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The Effect of a Commercially Available Chlorhexidine Mouthwash Product on Human Osteoblast Cells

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Abstract *- Chlorhexidine mouthwash (CMW) is used for decontamination of tooth, implant or prosthetic surfaces to treat or prevent local infection. A cell culture model was used to investigate cytotoxicity of CMW employing an MTT assay to record cell activity. Human osteoblast-like cells (HOS TE 85) were seeded. Dilutions of CMW (1:1 to 1:128) were made up with culture medium. Positive and negative controls were prepared. Cells were incubated, exposed to CMW, for 5 min to 4 h. Diluted tetrazolium salt solution was added. Plates were incubated for a further 4 h. Medium was removed, dimethylsulphoxide was added, and absorbance at 570nm read. Undiluted CMW caused total cytotoxicity, similar to positive control. Progessive dilution of CMW was associated with elevated cell survival. Cytotoxicity increased with longer time exposures. It was concluded that CMW can be cytotoxic in high concentrations and when applied for long time periods. Work is needed to determine effects on other cell types and clinical significance of these findings.*

KEYWORDS: Chlorhexidine, Cytotoxicity, Human, Osteoblast cells

INTRODUCTION

Chlorhexidine digluconate has been used by the dental profession for over 25 years. It is a symmetrical cationic molecule containing two 4-chlorophenyl rings and two biguanide groups connected by a central hexmethylene chain [1]. This well recognised antimicrobial agent has a wide spectrum of activity, encompassing gram-positive and gram-negative bacteria [2], yeasts and fungi [3], and some lipophilic viruses [4].

The antimicrobial agent has a membrane-active type of activity, which damages the inner (cytoplasmic) membrane. The antibacterial mode of action is thought to be as follows: the cationic chlorhexidine molecule is rapidly attracted to the negatively charged bacterial cell surface, with specific and strong adsorption to phosphate-containing compounds. This alters the integrity of the bacterial cell membrane and allows the chlorhexidine access to the inner membrane; here it binds with the phospholipids and leads to an increased permeability of the inner membrane and leakage of low molecular weight components, such as potassium ions.

At this stage the effects of chlorhexidine are reversible; the removal of chlorhexidine by neutralisers will allow the bacterial cell to recover [5]. Lesser damage is caused to the cell membrane by low levels of chlorhexidine, but as the concentration increases, greater damage to the cell membrane is observed, since higher molecular weight components are lost from the cell. Thus, at low concentrations chlorhexidine is bacteriostatic and at higher concentrations it is rapidly bactericidal [4]. The properties of chlorhexidine have led it to be considered a gold standard among chemical anti-plaque agents [5] and to have a range of proposed uses.

Despite the vast progress in treating patients with implants, maintaining healthy peri-implant tissues remains a challenge for some patients. It is generally accepted that if osseointegration is to be achieved, with minimum infectious complications, a strict approach to minimise bacterial contamination should be used during the insertion of endosseous oral implants [6,7,8]. There is general agreement that good oral hygiene and maintenance is essential in preventing local problems because implants, like teeth, can accumulate plaque and calculus [9]. The term "peri-implantitis" was introduced to describe a destructive inflammatory process affecting the soft and hard tissues around osseointegrated implants, leading to the formation of a peri-implant pocket and loss of supporting bone. Peri-implantitis usually produces a saucer shaped defect which is well demarcated. The bottom of the implant retains osseointegration as it is not affected, thus allowing bone destruction to proceed without any signs of implant mobility until osseointegration is completely lost [10]. The main aims of therapy for this condition are to remove any bacterial biofilm and associated toxins, to eliminate local inflammation and if possible to regain osseointegration between implant and bone.

Numerous methods have been used to clean the implant surface, including topical applications of saline, of chlorhexidine, or of citric acid, and use of carbon dioxide (CO_2) laser or air-powder abrasion. However, no consensus has been reached about the most adequate method [11].

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Contaminants including bacteria, bacterial products, calculus and soft tissue cells should be removed with or without modifying the implant surface or compromising the healing potential of the peri-implant tissues [12]. The pre-operative use of chlorhexidine rinse has been shown to be effective in reducing the incidence of oral infections associated with dental implants and to improve implant success rate [7,8]. Some operators may also bathe prosthetic implant components in chlorhexidine solutions during removal and replacement when prostheses are undergoing maintenance or repair.

