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ORIGINAL ARTICLE

Assessment of cytogenetic and cytotoxic effects of chlorhexidine digluconate on cultured human lymphocytes

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Abstract

Objective. The aim of this study was to assess the genetic and cellular toxicity of Chlorhexidine digluconate (CHX) on peripheral human lymphocytes *in vitro.* ***Materials and methods.*** Micronucleus assay was used to investigate the genotoxicity, while the cell viability and proliferation were evaluated by Trypan blue exclusion test and Nuclear Division Index in control and CHX-treated (0.05, 0.1, 0.2, 0.4, 0.5 mg/ml) human blood cultures. ***Results.*** A dose-dependent toxic effect was found depending on CHX incubation on the genetic and cell viability of the lymphocytes. Micronucleus frequency was found to be statistically higher at 0.5 mg/ml concentration compared to lower doses and the control group ($p < 0.05$). A significant reduction was shown in the cell viability and cell proliferation of the exposed lymphocytes at the concentrations of 0.4 and 0.5 mg/ml ($p < 0.05$), while no significant toxicity was found at lower concentrations compared to control ($p > 0.05$). ***Conclusion.*** This study showed dose-dependent genotoxic and cytotoxic effects of CHX on human lymphocytes *in vitro.* It should be considered during periodontal irrigation or novel CHX products at lower concentrations should be manufactured for clinical usage.

Key Words: *chlorhexidine, lymphocyte, toxicity*

Introduction

Topical antiseptic chemotherapeutic approaches are unique in the treatment and maintenance phase of oral and periodontal diseases [1–3]. Chlorhexidine digluconate (CHX) is a common chemotherapeutic agent used in dental practice owing to its wide spectrum of bactericidal capability [4–7]. As an effective antibacterial agent, it has been reported to largely increase the benefits obtained by periodontal treatment [8,9] and still remains the gold standard in the periodontal maintenance [10,11]. Although clinical benefits of this compound have been proved in several trials, publicly, information about its genetic and cellular toxicity is still conflicting [4,5,12].

Indeed, there is various evidence that this compound may have adverse effects at clinical concentrations. Even though it is considered to have low toxicity due to its poor absorption via oral administration [13], several authors have suggested that this compound is a cytotoxic agent on oral tissues and cells at the concentrations used clinically [14,15]. It has been stated that CHX has cytotoxic activity on cultured alveolar bone and gingival epithelial cells [14,16]. Genotoxic side-effects were also found on epithelial and blood cells when used for mouth rinsing in clinical trials [17,18]. Its topical application was also reported to result in penetration through the epithelial barrier, causing tissue damage [19]. Furthermore, the breakdown products of CHX, i.e. parachloroaniline, have also been considered to be mutagenic [20,21]. Nevertheless, it is still recommended in a number of periodontal clinical procedures such as full mouth disinfection technique and for individual usage [6]. Whereas, it was stated that only very high concentrations (0.5–2% for 10 min) of CHX could achieve substantial bactericidal effects against periodontopathogens [22]. Hence, the potential benefits and toxic effects of this compound should be considered

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during clinical and individual usage. Although its toxic potential on several cell types has been evaluated [15,16,18,20], there is a lack of data in the current literature about the human lymphocytes. Lymphocytes are the principle components of the cell-mediated immune system and play a fundamental role in the pathogenesis of periodontal destruction [23,24]. They are responsible for mediating the immune and inflammatory response against periodontopathogens in the disease pathogenesis [25]. The lymphocytes are interactive with several mediators and other important cell types involved in cell-mediated immunity, such as neutrophiles and macrophages [23]; thus, dysfunction of these cells may cause imbalances in regulation of the local immune response in diseased periodontal tissues. Therefore, it was aimed in this study to investigate the toxic effects of CHX on genetic and cellular structures of cultured human lymphocytes *in vitro*.

