *et al.* 1996). In addition, a combination of Ca(OH)₂ and CHX is more effective in eradication of *Enterococcus faecalis* within dentine blocks (Zehnder *et al.* 2003) and in root canal retreatment cases (Zerella *et al.* 2005). It would be of interest to know the interaction between CHX and other antiseptics such as H₂O₂ and Ca(OH)₂ that are used in periodontal and endodontic treatment.

Though reactive oxygen species (ROS) production by neutrophils have recently been shown to play a critical role in the host-defence response against invading microorganisms, these ROS are also critical in the pathogenesis of pulpal and periodontal disease (Marton *et al.* 1993, Chapple 1997, Alacam *et al.* 2000). Human leucocytes may generate excessive amounts of ROS such as the hydroxyl radical, the superoxide radical, H₂O₂ and hypochlorite in inflammatory tissue (Nakamura *et al.* 1998, Yasunari *et al.* 2006). ROS can kill bacteria but may also destroy the adjacent infected host tissues. The inhibitory effect on 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) cation (ABTS⁺) radical production by a number of antiseptic mouth rinses including CHX, has been elucidated (Battino *et al.* 2002). Moreover, recent reports suggested CHX is a potential genotoxic agent toward leucocytes, oral mucosal cells and lymphocytes (Eren *et al.* 2002, Ribeiro *et al.* 2005). The purposes of this study were (i) to evaluate the anti-oxidative and pro-oxidative properties of CHX and its interaction with DNA to evaluate the safety of its use in dental treatment, (ii) whether interactions by CHX and Ca(OH)₂ may generate toxic ROS, which may have antimicrobial effects.

Materials and methods

Materials

Lucigenin, H₂O₂, FeCl₂, xanthine, xanthine oxidase and CHX were obtained from Sigma (St Louis, MO,

USA.). Ethidium bromide and agarose were purchased from HT Biotechnology (Cambridge, UK). PUC18 plasmid DNA was purchased from Bayou Biolab (Los Angeles, CA, USA).

Scavenging of superoxide radicals by CHX

Superoxide radicals were generated by the reaction of xanthine with xanthine oxidase as described previously (Chang *et al.* 2002, Jeng *et al.* 2002, Yeung *et al.* 2002). Briefly, superoxide radicals were produced in a reaction mixture containing 150 µL of water, 60 µL of 1 mmol L⁻¹ lucigenin and various concentrations of CHX (5 µL, final concentration = 0.00002–0.02%). Aliquots of 10 µL of xanthine oxidase (0.2 U mL⁻¹) were added into each well. The reaction was started by injection of 10 µL of xanthine (3.33 mmol L⁻¹) and the lucigenin chemiluminescence was measured immediately by a microplate luminometer (Orion Microplate Luminometer; Berthold DS, Tforzheim, Germany). The level of emitted chemiluminescence during 30 s of measurement was recorded and averaged for comparison.

Effects of CHX and its interaction with H₂O₂ or hydroxyl radicals on DNA conformation

The reaction was conducted in an Eppendorf tube at a total volume of 30 µL containing 5 µL of 50 mmol L⁻¹ Tris buffer (pH 7.5), 5 µL of PUC18 plasmid DNA (5 µg) and 5 µL of CHX with or without subsequent addition of 5 µL of 30% H₂O₂ (final 1%) or H₂O₂ plus FeCl₂ (final 45 µmol L⁻¹) and incubated for further 30 min. The reaction mixture was then subjected to 0.8% agarose gel electrophoresis using a Mupid-2 electrophoresis apparatus run at 100 V. DNA bands were stained with ethidium bromide, visualized and photographed with Alpha Image IS-3300 (Alpha Innotech Corp., San Leandro, CA, USA) (Jeng *et al.* 2002).