Despite the wide use of chlorhexidine for impairing plaque formation, controlling gingivitis and disinfecting root canals, evidence of its genotoxicity is fairly consistent. A review of literature has shown chlorhexidine to be harmful to a number of mammalian cells at doses similar to or below those introduced into the oral cavity; for example sperm [13], polymorphonuclear leukocytes [14], macrophages [15, 16], epithelial cells [17 - 22], erythrocytes [17] and gingival and dermal fibroblasts [18-21]. In addition chlorhexidine application can reduce production of collagen and other proteins by gingival fibroblasts at sub-cytotoxic levels, implying that it may influence wound healing [22-24].

Chlorhexidine has been shown to be highly cytotoxic to periodontal ligament cells, inhibiting double-stranded nucleic acid content, protein synthesis and mitochondrial activity [25]. Increased DNA damage in buccal mucosal cells was detected by a single cell gel assay in individuals that rinsed their mouths with chlorhexidine [1]. Genetic mutations were also induced by the breakdown products of chlorhexidine in micro-organisms [26]. It has been proposed that chlorhexidine may have a general inhibitory effect on cells or it may specifically affect protein biosynthesis [25].

However, most of these studies relate to preparations of chlorhexidine in an aqueous vehicle. This agent is not however available alone: widely used commercially available formulations include a number of other components including ethanol, polyoxyl hydrogenated castor oil, peppermint oil, Ponceau 4R (E124, a synthetic red azo dye) and sorbitol. These may act to potentiate or reduce both the antibacterial effect and the cytotoxicity of the complete product. In this setting the chlorhexidine is diluted by the other agents, and although ethanol is known to be cytotoxic, there are little data available in the literature regarding the toxicity of the other agents in question when applied at the concentrations seen in undiluted or diluted mouthwashes. As a result, it is important to investigate if chlorhexidine mouthwashes may have deleterious effects if used in such formulations, since this would relate to their clinical application. Eren *et al* [1] have reported DNA damage caused to buccal epithelial cells after rinsing with chlorhexidine mouthwash but did not investigate which mouthwash elements were responsible, or the phenotypic effects of such damage.

The ultimate goal of using chlorhexidine in periodontology and implantology is as a disinfectant; whether it is as an adjunct in the treatment of peri-implantitis, when attempting to regain osseointegration, or its use pre-operatively, in reducing the incidence of oral infections associated with dental implants [8]. The objective of this investigation was to evaluate the cytotoxicity of chlorhexidine mouthwash over a range of concentrations and exposure time using a cell culture model. We were primarily interested in the effect of the whole product, ie a widely used chlorhexidine mouthwash in the UK, as opposed to the potential cytotoxic effect of chlorhexidine alone (as was carried out by Chang *et al* in 2001 [25].

MATERIALS AND METHODS

The tests in this study were carried out according to the guidelines of the International Standard (ISO 10993 - Biological evaluation of medical devices – Part 5: Tests for cytotoxicity *in vitro* methods) [27].

A human osteoblast-like cell line (HOS TE85, ECACC No. 87070202) was used for the study. Numerous *in vitro* models using human, animal, non-transformed and transformed cells have been described for studying biocompatibility. For example, primary cultures of osteoblast cells are advantageous for studies of bone cell metabolism and differentiation because they have not been transformed and retain a normal genotype [27-29]. However, human transformed osteoblast-like cell lines such as MG-63, HOS TE-85, are readily available and widely used as representative cell models. Although differences have been noted within species and between transformed and non-transformed cells [30-32], cell lines provide a readily available, safe source, of cells for screening studies such as cytotoxicity testing. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% Foetal Calf Serum, 0.02M HEPES, [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 2mM L-Glutamine, 1% Penicillin/streptomycin (all Life technologies) and 150µg/ml Ascorbate (HOS medium). Cells were grown to confluence at 37ºC with 5% CO_2 in a humidified atmosphere and were trypsinised using Trypsin (Sigma) 0.02% in Phosphate Buffered Saline (PBS), (Sigma) with HEPES (Life technologies). The cells were resuspended in DMEM (10% FCS) and seeded at a density of 1x 10^4/ml into each well 96 well plates. Four plates were set up for the different time periods studied.

