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Original Article

Assessment of the cytotoxicity of chlorhexidine by employing an *in vitro* mammalian test system

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Abstract

Background/purpose

Chlorhexidine (CHX), a chlorophenyl biguanide with broad antibacterial action, has been widely used in dentistry. The initial uses of CHX in dentistry were to wash operation site and to disinfect root canals. Recently, the addition of CHX into many dental materials has improved the overall therapeutic efficacy. The aim of this study was to evaluate the potential toxicological implications of CHX employing an *in vitro* mammalian test system.

Materials and methods

Cytotoxicity, mode of cell death, and generation of superoxide anion were performed to elucidate the toxic effects of CHX on Chinese hamster ovary cells. Cytotoxicity was judged using tetrazolium bromide reduction assay. The mode of cell death was determined by flow cytometry. Superoxide anion generation was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome *c*.

Results

from apoptosis to necrosis as the concentrations of CHX elevated. CHX demonstrated a significant superoxide anion generation in a dose-dependent manner ($P < 0.05$). The addition of superoxide dismutase decreased the cytotoxicity induced by CHX ($P < 0.05$).

Conclusion

CHX was demonstrated to exhibit cytotoxicity that could disrupt the stable cellular redox balance, resulting in increasing levels of free radical generation and subsequent cell death. CHX has significant potential for cytotoxicity.

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Keywords

cell death; chlorhexidine; cytotoxicity; superoxide anion

Introduction

Chlorhexidine (CHX) is a synthetic cationic molecule containing two 4-chlorophenyl rings and two biguanide groups connected by a central hexamethylene chain. CHX is a broad-spectrum antiseptic currently used as a local antiseptic in daily clinical practice. CHX has been introduced in different concentrations and formulations in several commercial products for dental hygiene such as toothpaste, mouthwash, gels, sprays, and chewing gums.[1] In recent years, CHX has been recommended as an endodontic irrigant,[2] and supplemented into calcium hydroxide based root canal sealer[3] and mineral trioxide aggregate[4] to improve the overall therapeutic efficacy of endodontic treatment.

Unfortunately, the toxic qualities of CHX do not seem to be reserved entirely for bacteria.[5] In addition, we know that CHX application directly to surgical wounds in the oral cavity can delay and alter wound healing.[6] Numerous studies have indicated that CHX is cytotoxic to many cells, such as murine fibroblast cell lines,[7,8] human dermal fibroblasts,[9] human gingival fibroblasts,[10,11] human periodontal ligament cells,[12] human alveolar bone cells,[13] and human osteoblastic cell line.[7,14] However, the

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death occurs in eukaryotic cells: necrosis (reproductive cell death) and apoptosis (programmed cell death).[15,16] Cell death is controlled by several factors within the cell, including the disruption of a stable redox balance between reactive oxygen species (ROS) and antioxidant protective systems.[17] In addition, the production of superoxide anion and its reactive metabolites are important in bacterial killing. In this study, cytotoxicity, the mode of cell death, and superoxide anion generation were used to investigate the effects of CHX by mammalian test system using Chinese hamster ovary (CHO) cells.

Materials and methods

CHX, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and ferricytochrome *c* were obtained from Sigma (Sigma Chemical Co., Saint Louis, MO, USA). All tissue culture reagents were purchased from Gibco Laboratories (Grand Island, NY, USA). Reagents for flow cytometry analysis were obtained from Becton Dickinson, San Jose, CA, USA.

Cell culture

CHO cells were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Eagle's minimum essential medium (MEM) with 10% fetal calf serum, 100 units/mL penicillin and 100 mg/L streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 3 minutes, and aliquots of separated cells were subcultured. The cells were subcultured at 1:4 splits every 3 days.[18,19]