ROS production by interaction of CHX with 0.1 N NaOH or saturated Ca(OH)₂ solution

A reaction mixture (245 µL in volume) was prepared comprising 150 µL water, 60 µL 1 mmol L⁻¹ lucigenin and various concentrations of CHX (final 0.00004–0.04%). The reaction was started by auto-injection of 10 µL 0.1 N NaOH (final pH 12.76). The changes in lucigenin chemiluminescence were detected using a Microplate Luminometer as described previously (Chang *et al.* 2002, Jeng *et al.* 2002, Yeung *et al.*

2002). The nature of specific ROS production was confirmed by addition of superoxide dismutase (SOD) and catalase. In addition, 0.5 g Ca(OH)₂ was dissolved in 50 mL double distilled-water with agitation for 1 h and centrifuged at 2000 rpm to give a saturated Ca(OH)₂ solution. The solution was filtrated through Whatman filter papers and the pH value was measured to be 12.5. A reaction mixture (215 µL) was prepared comprising 60 µL 1 mmol L⁻¹ lucigenin and various concentrations of CHX (with final concentrations ranging from 0.00002% to 0.02%). The reaction was started by injection of 150 µL saturated Ca(OH)₂ solution. The lucigenin chemiluminescence was measured by a microplate luminometer for 30 s as previously described. The level of emitted chemiluminescence over this period was recorded and averaged. SOD or catalase was added to clarify the nature of ROS through the experiment.

Statistical analysis

The ROS-induced lucigenin chemiluminescence was recorded as relative light units (RLU). In some experiments, the RLU values of the experimental groups were divided by the RLU value of the untreated solvent control (NaOH or saturated Ca(OH)₂ solution) and data presented as a percentage of the control. Paired Student's *t*-test was used to compare the difference between groups. A *P*-value <0.05 was considered to show significant difference between groups.

Results

Chlorhexidine was an effective superoxide radical scavenger

Reaction by xanthine and xanthine oxidase may generate superoxide radicals as revealed by an increase in lucigenin chemiluminescence for 30 s (Fig. 1a). The RLUs increased rapidly within the first 6 s and increased further during the incubation period. In the presence of CHX (0.00002–0.02%), superoxide-induced lucigenin chemiluminescence was inhibited by 56–88% (Fig. 1b).

Interaction of CHX with DNA in the presence of absence of H₂O₂ with/without FeCl₂

Commercially available PUC18 DNA was generally in supercoil form (form I) in conformation. Mild DNA breaks will change the conformation of supercoil form

of DNA to become the nick-relaxed form (form II). When DNA damage was severe, double DNA breaks will lead to the linear form (form III) and even DNA smear with marked fragmentation (Zhang *et al.* 2004). Cross-links of DNA may retard the mobility of DNA in agarose gels (Chen *et al.* 1994).

Incubation with CHX (0.083–0.17%) led to DNA breaks with formation of form II DNA and inhibition of the migration of DNA as revealed by high molecular weight DNA, suggesting the presence of DNA conformation changes or DNA–DNA crosslinks (Fig. 2a). At concentrations higher than 0.17%, CHX caused DNA breaks with loss of all DNA image in agarose gels (data not shown). H₂O₂ (1%) alone showed little effect on DNA conformation. No obvious change in DNA conformation was seen with a combination of CHX and H₂O₂ (1%) (Fig. 2b). In the presence of transition metals such as Fe²⁺, H₂O₂ may generate potent hydroxyl radicals leading to DNA breaks (Leonard *et al.* 2000). Using this model system, it was found that the reaction of H₂O₂/FeCl₂ with DNA led to severe DNA breaks as indicated by loss of all DNA images (Fig. 2c, lane 4). Catalase, which may degrade H₂O₂, effectively prevented the H₂O₂/FeCl₂-induced DNA breaks (Fig. 2c, lane 11). CHX (0.02–0.09%) partially prevented the H₂O₂/FeCl₂-induced DNA breaks (Fig. 2c, lane 6–9). However, CHX at a concentration of 1.8% had little preventive effect, possibly because of the DNA-breaking effect of CHX itself.

ROS production by reaction of CHX with NaOH solution

Chlorhexidine may induce ROS production in the alkaline environments (0.1 N NaOH, pH = 12.7) with a biphasic response (Fig. 3a). CHX increased the ROS production by 7.3- to 18.9-folds at concentrations of 0.0004% and 0.02% in the alkaline environment. Interestingly, catalase (5 µL, 100 U µL⁻¹) and SOD (10 µL, 50 U µL⁻¹) effectively prevented CHX-mediated chemiluminescence in the alkaline environment (Fig. 3b).

ROS production by reaction of CHX with saturated Ca(OH)₂ solution

Under similar experimental conditions, it was found that CHX also induced excessive lucigenin chemiluminescence in the presence of Ca(OH)₂ which increases by 78.7- and 32-fold at concentrations of 0.001% and 0.02%, relative to the solvent control (Fig. 4a).