Sequential dilutions of sterilised chlorhexidine mouthwash (Corsodyl, 0.2%) from 1:1 to 1:128 were made up to 100 µl with HOS cell culture medium. Controls were also prepared, the negative control was HOS medium alone and the positive control was 30% alcohol.

The HOS cell media was then tipped out of the 4 plates with care to prevent contamination of the plates, the different dilutions of mouthwash were added to the HOS cells on each of the 96 well plates. The negative control comprised HOS cells with culture media alone, and the test control was neat mouthwash with no HOS cell and the positive control was 30% alcohol. The test samples and controls were incubated at 37°C with 5% CO_2 for the experimental time periods of 5 min, 10 min, 1h, 2h and 4h. The MTT assay was then carried out to determine cell metabolic activity in the presence of the test samples. 100µl of DMEM medium (without ascorbate) was added to each well with 10µl of MTT at a concentration of 5mg/ml giving a final concentration of the MTT of 5µg/ml. The ascorbate was removed from the medium because it interferes with the assay. The plates were incubated for 4 hours and the contents removed by inverting the plate. The crystals were solubilised with 100µl of DMSO (Sigma) and shaken gently for 5 min until all the crystals had dissolved. Absorbance

was measured on a Dynatech MR700 micro plate reader at test wavelength: 570nm and reference wavelength: 630nm. This protocol was repeated for a total of 12 sets of wells and plates.

Statistical methods

Collected data was analysed using Stata 8 for PC (Stata Corporation, College Station, Texas). Mean absorbance value was calculated for each dilution (n=12) and for all five time periods. Frequency distributions were determined for all variables. The variables could not all be satisfactorily transformed to a normal distribution by simple arithmetic functions, and some were handled as categorical data. Data were compared between different exposure times and concentrations using analysis of variance and linear regression, to identify the effect of these factors on cell survival after exposure.

RESULTS

Table 1 shows the relative absorbencies for all the test samples and controls. Figure 1 shows the effect of time and dilution on cytotoxicity of the mouthwash solution on HOS cell activity. The results show that both the concentration of mouthwash and the exposure time have an effect on cell metabolic activity. As the concentration of mouthwash decreased, an increase in metabolic activity of the cells was observed. This trend was observed for all dilutions of mouthwash tested. Cells exposed for 5 minutes had significantly lower activity (p less than or equal to 0.024) than those exposed for 10 minutes at each dilution with the exception of 1/8 dilution (p=0.24). However, it can be seen that with each subsequent sequential dilution the cell activity increases until dilutions of 1:128 gave results similar to negative control samples. Exposure to undiluted chlorhexidine mouthwash caused total cytotoxicity, similar to positive control samples. Cytotoxicity was seen to increase with an increase in exposure times. It may also be seen that a 4 hour exposure curtailed most cell activity regardless of dilution.

These differences were statistically significant for different dilutions and different exposure times (after exclusion of controls): one way ANOVA for variations in dilutions for standardised exposure time and one way ANOVA for variations in exposure time for each dilution both showed statistically significant associations with absorbance output and hence cell vitality ($p<0.001$). Linear regression analysis of absorbance readings by dilution and exposure was also carried out after exclusion of controls and confirmed these findings ($p<0.0001$, $r^2=0.34$). Further analysis using t-tests revealed that there were differences in cell vitality between exposures of 5 and 60 minutes at dilutions of 1/16 ($p<0.0001$), 1/32 ($p=0.0826$), 1/64 ($p=0.0115$), and between exposures of 5 min and two hours for these dilutions ($p<0.0001$, $p<0.0001$, $p=0.0001$, $p<0.001$ respectively) and between 1 and 2 hours for 1/128 dilution ($p=0.0002$). The pattern of results illustrated in Figure 1 was assessed and t-testing suggested that significant reductions in cellular vitality occurred at all concentrations for a 5 minute exposure ($p\leq0.0043$ for each concentration compared to negative control). For exposures of 5 minutes, progressive significant increases in cell survival occurred as dilutions increased from 1/8 to 1/128 and to negative control ($p\leq0.033$, with the exception of 1/64 vs 1/128 dilutions, where $p=0.084$). Similar changes were seen for progressive dilutions at 10 minute exposures ($p\leq0.0041$). For exposures of 1 and 2 hours, progressive dilutions gave significant increases in cell survival only as dilutions increased from 1/16 to 1/128 and to negative control ($p\leq0.0471$).