Materials and methods

Experimental design

This study was carried out according to the approval of the Ethical Committee of the Faculty of Dentistry, Ataturk University, and informed consent was obtained from each donor. Whole blood samples were collected at the Department of Periodontology from 10 healthy non-smoker donors (five male and five female, between 20–24 years old) with no history of exposure to any genotoxic agent. For all the volunteers, hematological and biochemical parameters were analyzed and no disease was detected. Blood cultures were set up at the Molecular Biology and Genetic Laboratory, Faculty of Science, Ataturk University (by H.T) according to a slight modification of the protocol as described by Evans and O'Riordan [26]. The peripheral blood lymphocytes (0.5 ml) were cultured in 5 ml of culture medium (Chromosome Medium B, Biochrom, Berlin, Germany) with its standard content (85 ml minimum essential medium, 15 ml fetal bovine serum, 2500 E heparin, 7500 E penicillin, 5 mg streptomycin, 0.25 mg phytohemagglutinin, 0.5 mg ascorbic acid and 0.5 mg glutathione). Chlorhexidine digluconate (CAS No. 7732-18-5; $C_{22}H_{30}Cl_2N_{10}{\cdot}2(C_6H_{12}O_7)$) was purchased from Sigma Chemical Co. (St. Louis, MO). The CHX compound in concentrations of 0.05, 0.1, 0.2, 0.4 and 0.5 mg/ml according to previous studies [27,28] was added to the cultures just before incubation for analysis as mentioned below. Each individual lymphocyte culture without CHX was studied as a control group, while Mitomycin C (10^{-7} M) was used as the positive control in all assays. Experiments conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki).

Cytogenetic analysis

Micronucleus assay. The micronucleus (MN) originates from chromosome fragments or whole chromosomes that lag behind at anaphase stage of nuclear division. The MN rates in human cells have become one of the standard cytogenetic tests for genotoxicity testing. The MN assay provides a convenient and reliable endpoint of both chromosome breakage and chromosome loss. Because MN formations are expressed in cells that have completed nuclear division, they are ideally scored in the binucleated (BN) stage of the cell cycle. The MN assay is the preferred method for measuring MN in cultured human cells because scoring is specifically restricted to once-divided cells. These cells are recognized by their BN appearance after inhibition of cytokines by cytochalasin-B. Over the past 25 years the MN assay has evolved into a comprehensive method for measuring chromosome breakage, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis [29,30].

In this study, the Micronucleus test (MN) was performed by adding cytochalasin B (Sigma; final concentration of 6 μg/ml) after 44-h of culture. At the end of the 72-h incubation period, the lymphocytes were fixed with ice-cold methanol:acetic acid (1:1). The fixed cells were put directly on slides using a cytospin and stained with Giemsa. All slides were coded before scoring. The MN was scored according to the criteria as described by Fenech [31]. At least 1000 binucleated lymphocytes were examined per concentration for the presence of one, two or more MN.

Cytotoxicity analysis

Trypan blue exclusion test. After exposure, cell viability was determined by trypan blue exclusion test. Cells were counted in a Neubauer Chamber. Viable cells were detected based on their ability to exclude the dye and non-viable cells turned blue due to defects in the cell membrane. We determined the percentage of viable cells in 16 counting fields.

Nuclear division index. For cell cycle analysis, 400 cells per treatment group were scored for the presence of one, two or more than two nuclei and the nuclear division index (NDI) was calculated as follows:

$$\mathrm{NDI} = \left[1\mathrm{N} + (2 \times 2\mathrm{N}) + (4 \times {>}2\mathrm{N})\right]/\mathrm{C}$$

where 1N is number of cells with one nucleus, 2N with two nuclei and >2N with more than two nuclei, C is number of cells examined [32].

Statistical analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) and Fisher's LSD

test using the S.P.S.S. 13.0 software. A p-value < 0.05 was considered statistically significant for all tests.

Results

The cytogenetic effects of CHX measured by MN frequency are given in Figure 1 according to the dose–response characteristics of the cultures. The MN frequency in the concentration interval 0.05–0.4 mg/ml was not statistically higher with respect to the control group ($p > 0.05$). However, a dose-dependent increasing was shown in the MN frequency and it was statistically higher at 0.5 mg/ml concentration compared to lower doses and the control group ($p < 0.05$). The cell viability and cell death of the exposed lymphocytes was evaluated by trypan blue exclusion test and a dose-dependent cellular toxicity was found. At the concentrations between 0.05–0.2 mg/ml, CHX was not found to be toxic on the cell viability and the values were similar with the control group. However, the trypan blue test showed ~ 40% and 50% reduction in cell viability at the concentrations 0.4 and 0.5 mg/ml, respectively. Cell death at these concentrations was also significantly higher compared to other concentrations and the control group ($p < 0.05$) (Figure 2). Figure 3 shows the cell proliferation rate of the lymphocytes measured by NDI at the end of the 72 h incubation period. The results demonstrated that CHX did not affect the cellular proliferation of the lymphocytes at the concentrations 0.05, 0.1 or 0.2 mg/ml. In contrast, a statistically significant reduction was found in the cultured cell proliferation at the concentrations 0.4 and 0.5 mg/ml with respect to other lower concentrations ($p < 0.05$).