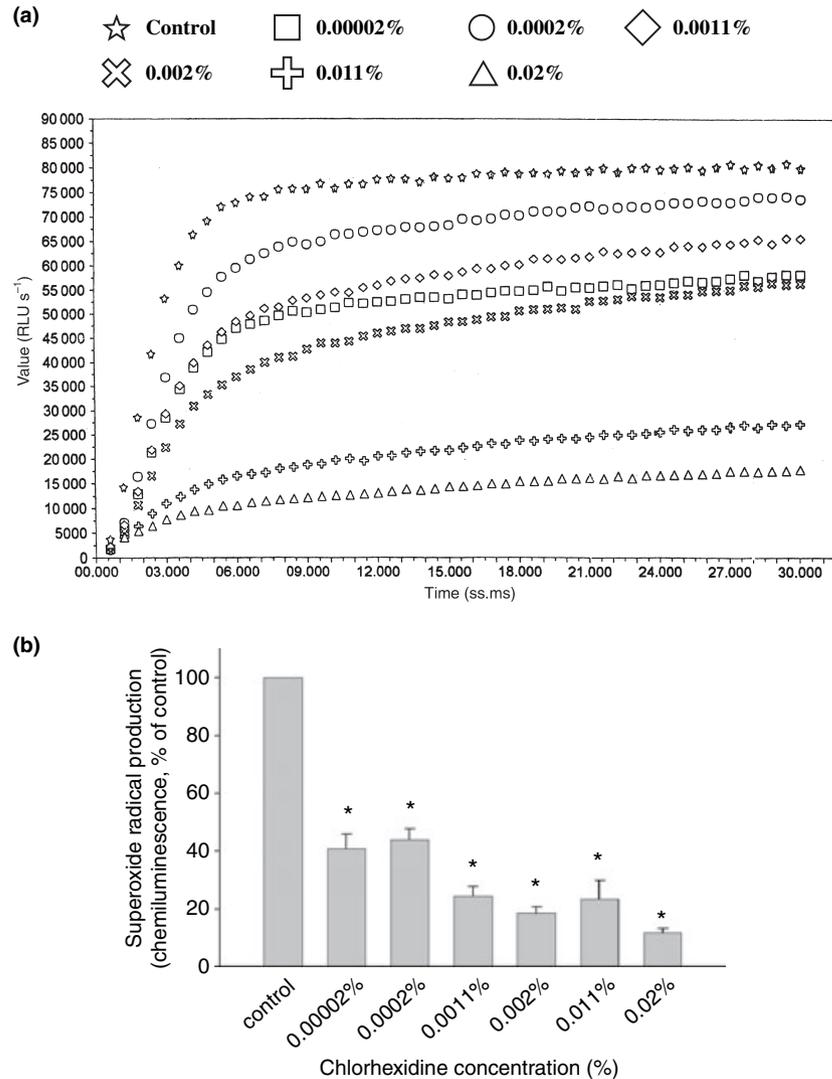


Figure 1 Scavenging properties of chlorhexidine (CHX) towards superoxide radicals generated by xanthine and xanthine oxidase. (a) One representative picture of superoxide-mediated chemiluminescence production (relative light unit) and its prevention by various concentrations of CHX (final 0.00002–0.02%). (b) Quantitative analysis of chemiluminescence generated by xanthine and xanthine oxidase (as 100%) and its inhibition by various concentrations of CHX. Results are expressed as a percentage of control (Mean ± SE, n = 14). *denotes a significant difference when compared with the control (P < 0.05).

Catalase and SOD effectively prevented the CHX-generated chemiluminescence in saturated Ca(OH)₂ solution (Fig. 4b).