Table 1. *Variations in mean absorbance of HOS cells with changes in exposure time and dilution of chlorhexidine*

Dilution	*1*		*1/4*		*1/8*		*1/16*		*1/32*		*1/64*		*1/128*		*negative control*		*positive control*		*corsodyl no cells*	
Time, minutes	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
5	0.022	0.003	0.040	0.006	0.061	0.009	0.149	0.040	0.202	0.042	0.280	0.063	0.317	0.058	0.398	0.126	0.025	0.002	0.016	0.002
10	0.019	0.003	0.052	0.016	0.061	0.015	0.200	0.033	0.267	0.052	0.332	0.053	0.427	0.094	0.467	0.081	0.027	0.002	0.016	0.001
60	0.018	0.001	0.041	0.007	0.032	0.004	0.037	0.005	0.180	0.032	0.216	0.061	0.316	0.078	0.419	0.090	0.029	0.003	0.021	0.002
120	0.019	0.002	0.025	0.005	0.022	0.005	0.020	0.006	0.094	0.031	0.167	0.052	0.189	0.064	0.313	0.072	0.027	0.002	0.019	0.001
240	0.023	0.003	0.032	0.001	0.032	0.002	0.029	0.002	0.029	0.002	0.081	0.011	0.214	0.051	0.279	0.050	0.030	0.003	0.010	0.001

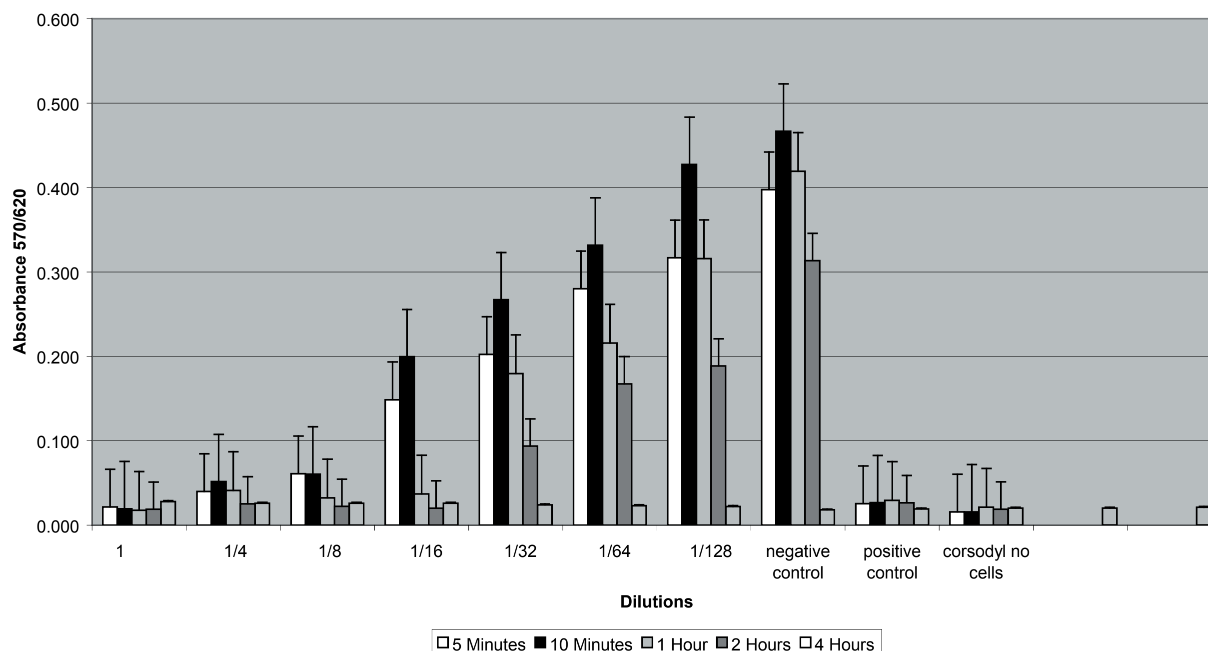

Figure 1. *The Effect Of Time And Dilution On Cytotoxicity Of Chlorhexidine Solution To Hos Cells*

DISCUSSION

The MTT assay is used to measure cell activity. This assay uses a tetrazolium salt and detects only living cells hence, it is a measure of cell metabolic function, dependent on the intact activity of a mitochondrial enzyme, succinate dehydrogenase, which is impaired after exposure of cells to toxic surroundings and the signal detected is dependent on the metabolic activity of the cells in question [33-35]. The MTT is converted to a blue formazan by mitochondrial dehydrogenases. This enzyme is only present in intact, living cells; hence the blue colour produced and absorbed should be proportional to the number of viable cells present.