Cytotoxicity assay

Dehydrogenase activity was measured by MTT colorimetric assay to monitor mammalian cell survival and proliferation. Cells were diluted in fresh complete medium and seeded in 96-well plates (2×10^4 cells/well). After overnight attachment, cells were treated with various concentrations (0%, 10^{-5}%, 5×10^{-5}%, 10^{-4}%, and 5×10^{-4}% w/v) of CHX for each time point (1 hour, 2 hours, and 4 hours), then 50 µL MTT dye was added to each well. Plates were incubated in a CO_2 incubator for 4 hours. Optical density was determined by eluting the dye with dimethyl sulfoxide and the spectrophotometric absorbance measured at 550 nm using a spectrophotometer (Hitachi, Tokyo, Japan).[20] Cells treated with 0% of CHO were used as denominator for

each (100%

Detection of apoptosis and necrosis by flow cytometry

Apoptosis and necrosis were discriminated by Annexin V-FITC and propidium iodide (PI; Annexin V Assay Kits; BioVision, Mountain View, CA, USA). The processes of detection were according to manufacturer's instructions. Briefly, after incubation with serum-free medium and various concentrations (10^{-5}%, 5×10^{-5}%, 10^{-4}%, and 5×10^{-4}%) of CHX for 1 hour, CHO cells were collected by trypsination and washed once with ice-cold phosphate buffered saline, and 1×10^5 cells were incubated in 100 µL binding buffer containing Annexin V-FITC and PI for 30 minutes at room temperature in the dark. The samples were analyzed on a FACS flow cytometer (Becton, Dickinson, and Company). Data analysis was performed with CellQuest software (Becton, Dickinson, and Company), which allowed assessment of only specific populations, individualized by gates according to size, granularity, and fluorescence parameters. Percentages of viable cells (annexin V−; PI−), early apoptotic cells (annexin V+; PI−), necrotic cells (annexin V−; PI+) and late apoptotic cells (annexin V+; PI+) were determined as described in detail elsewhere.[21–23]

Measurement of superoxide anion generation

Various concentrations of CHX on CHO cells induced superoxide anion generation were determined by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*.[24] The assay cuvette contained a suspension of CHO cells (1×10^5 cells) and 40 µM of ferricytochrome *c* was brought up to a final volume of 1.5 mL, and the reference cuvette also received 17.5 U/mL of superoxide dismutase. The indicated concentrations of CHX were added to both cuvettes. Absorbance changes in the reduction of ferricytochrome *c* were monitored continuously for 5 minutes at 550 nm in a double-beam spectrophotometer (Hitachi, Tokyo, Japan). The amount of superoxide anion in the reaction mixture was calculated from the formula: superoxide anion (nmol) = 71.55 × absorbance.[25]

Effect of superoxide dismutase on CHX induced cytotoxicity

To further elucidate the link between cell viability and cellular ROS production, 10 units, 100 units, or 1000 units SOD were added to culture. CHO cells were exposed to SOD for 15 minutes prior to the addition of 50% inhibition concentration (IC_{50}) of CHX, then coincubated for 1 hour. Cytotoxicity was judged using MTT assay analysis as described previously.

Statistical analysis

Triplic
repea
from control and treated groups were statistically analyzed by paired t test.

Results

The MTT assay is a colorimetric assay based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt to blue formazan crystals. As shown in Fig. 1, CHX demonstrated a cytotoxic effect on CHO cells. CHX reduced the activity of dehydrogenase of CHO cells in a dose-dependent and a time-dependent manner ($P < 0.05$).

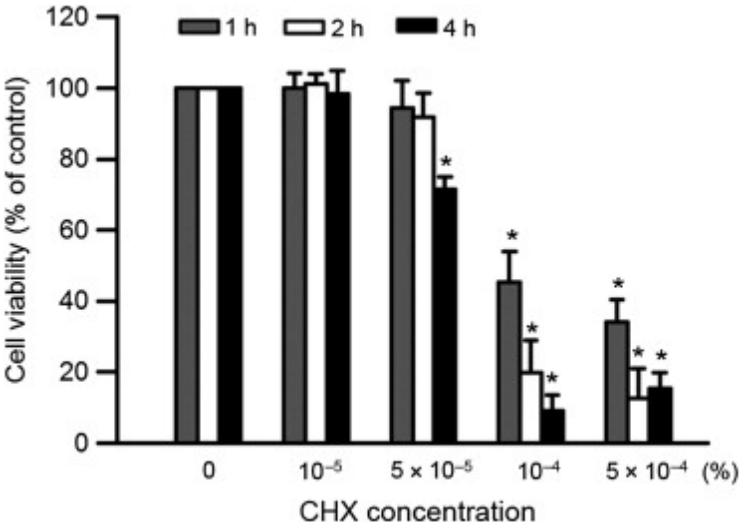

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Figure 1. Viability of Chinese hamster ovary cells after exposure to various concentrations of chlorhexidine for 1 hour, 2 hours, and 4 hours. Results are expressed as percentage of cell viability relative to the untreated controls. Data are shown as mean ± standard deviation. * Statistically significant difference when compared with control ($n = 3$).