Discussion

Chlorhexidine is a large dicationic molecule, 1,6-di(4-chlorophenyl-diguanido)-hexane and is a commonly-used antiseptic agent for wound sterilization and disinfection against various microorganisms. It is also

widely used as a mouthrinse solution to control dental plaque formation and as an endodontic irrigant or medicament to reduce the root-canal microbial load. Periodontal and periradicular disease, as infectious diseases with inflammatory cell infiltration, may generate various kind of ROS, which may destroy adjacent tissues (Nakamura *et al.* 1998, Yasunari *et al.* 2006). A decrease in total antioxidant capacity and glutathione level in gingival crevicular fluid of periodontitis patients has been reported (Chapple *et al.* 2002, Brock *et al.*

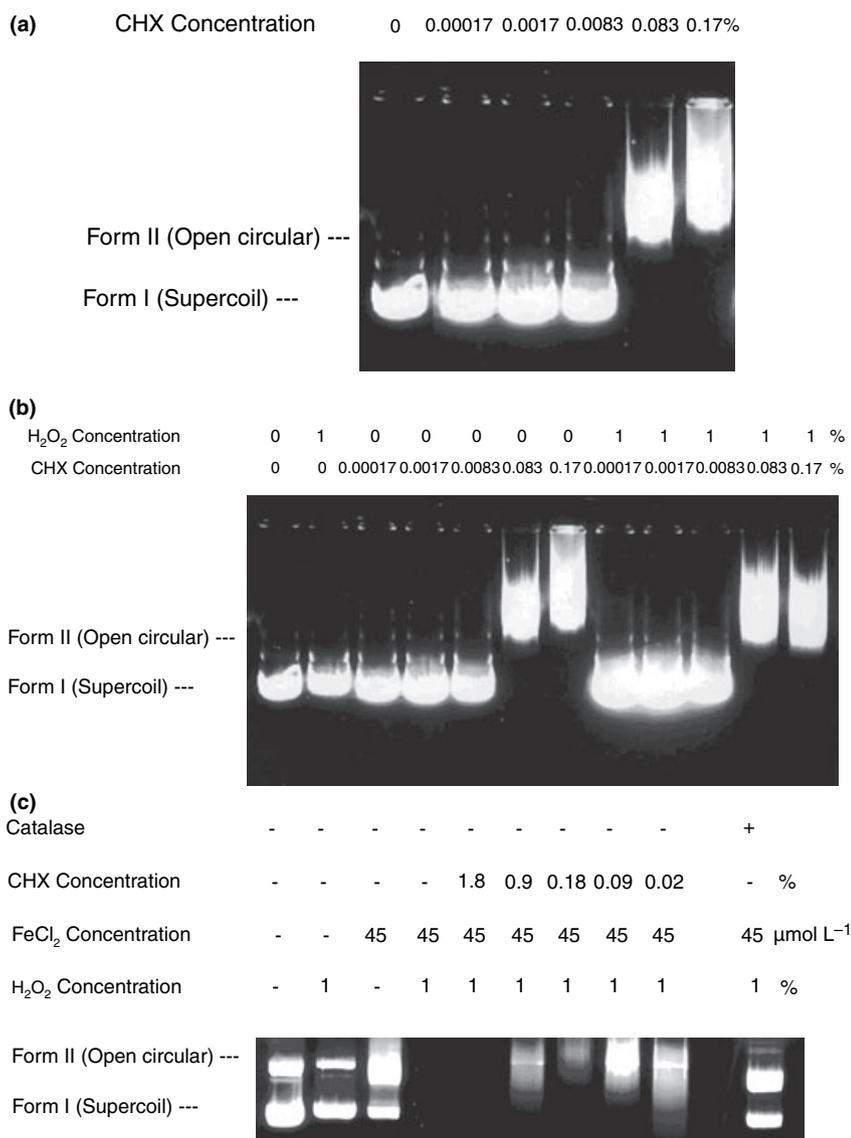


Figure 2 Interaction of chlorhexidine (CHX) with DNA in the presence or absence of 1% H₂O₂ and/or FeCl₂. (a) Interaction of various concentrations of CHX with PUC18 plasmid DNA. (b) Interaction of CHX with/without H₂O₂ on DNA conformation. (c) Interaction of CHX with DNA in the presence or absence of H₂O₂ and FeCl₂ (fenton reaction) to generate hydroxyl radicals. Catalase, which can degrade H₂O₂ was used as the control.

2004), suggesting enhanced ROS production in diseased sites. ROS is important in pathogenesis of pulpal and periodontal diseases and thus the development of ROS scavengers is important for disease control and prevention.