Discussion

Long-term application of CHX is still recommended in several periodontal mechanical treatment modalities including full mouth disinfection technique for subgingival irrigation of deep periodontal pockets [6,7]. Thus, it is possible to hypothesize that CHX may be absorbed by the connective tissues penetrating through the gingival sulcus and may potentially cause toxic effects on the exposed cells. The current study was designed to evaluate the adverse effects of CHX at different concentrations on the genetic structure and cell viability of human lymphocytes. The cytogenetic effects on the cultured cells were analyzed by the MN test. The results showed no significant genotoxic potential at the concentrations lower than 0.5 mg/ml (0.05, 0.1, 0.2, 0.4 mg/ml), while it was found that CHX had significant genotoxicity on the cultured lymphocytes at 0.5 mg/ml concentration. In other studies on genotoxicity of CHX, significant side-effects have been reported on epithelial and blood cells when used for mouth rinsing [20,22]. Similarly, CHX has also been reported to be able to induce primary DNA damage in leukocytes and oral mucosal cells in rat models [18]. In this study, the cytotoxic potential of CHX was examined via the trypan blue exclusion test and NDI assay by testing the cell

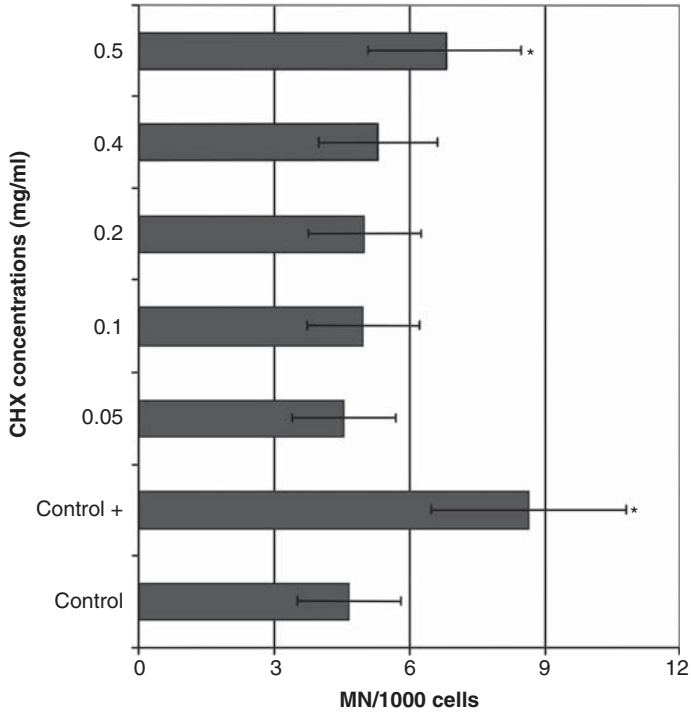

Figure 1. Effects of CHX on micronuclei frequency at various concentrations in cultured peripheral blood lymphocytes for 72 h. MN, micronucleus. *Significantly different from the control at the $p < 0.05$ level.

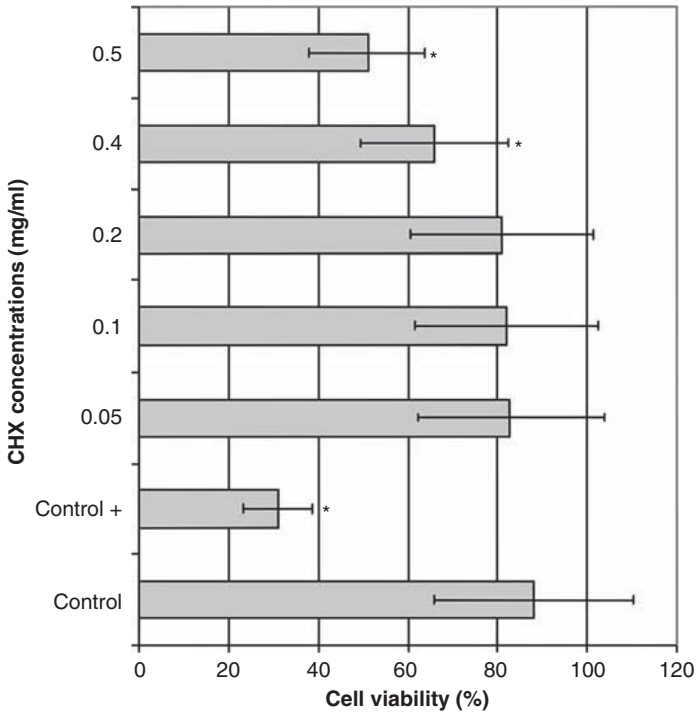

Figure 2. Percentage of cell viability of the CHX-exposed lymphocytes at different concentrations. *Significantly different from the control at the $p < 0.05$ level.