Fig. 1 shows that, when CHO cells were treated with CHX at various concentrations lower than 10^{-4}%, the cell viability is in negative correlation with treated time. However, there was significant difference in cellular viability for cells treated with 10^{-4}% CHX for over 1 hour, which suggests that the maximum effect is achieved at this point. Therefore, all subsequent experiments were treated with 10^{-4}% CHX and the maximum time frame was set at 1 hour.

In order to investigate the mode of cell death of CHX, the cells were processed for

apoptosis was about 1.62%, 3.51%, and 3.47% at the concentration of CHX 5×10^{-5}%, 10^{-4} %, and 5×10^{-4}%, respectively. The amount of cell necrosis was 2.38%, 8.62%, and 22.50% at the concentration of CHX 5×10^{-5}%, 10^{-4} %, and 5×10^{-4}%, respectively (Table 1). CHX-induced cell death by necrosis rather than apoptosis when the cytotoxicity was enhanced.

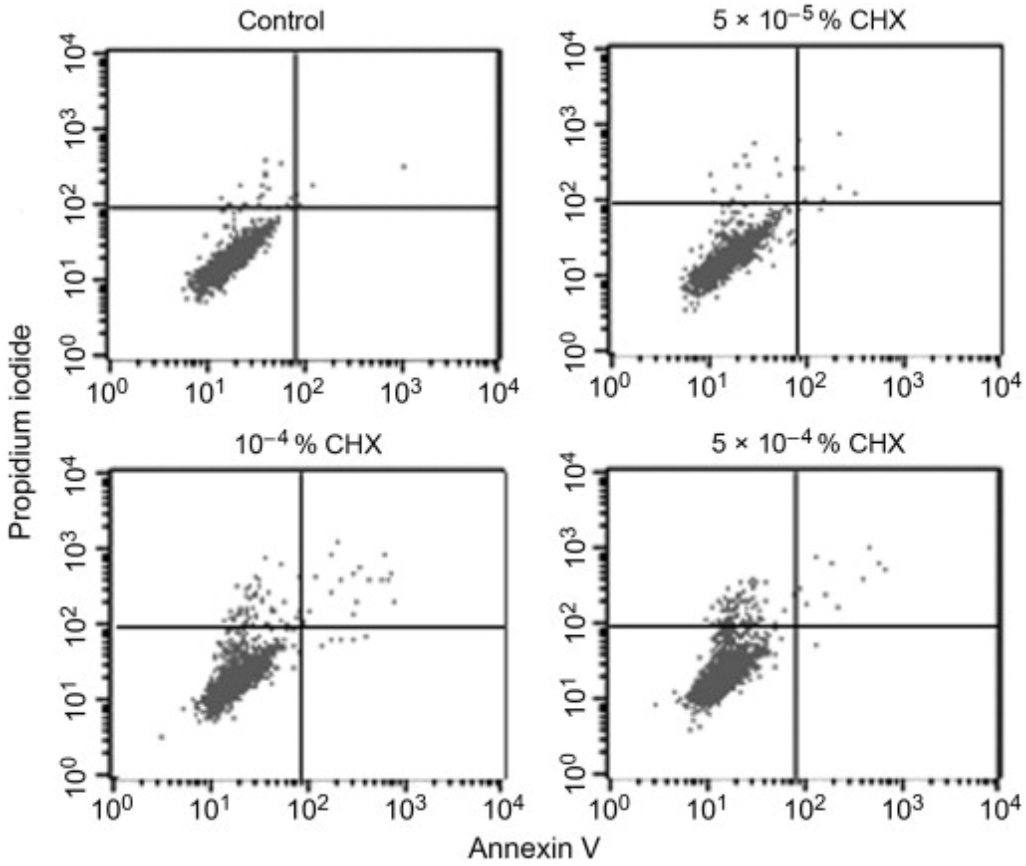

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Figure 2. The dot plots are representative of control and Chinese hamster ovary cells exposed to various concentrations of chlorhexidine, representing the populations of viable [bottom left: annexin V–, propidium iodide (PI–)], apoptotic (bottom right: annexin V+, PI– and top left: annexin V+, PI+), and necrotic (top right: PI+) cells. The x-axis is the fluorescence intensity of annexin-V; the y-axis is the fluorescence intensity of PI.