Chlorhexidine showed little scavenging effect toward the H₂O₂-induced DNA damage as analysed by comet assay (Battino *et al.* 2002). Beside its antiseptic effect, nontoxic CHX (0.1–1 μg mL⁻¹) was found to inhibit

superoxide radical production by neutrophils (Goultschin & Levy 1986). This event may be due to scavenging of superoxide radicals or inhibition of NADPH oxidase, which mediates neutrophil ROS production. In the present *ex vivo* experiments, it was found that CHX may effectively scavenge the superoxide radical and exhibits some antioxidant property to hydroxyl radicals. These results suggest CHX exhibits antioxidant properties and might have some preventive

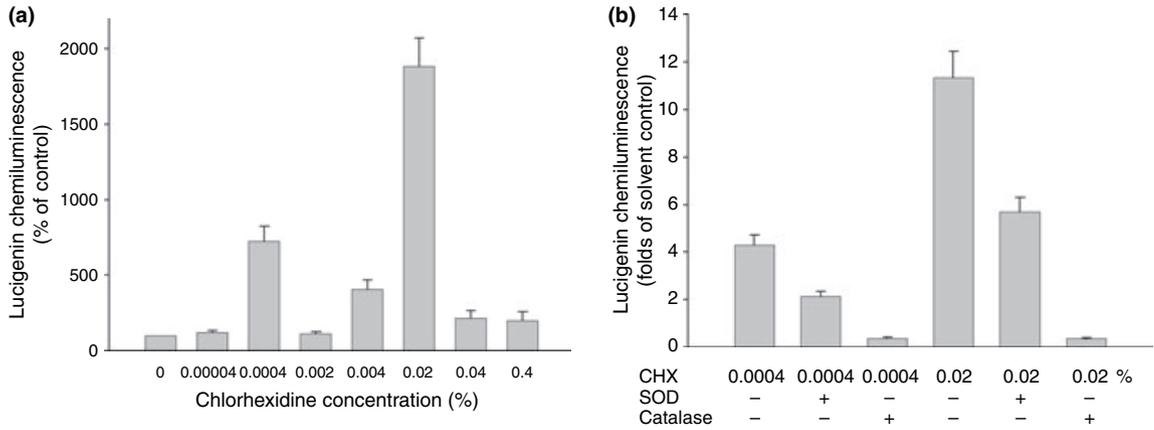


Figure 3 (a) Chlorhexidine (CHX) may generate reactive oxygen species (ROS) production in the alkaline environment (0.1 N NaOH, pH = 12.76), especially at a final concentration of 0.02% ($n = 5$). (b) The ROS production by CHX in 0.1 N NaOH solution may be scavenged by catalase and superoxide dismutase respectively ($n = 4$).

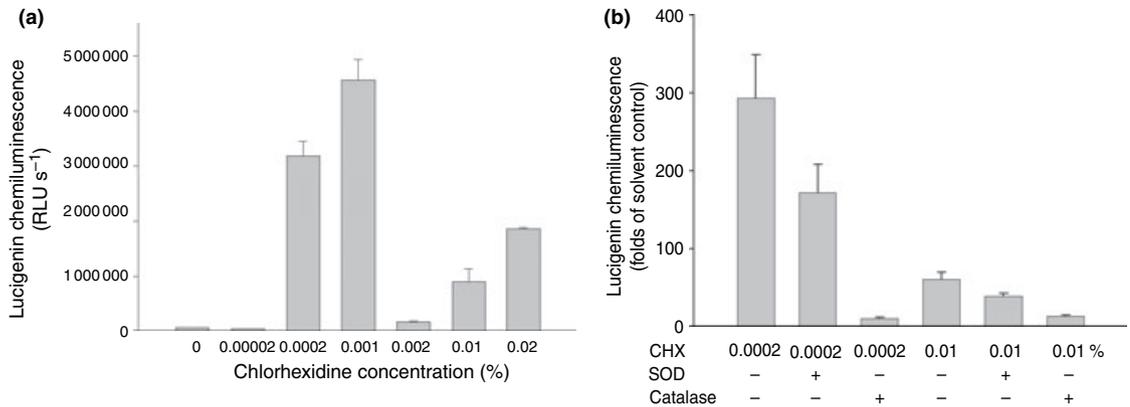


Figure 4 (a) Chlorhexidine (CHX) produced excessive reactive oxygen species (ROS) in the saturated Ca(OH)₂ solution (pH = 12.5), especially at final concentrations ranging from 0.0002% to 0.001% ($n = 5$). (b) The ROS produced by CHX in saturated Ca(OH)₂ solution can be scavenged by catalase and superoxide dismutase respectively ($n = 6$).

effect on inflammatory periodontal and periapical destruction.