The graph presents a general trend exhibiting an increase in cell metabolic activity with each sequential dilution; thus indicating that toxicity of chlorhexidine mouthwash can be reduced when diluted. The results also suggest that an increase in exposure time of cells to the mouthwash can have a direct effect on cell viability and metabolic activity. The decrease in metabolic activity implies that chlorhexidine mouthwash has induced an effect on cell membrane integrity resulting in cell damage and subsequent death.

As stated above, we were interested in the effects of a combined formulation of chlorhexidine with other chemicals: in order to test the degree of toxicity for which chlorhexidine itself is responsible, it would be necessary to test the effect of the dissolving solution, containing all the other mouthwash components, on the cells. It is known that polyethoxylated castor oil is of relatively low cytotoxicity compared to antiseptics such as benzalkonium chloride or cetylpyridinium chloride [36]. Even so, the results seen here mirror those reported by others [19, 25, 37, 38] for cytotoxicity to periodontal fibroblasts following exposure to chlorhexidine alone, with similar relative reductions in cellular vitality for similar concentrations of chlorhexidine. Wilken *et al.* [39] have suggested that the cytotoxicity of chlorhexidine-containing oral preparations relates more to the effects of chlorhexidine than other elements such as alcohol. Zhang *et al.* [35] have also reported similar results for a commercial chlorhexidine preparation with 0.12% chlorhexidine gluconate (as well as other ingredients), and Shahan *et al.* [23] have reported that wound irrigation with such a preparation may impair wound healing in a murine model, although this may only be for 48 hours postoperatively. This is at odds with the results of Mobracken and Wengstrom [40], who suggested that slower healing was apparent for up to 7 days when a site was treated using chlorhexidine acetate solution. Alleyn et al. [41] also elegantly illustrated how the pre-treatment of dentine surfaces could adversely influence the responses of fibroblasts subsequently exposed to these surfaces by assessing cell attachment. No other assessments of vitality were carried out.

Cytotoxicity screening, as indicated by the ISO-10993-Part 5 guidelines [27], recommends an indirect method as a rapid and effective way of assessing the effect of any toxic leachable or agent on cells in vitro. In addition, the guidelines recommend that both a positive control (one that causes a detrimental effect) and a negative control (one which does not have a toxic effect) should be included in the tests. In this study, alcohol was used as the positive control. This satisfied both the ISO recommendations and also represents a major constituent of the chlorhexidine mouthwash (which contains 5.7% ethanol). Human osteoblast-like cells have been used in this study, and although this may not seem comparable with native cells involved in the clinical situation, the protocol used is consistent with standard ISO tests for cytotoxicity. Chlorhexidine has been proposed as a means of disinfecting the surfaces of failing implants as part of a process to try to develop re-osseointegration, and so it is not impossible that residues of such agents may come into contact with bone cells at some point. Even so, these potential complications must be weighed against the proven antibacterial effects of such agents and their ability to prevent plaque formation. Furthermore, issues of clearance of chlorhexidine from a surgical site have not been addressed in this study, and further work is needed

to equate the results reported here to the dynamics of blood flow and chemical clearance in a healing wound area *in vivo*.

In previous investigations using cells from the periodontium, chlorhexidine has been shown to affect cell proliferation, as well as total protein production *in vitro* [19]. The study undertaken by Mariotti and Rumpf [24] showed that the effects of chlorhexidine on retarding proliferation of gingival fibroblasts occur rapidly. Other *in vitro* studies also demonstrated that a short exposure to chlorhexidine was equally effective in impairing human gingival fibroblast function as longer exposure times [41] and that short exposure was sufficient to damage epithelial cells [19]. Similar results for impaired cell function (expressed as nitric oxide production) at low doses, followed by cytotoxicity for higher doses have also been described for murine peritoneal macrophages [16].