Table 1. The percentage of apoptotic and necrotic cells in the cultures were assessed by using flow cytometry analysis after exposure to various concentrations of CHX (*n* = 3).

0	97.09 ± 1.34	0.31 ± 0.18	0.01 ± 0.02	2.34 ± 0.32
5×10^{-5}	95.99 ± 1.57	1.42 ± 0.89*	0.11 ± 0.76	2.38 ± 0.63
1×10^{-4}	85.87 ± 4.84*	5.43 ± 2.13*	0.19 ± 1.72	8.62 ± 1.92*
5×10^{-4}	71.29 ± 6.47*	5.57 ± 1.47*	0.15 ± 2.16	22.50 ± 3.93*

*Statistically significant difference when compared with control, $P < 0.05$.

To examine whether CHX interferes with the assay for superoxide anions, CHX was administered to a reaction solution containing ferricytochrome *c*; CHX did not directly interact with ferricytochrome *c* (data not show). As shown in Fig. 3, the administration of CHX to CHO cells increased the amount of cytochrome *c* reduction in a dose-dependent manner ($P < 0.05$). The levels of superoxide anion induced by CHX were 0.96 nmol, 1.35 nmol, 2.03 nmol, 1.95 nmol, and 1.54 nmol at the concentrations of CHX 0, 10^{-5}%, 5×10^{-5}%, 10^{-4} %, and 5×10^{-4}%, respectively.

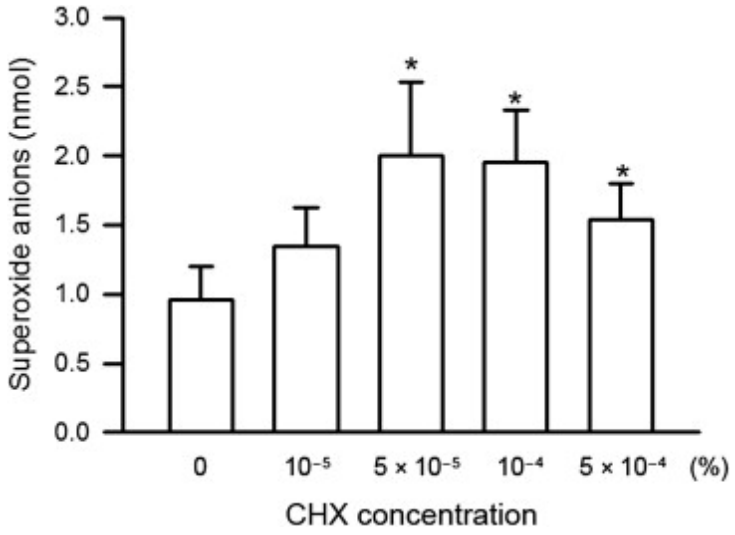

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Figure 3. Superoxide anion generation after exposure to various concentrations of chlorhexidine by the reduction of cytochrome *c* was monitored continuously for 5 minutes. Data are shown as mean ± standard deviation. *Statistically significant difference when compared with control ($n = 3$).

units, reduce cells from CHX-induced cytotoxicity ($P < 0.05$). CHX at a concentration of 10^{-4}% caused about 50% of cell death over the 1-hour incubation period. The addition of 10 units, 100 units, and 1000 units SOD enhanced the cell viability up to 64%, 88%, and 92%, respectively.