viability and proliferation of the lymphocytes, respectively. Previous studies showed cytotoxic effects, especially on cultured alveolar bone and on a variety of cell types including gingival epithelial cells, macrophages, neutrophils and red blood cells in culture [14,16]. Giannelli et al. [15] examined cell viability and cell death in osteoblastic, endothelial and fibroblastic cell lines exposed to various concentrations of CHX (0.0025%, 0.005%, 0.0075%, 0.01% and 0.12%) and showed a significant reduction of cell viability at higher concentrations. Almazin et al. [27] also found toxic effects on the cell viability of osteoblasts

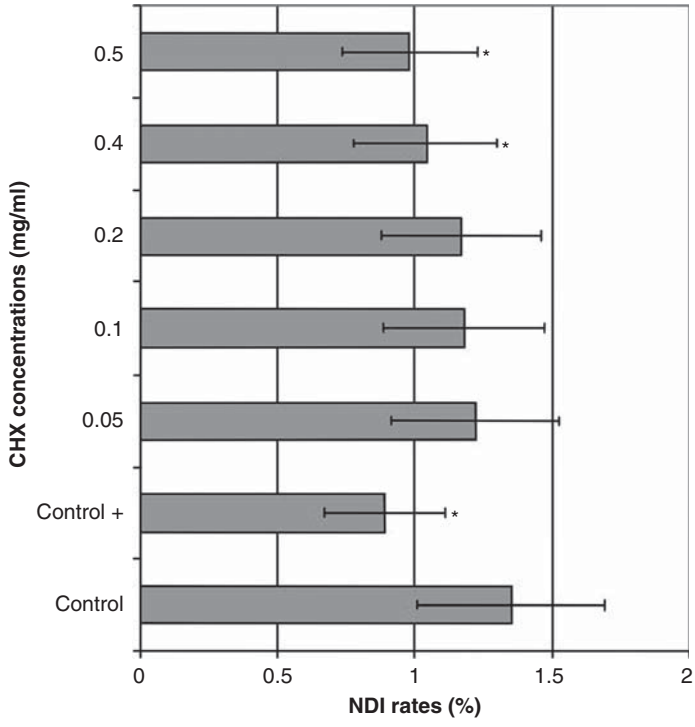

Figure 3. Cell proliferation activity of the cultured lymphocytes under CHX incubation for 72 h. NDI, nuclear division index. *Significantly different from the control at the $p < 0.05$ level.

at a concentration of 0.5 mg/ml. The results of this *in vitro* study agreed with their findings on cultured lymphocytes and indicated a statistically significant reduction in the cell viability of the exposed cells at higher concentrations of CHX (0.4 and 0.5 mg/ml). However, our results were not consistent with the findings of Chen et al. [28], who found a 50% reduction in cellular viability of periodontal ligament cells under CHX incubation at a very low concentration (15 μg/ml). Our results were also found to be conflicting with the findings of Lessa et al. [33] that CHX had high direct cytotoxic effects on the odontoblasts, even at very low concentrations (0.06–2%). In addition, the results of this study showed that CHX induced a dose-dependent reduction on lymphocyte proliferation and was consistent with the previous studies performed on human fibroblasts [34–36].

This study showed that CHX had dose-dependent genotoxic and cytotoxic effects on the human lymphocytes *in vitro*. However, the major limitation of this paper is that the data obtained from *in vitro* cultures may not reflect the *in vivo* condition. Therefore, further *in vivo* studies are needed to confirm the results of this study. It is possible that CHX may be absorbed by the gingival connective tissue cells by penetrating through gingival sulcus during subgingival irrigation of periodontal pockets. Hereby, it may interfere with the cellular immune and inflammatory processes depending on the mentioned toxic effects, especially at higher concentrations. It should be highlighted that the CHX concentrations which lead to toxic effects in this study was lower than the concentration of CHX products used as mouthrinsing solution. Therefore, it should be considered when using for pocket irrigation during periodontal mechanical procedures either novel CHX products at lower concentrations should be manufactured for clinical usage.

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