Chlorhexidine is unable to induce liberation of reactive oxygen/nitrogen intermediates in murine macrophages (Bonacorsi *et al.* 2004). However, in the present *ex vivo* experiments, it was found that CHX may induce ROS production in the alkaline environment (0.1 N NaOH or saturated Ca(OH)₂ solution) with a biphasic response. The precise reason for the biphasic ROS generation by CHX is not clear. The chemical structure of CHX (N,N1-Bis(4-chlorophenyl)-3,12-dimino-2,4,11,13, tetraazatetra decadediimidamide) contains two chlorophenyl guanide moieties at both ends, which are separated by a long-chain aliphatic nitrogenated structure. Whether different structural moiet-

ies in the chemical structure of CHX are responsible for its antioxidant and pro-oxidant properties should be further addressed. Clinically, Ca(OH)₂ is often used as an intracanal medicament. Combined use of CHX and Ca(OH)₂ in the root canal may generate excessive ROS, which may potentially kill various root-canal pathogens. This may partly explain why the combined use of Ca(OH)₂/CHX mixture shows more efficacy in eliminating endotoxin and for endodontic retreatment of failed cases associated with *E. faecalis* infection (Buck *et al.* 2001, Zehnder *et al.* 2003, Zerella *et al.* 2005). The precise reasons should be further addressed. Interaction of CHX and Ca(OH)₂ may generate ROS, which can be inhibited by SOD and catalase, indicating the produced ROS are H₂O₂ and superoxide radicals.

This ROS production may possibly destroy the cell wall and membrane structure of microorganisms.

Previous reports indicate that CHX does not induce chromosome aberrations in Syrian hamster embryo cells (Yamaguchi & Tsutsui 2003, Hikiba *et al.* 2005) and genotoxicity toward Chinese hamster ovary cells as analysed by Comet assay (Ribeiro *et al.* 2004), whereas H₂O₂ and NaOCl may stimulate transformation of Syrian hamster embryo cells (Yamaguchi & Tsutsui 2003). Another report indicates that CHX is able to induce primary DNA damage in leucocytes and in oral mucosal cells (Ribeiro *et al.* 2004) and was cytotoxic to neutrophils, epithelial cells and red blood cells (Heyden *et al.* 1971, Gabler *et al.* 1987). CHX also causes membrane damage to neutrophils and macrophages with the release of intracellular enzymes (Kenney *et al.* 1972, Knuuttila & Soderling 1981). The effect of CHX on DNA conformation and structure was therefore studied under different experimental conditions. In the present study, CHX may have induced DNA conformation changes or DNA–DNA cross-links. CHX further induced obvious DNA breaks at higher concentrations as indicated by loss of all DNA bands after exposure. The routine concentration of CHX solution for oral rinsing is about 0.2% and is above the concentration that may not lead to DNA smearing *ex vivo*. Further clinical studies are needed to evaluate the micronuclei or chromosomal aberrations in individuals using CHX for routine mouth rinsing, because a daily oral rinse with CHX for 18 days has shown to significantly induce DNA strand breaks of peripheral blood lymphocytes and buccal epithelial cells (Eren *et al.* 2002).

The present results indicate that CHX exhibits both antioxidant and pro-oxidant properties in different experimental conditions, possibly due to different structural moiety. These may partly explain why CHX acts as an effective dental chemical adjunct in routine periodontal plaque control and as an irrigant and medicament for root-canal disinfection. However, higher concentrations of CHX may generate DNA breaks, DNA conformation changes as well as potential DNA–DNA cross links. Reaction of CHX with 0.1 N NaOH or Ca(OH)₂ may induce massive production of ROS, which may be useful for specific root-canal disinfection. Concentrations of 0.1–0.2% of CHX are used clinically for endodontic and periodontal treatments, this is higher than the concentrations tested in this study. As pulpal infection and medication may affect the healing of the marginal periodontium (Blomlof *et al.* 1988, 1992), more studies are needed to evaluate the effects of combination of root-canal medicaments such

as CHX, Ca(OH)₂ and others on the root-canal microorganisms and periradicular tissue.

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