Ribeiro et al [42] demonstrated that chlorhexidine digluconate intake induces primary DNA damage in rat leukocytes and oral mucosal cells, and hypothesised that chlorhexidine may bind to proteins, contribute to alterations of cellular functions and induce DNA damage.

The results for 5 minute exposure time point appear to be an anomaly, with cells having a lower activity than at 10 minutes. This does not correlate with the rest of the findings where an increase in exposure time resulted in a fall in cell metabolic activity. A possible explanation could be that the short exposure time period involved several technical steps, and, as a result, the cells were disturbed and it is possible that the short time period was insufficient for cells to recover resulting in a lower activity as recorded by the absorbance readings.

The negative control without mouthwash showed the highest metabolic activity for each time point studied, indicating no deleterious effects on the cells and normal metabolic activity. The positive controls revealed a fall in activity at most time points, as predicted. The studied also showed that reduction in cellular metabolic activity in the presence of neat mouthwash was greater than for the positive control, confirming its cytotoxicity.

Therefore, within the limitations of this study, it would appear that exposure of cell lines to this formulation of complete chlorhexidine mouthwash may have cytotoxic effects. Obviously these results cannot be fully extrapolated to the clinical situation without further work, and the degree to which this agent may influence clinical healing is uncertain. In addition, the development of newer, combined chlorhexidine-containing mouthwashes [43-46] may lead to less cytotoxic preparations, but this has not yet been investigated in published literature. The effect of any such toxicity may be more significant if the agent is applied to exposed connective tissue rather than surgical wounds closed by sutures.

CONCLUSION

This study suggests that chlorhexidine mouthwash can be cytotoxic in high concentrations and when applied for long periods of time. The ability of chlorhexidine to non-selectively kill oral microorganisms makes it the gold standard of antimicrobials, explaining its wide usage. However, chlorhexidine-induced effects on cells suggest that this drug is toxic to cells at concentrations far below those that are introduced into the oral cavity. This may have implications if such agents are employed to disinfect the surfaces of implants and prostheses *in situ*.

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REFERENCES

1. Eren, K., Ozmeric, N. and Sardas, S. Monitoring of buccal epithelial cells by alkaline comet assay (single cell electrophoresis technique) in cytogenic evaluation of chlorhexidine. *Clin. Oral Invest.* 2002; **6**: 150-154.
2. Davies, G., Francis, J., Martin, A., Rose, F.L. and Swain, G. 1:6Di-4–chlorophenyl-diguanidohexanane. Laboratory investigation into a new antibacterial agent of high potency. *Br. J. Pharmacol.* 1954; **9**: 192-196.
3. Budtz-Jorgensen, J. and Löe, H. Chlorhexidine as a denture disinfectant in the treatment of denture stomatitis. *Scand. J. Dent. Res.* 1972; **80**: 457-464.
4. Denton, G.W. Chlorhexidine. In: Block SS, (Ed.): *Disinfection, sterilisation and preservation*. 4th ed Philadelphia: Lea and Febiger, 1991: 274-289.
5. Jones, C.G. Chlorhexidine: is it still the gold standard? *Periodontol. 2000* 1997; **15**: 55-62.
6. Adell, R., Lekholm, U. and Branemark, P.-I. *Tissue integrated prosthesis. Surgical procedures,* 1st ed. Chicago: Quintessence, 1985; 211-232.
7. Lambert, P.M., Morris, H.F. and Ochi, S. The influence of 0.12% chlorhexidine digluconate rinses on infectious complications and implant success. *J. Oral Maxillofac. Surg.* 1997; **55**: 25-30.
8. Young, M.P.J., Korachi, M., Carter, D.H., McCord, J.F. and Drucker, D.B. The effects of an immediately pre-surgical chlorhexidine oral rinse on the bacterial contamination of bone debris collected during dental implant surgery. *Clin. Oral Impl. Res.* 2002; **13**: 20-29.
9. Orton, G.S., Steele, D.L. and Wolinsky, L.E. The dental professional's role in monitoring and maintenance of tissue-integrated prosthesis. *Int. J. Oral Maxillofac. Impl.* 1989; **4**: 305-310.
10. Mombelli, A. Microbiology and antimicrobial therapy of peri-implantitis. *Periodontol. 2000* 2002; **28**: 177-189.
11. Esposito, M., Lausmaa, J., Hirsch, J.M. and Thomsen, P. Surface analysis of failed oral titanium implants *J. Biomed. Mater. Res.* 1999; **48**: 559-568.
12. Schou, S., Holmstrup, P., Jørgensen, T. *et al.* Implant surface preparation in the surgical treatment of experimental peri-implantitis with autogenous bone graft and ePTFE membrane in cynomolgus monkeys. *Clin. Oral Impl. Res.* 2003; **14**: 412-422.
13. Louis, S.M. and Pearson, R.M. A comparison of the effects of