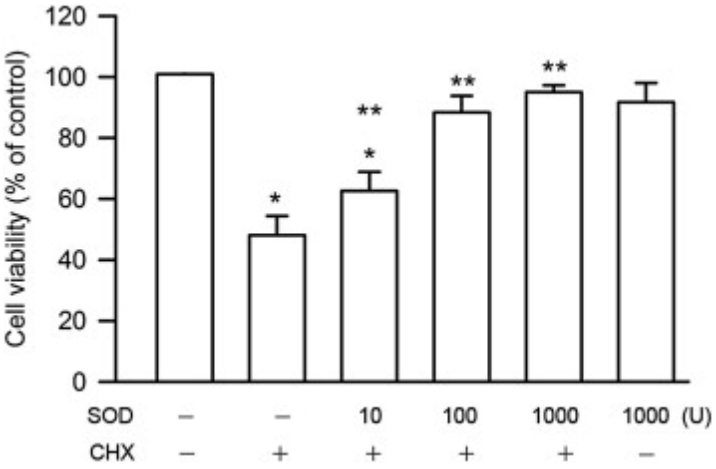

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Figure 4. Effects of superoxide dismutase (SOD) on chlorhexidine induced cytotoxicity to Chinese hamster ovary cells. Phosphate buffered saline or 10 units, 100 units, and 1000 units SOD were incubated for 15 minutes prior to administration of 10^{-4}% CHX. Data are shown as mean ± standard deviation. *Statistically significant difference when compared with control **Statistically significant difference when compared with no treatment with SOD (n = 3).

Discussion

Methods of assessing cytotoxicity *in vitro* are popular for initial biological screening as evidenced by the abundance of test methods. The advantages of *in vitro* tests include controllability, reproducibility, rapidity, and economy.[26] Our choice of cell line and use of cell in continuous culture permits an accurate evaluation of the changes, independently from factors such as age and metabolic and hormonal states of the donor that may influence the cells in primary culture. CHO cells have a small number of relatively large chromosomes, they grow fast, and reproducible results can be obtained from the same cell source if frozen cell stocks are maintained. In addition, CHO cells have been widely used to evaluate the cytotoxicity of various dental materials/chemicals.[18,19,27,28]

effects to CHO cells. Many types of cells have been applied to evaluate the cytotoxicity of CHX. CHX was found to be a cytotoxic agent to murine fibroblast cell lines,[7,8] human dermal fibroblasts,[9] human gingival fibroblasts,[10,11] human periodontal ligament cells,[12] human alveolar bone cells,[13] and human osteoblastic cell line.[7,14] Data suggest actual differences in cytotoxic levels with CHX on different type of cells. These results have clearly shown the cytotoxicity of CHX. In addition, the cytotoxicity of CHX is not cell type specific.

Apoptosis and necrosis have long been considered as two distinct mechanisms of cell death.[15] Our study showed that CHX induced both modes of cell death. However, the percentages of cell viability obtained from MTT and Annexin V staining assays differed, which may be due to varying sensibilities with different experimental approach. In flow cytometry, many dead cells are lost in the repetitive washing processes; thus it would demonstrate relatively higher cell viability. Increasing CHX concentrations can affect the mode of cell death. When cytotoxicity was raised, CHX-induced cell death by necrosis rather than apoptosis. Our results were in agreement with Faria et al[8] who reported that CHX induced apoptosis at lower concentrations and caused necrosis at higher concentrations on L929 fibroblasts. However, our results differed from those of Giannelli et al,[7] who found that the number of cells of the human osteoblastic cell line Saos-2 undergoing apoptotic nuclear fragmentation increased upon exposure to higher concentration, reaching almost 80% of the total cells. The reason for this contrary result is not clear. It may be due to the different origins of the cells or different experimental protocols used in each laboratory. The cellular effects of CHX may not necessarily be comparable in all tissues.

To further elucidate the mechanism underlying CHX-induced cell death, we next investigated the ability of this compound to provoke ROS generation in CHO cells. In this study, CHX was found to induce superoxide anion generation. Similar results were found by Giannelli et al,[7] who reported that CHX can generate ROS in Saos-2 osteoblastic cell line and Yeung et al,[29] who reported that CHX can induce ROS in the *ex vivo* experiments and act as a pro-oxidant. Consistently, our recent study reported that the cytotoxicity of CHX is related to intracellular glutathione levels.[14] Taken together, cytotoxicity of CHX may be partly related to ROS generation.

SOD, an extracellular superoxide free radical scavenger, is a defense system of redox-regulating molecule in mammalian cells.[30] SOD was added to clarify the

generation of super anions that ultimately leads to CHX-induced cell death via necrosis or apoptosis.

The cytotoxic effects of CHX on CHO cells depended on the exposure dose, frequency, and duration. In addition, CHX can induce two distinct modes of cell death. CHX-induced cell death may be via superoxide anion generation. Potential cytotoxicity when extruded into the periradicular tissue and at higher concentrations should be considered during endodontic practice.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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