nonoxynol-9 and chlorhexidine on cell motility. *Contraception* 1985; **32**: 199-205.
14. Kenny, E.B., Saxe, S.R. and Bowles, R.D. Effect of chlorhexidine on human polymorphonuclear leukocytes. *Arch. Oral Biol.* 1972; **17**: 1633-1636.
15. Knuuttila, M. and Soederling, E. Effect of chlorhexidine on the release of lysosomal enzymes from cultured macrophages. *Acta Odontol. Scand.* 1981; **39**: 285-289.
16. Bonacorsi, C., Raddi, M.S. and Carlos, I.Z. Cytotoxicity of chlorhexidine digluconate to murine macrophages and its effect on hydrogen peroxide and nitric oxide induction. *Brazilian J. Med. Biol. Res.* 2004; **37**: 207-12.
17. Helgeland, K., Heyden, G. and Rolla, G. Effect of chlorhexidine on animal cells in vitro. *J. Periodontol.* 1971; **62**: 434-438.
18. Damour, O., Hua, S.Z., Lasne, F., Villain, M., Rousselle,, P. and Collombel, C. Cytotoxicity evaluation of antiseptics and antibiotics on cultured human fibroblasts and keratinocytes. *Burns* 1992; **18**: 479-85.
19. Babich, H., Wurzburger, B.J., Rubin, Y.L., Sinensky, M.C. and Blau, L. An in vitro study on the cytotoxicity of chlorhexidine digluconate to human gingival cells. *Cell Biol. Toxicol.* 1995; **111**: 79-88.
20. Pucher, J.J. and Daniel, J.C. The effects of chlorhexidine digluconate on human fibroblasts in vitro. *J. Periodontol.* 1993; **62**: 526-532.
21. Boyce, S.T., Warden, G.D. and Holder, I.A. Cytotoxicity testing of topical antimicrobial agents on human keratinocytes and fibroblasts for cultured skin grafts. *J. Burn Care Rehabil.* 1995; **16**: 97-103.
22. Fraser, J.F., Cuttle, L., Kempf, M. and Kimble, R.M. Cytotoxicity of topical antimicrobial agents used in burn wounds in Australasia. *Aust. NZ J. Surg.* 2004; **74**: 139-142.
23. Shanan, M.H., Chuang, A.H., Brennan, W.A., Dirksen, T.R., Van Dyke, T.E. and McPherson, J.C. The effect of chlorhexidine irrigation on tensile wound strength. *J. Periodontol.* 1993; **64**: 719-722.
24. Marotti, A.J. and Rumpf, D.A.H. Chlorhexidine-induced changes to human gingival fibroblast collagen and non-collagen protein production. *J. Periodontol.* 1999; **70**: 1443-1448.
25. Chang, Y.C., Huang, F.M., Tai, K.W. and Chou, M.Y. The effect of sodium hypochlorite and chlorhexidine on cultured human periodontal cells. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 2001; **92**: 446-50.
26. International Standard (1992) Biological valuation of medical devices –Part 5: Tests for cytotoxicity: *in vitro* methods. ISO 10993-5.
27. Di Silvio, L. and Gurav, N. Osteoblasts, Chapter 11 In Koller, M.R., Palsson, B.O. and Masters J.R.W. (Eds): *Human Cell Culture* Vol 5,. Kluwer Academic Publishers, 2001; 221-241
28. Harris, S.A., Enger, R.J., Riggs, B.L. and Spelsberg, T.C. Development and Characterization of a Conditionally Immortalized Human Fetal Osteoblastic Cell line. *J. Bone Min. Res.* 1995; **10**: 178-186.
29. McAllister, R., Gardner, M.B., Greene, A.E., Bradt, C., Nichols, W.W. and Landing, B.H. Cultivation *in vitro* of cells derived from human osteosarcoma. *Cancer* 1971; **27**: 397-402.
30. Ng, K. W., Partridge, N. C., Niall, M. and Martin, T. J. Epidermal growth factor receptors in clonal lines of a rat osteogenic sarcoma and in osteoblast-rich rat bone cells. *Calc.Tiss.Intnl.* 1983; **35**: 298-303.
31. Ikeda, T., Futaesaku, Y. and Tsuchida, N. *In vitro* differentiation of the human osteosarcoma cell lines, HOS and KHOS. *Virchows Archiv B Cell Pathol.* 1992; **62**: 199-206.
32. Clover, J. and Gowen, M. (1994). Are MG-63 and HOS TE85 Human osteosarcoma
cell lines representative models of the osteoblastic phenotype? *Bone* 1994; **15**: 585-591
33. Mossmann, T. Rapid colorimetric assay for cellular growth and survival:application to proliferatin and cytotoxicity assays. *J. Immunol. Meth.* 1983; **65**: 55-63.
34. Denizot, F. and Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Meth.* 1986; **89**:271.
35. Zhang, W., Torabinejad, M. and Li, Y. Evaluation of cytotoxicity of MTAD using the MTT-tetrazolium method. *J. Endodont.* 2003; **29**: 654-7.
36. Burgalassi, S., Chetoni, P., Monti, D. and Saettone, M.F. Cytotoxicity of potential ocular permeation enhancers evaluated on rabbit and human corneal epithelial cell lines. *Toxicol. Lett.* 2001; **122**: 1-8.
37. Cline, N.V. and Layman, D.L. The effects of chlorhexidine on the attachment and growth of cultured human periodontal cells. *J. Periodontol.* 1992; **63**: 598-602.
38. Goldschmidt, P., Cogen, R. and Taubman, S. Cytopathologic effects of chlorhexidine on human cells. *J. Periodontol.* 1997; **48**: 212-215.
39. Wilken, R., Botha, S.J., Grobler, A. and Germishuys, P.J. In vitro cytotoxicity of chlorhexidine gluconate, benzydamine-HCl and povidone iodine mouthrinses on human gingival fibroblasts. *South African Dent. J.* 2001; **56**: 455-60.
40. Mobracken, H. and Wengstrom, C. Interference with healing of rat skin incisions treated with chlorhexidine. *Acta Dermatol.* 1974; **54**: 29-34.
41. Alleyn, C.D., O'Neal, R.B., Strong, S.L., Scheidt, M.J., Van Dyke, T.E. and McPearson, J.C. The effect of chlorhexidine treatment of root surfaces on the attachment of human gingival fibroblast in vitro. *J. Periodontol.* 1991; **62**: 434-438 .
42. Ribeiro, D.A., Bazo, A.P., da Silva Franchi, C.A., Marques, M.E.A. and Salvadori, D.M.F. Chlorhexidine induces DNA damage in rat peripheral leukocytes and oral mucosal cells. *J. Periodont. Res.* 2004; **39**: 358-361.
43. Quirynen, M., Avontroodt, P., Peeters, W., Pauwels, M., Coucke, W. and van Steenberghe, D. Effect of different chlorhexidine formulations in mouthrinses on de novo plaque formation. *J. Clin. Periodontol.* 2001; **28**: 1127-36.
44. Santos, S., Herrera, D., López, E., O'Connor, A., González, I. and Sanz, M. A randomized clinical trial on the short-term clinical and microbiological effects of the adjunctive use of a 0.05% chlorhexidine mouth rinse for patients in supportive periodontal care. *J Clin Periodontol* 2004; **31**: 45-51.
45. Quirynen, M., Soers, C., Desnyder, M., Dekeyser, C., Pauwels, M. and van Steenberghe, D. A 0.05% cetyl pyridinium chloride/0.05% chlorhexidine mouth rinse during maintenance phase after initial periodontal therapy. *J. Clin. Periodontol.* 2005; **32**: 390-400.
46. van Strydonck, DA, Timmerman, MF, van der Velden, U, van der Weijden, GA. Plaque inhibition of two commercially available chlorhexidine mouthrinses. *J. Clin. Periodontol.* 2005; **32**: 305-9.