In Vitro Cytotoxicities of Mouthwashes Including Chlorhexidine

Klorheksidin İçeren Gargaraların *İn Vitro* Sitotoksisiteleri

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Geliş Tarihi/*Received:* 02.05.2012
Kabul Tarihi/*Accepted:* 05.09.2012

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ABSTRACT Objective: Chlorhexidine (CHX) is the most widely used antiseptic because of its high bactericidal capability and efficacy in the treatment of oral infections. However, there are emerging evidences suggesting that this compound may also have adverse effects on oral tissues and cells at the concentrations used clinically. In this study, the cytotoxic effects of CHX at different concentrations on L929 fibroblast cells *in vitro* were determined. **Material and Methods:** In this study, L929 cells were exposed to 0.2%, ten fold diluted 0.02% and one hundred fold diluted 0.002% concentrations of the mouthwashes including CHX for 1h, 3h and 24h to determine cell viability ratios by MTT assay. **Results:** Findings were evaluated by one-way analysis of variance which was followed by Bonferroni's post hoc comparison tests, $p<0.05$ were considered to be statistically significant. There was no statistically significant difference among mouthwashes in 0.2% and 0.02% concentrations in any exposure period ($p>0.05$). However, there were statistically significant differences among mouthwashes at 0.002% concentrations. According to the cell viability studies at the 0.2% and 0.02% concentrations at all exposure time periods, the cell viability ratio was around 20% when the control cell viability was considered to be 100. Cell viability ratio was higher than 50% in one hundred fold diluted 0.002% mouthwashes including CHX. **Conclusion:** Thus, clinically used CHX concentration must be carefully determined and reduced CHX concentrations must be used in periodontology and endodontics until more data are available related to CHX and its toxicity in vital tissues.

Key Words: Chlorhexidine; *in vitro*; drug toxicity; mouthwashes

ÖZET Amaç: Klorheksidin (CHX) yüksek bakterisidal etkisi ve oral enfeksiyonların tedavisindeki etkiniği nedeniyle en fazla kullanılan antiseptiktir. Fakat, bu bileşenin klinik olarak kullanıldığı konsantrasyonlarda oral dokular ve hücreler üzerinde yan etkileri olduğuna dair deliller bulunmaktadır. Bu çalışmada, ticari olarak mevcut CHX içeren gargaraların *in vitro* sitotoksisitesi, L929 fibroblast hücreleri kullanılarak belirlenmiştir. **Gereç ve Yöntemler:** Bu çalışmada, L929 hücreleri, ticari olarak mevcut %0,2 konsantrasyonda CHX içeren, on kat seyreltilmiş %0,02 konsantrasyonda ve yüz kat seyreltilmiş %0,002 konsantrasyonlarda CHX içeren gargaralarla, 1 saat, 3 saat ve 24 saat boyunca muamele edilmişler ve hücre canlılıkları MTT test sistemi ile belirlenmiştir. **Bulgular:** Elde edilen sonuçlar, tek yönlü varyans analizi ve Bonferroni düzeltmesi ile incelenmiştir, $p<0,05$ değerleri istatiksel olarak anlamlı bulunmuştur. Hiç bir muamele süresinde, %0,2 konsantrasyonda CHX içeren ve on kat seyreltilmiş %0,02 konsantrasyonda CHX içeren gruplar arasında istatiksel olarak anlamlı bir fark saptanmamıştır ($p>0,05$). Fakat yüz kat seyreltilmiş %0,002 konsantrasyonlarda CHX içeren gruplarda gargaralar arasında anlamlı farklılıklar saptanmıştır ($p<0,05$). Hücre canlılık sonuçlarına göre, muamele edilmemiş hücrelerin canlılığı %100 kabul edildiğinde %0,2 konsantrasyonda CHX içeren ve on kat seyreltilmiş %0,02 konsantrasyonda CHX içeren gruplarda hücre canlılık oranı %20 civarında iken yüz kat seyreltilmiş %0,002 konsantrasyonda CHX içeren gruplarda, hücre canlılık oranları %50'nin üzerindedir. **Sonuç:** Bu sonuçlar göstermektedir ki CHX *in vitro* ortamda hücrelere sitotoksik etki göstermektedir. Bu bakımdan, klinik kullanımda daha düşük konsantrasyonlarda CHX'in kullanılması ve bu ajanın sitotoksik özelliklerinin canlı dokularda da daha detaylı bir şekilde belirlenmesi gerekmektedir.

Anahtar Kelimeler: Klorheksidin; canlı dışında; ilaç toksisitesi; gargaralar

Turkiye Klinikleri J Dental Sci 2012;18(3):277-82

Chlorhexidine (CHX) has been the most widely used and studied antiplaque agent within the variety of different oral antimicrobials that are now available in the market.[1,2] This cationic antimicrobial agent is active against gram-positive and gram-negative bacteria, facultative anaerobes and aerobes, moulds, yeasts and viruses. Its action is by adsorbing onto the cell wall of the microorganisms. CHX causes leakage of intracellular components that leads to cell death.[3]

0.2% CHX solution was accepted to be clinically effective mouthwash that inhibited supragingival plaque formation and thus the development of chronic gingivitis and caries.[4] CHX has been incorporated into several dental agents such as mouthwashes, dental gels, root canal irrigants and varnishes.[5,6] The concentration of CHX may change where it is used at 0.12% and 0.2% as a mouthwash agent and in higher concentrations such as 40% in varnishes.[7] It may be used in high concentrations as a root canal irrigant as well.[6]

CHX has cytotoxic effects on a variety of eukaryotic cells which can alter membrane permeability, protein synthesis, lysosomal enzyme release, mitochondrial function disturbance, intracellular Ca increase and oxidative stress.[8-10] Faria et al. reported that CHX elicits accumulation of proteins in the endoplasmic reticulum, which causes ER overload, resulting in ER stress and cell death either by necrosis or apoptosis.[11] Several studies have reported that CHX has cytotoxic effects on mammalian cells even at the conditions used clinically. Patel et al. reported that commercially available mouthwash had cytotoxic effects on human osteoblast like cells in the concentrations used clinically and cytotoxicity might be increased when used for longer time periods.[12] It is reported that CHX is able to induce primary DNA damage in leukocytes and in oral mucosal cells in rats treated with 0.12% CHX twice daily during 8 days.[13] In addition, in cultured cells, cytotoxicity of CHX has been shown for blood cells, keratinocytes, fibroblasts, osteoblasts and osteoclasts and macrophages.[7] CHX has been tested for its genotoxic effects in terms of DNA damage by single cell electrophoresis and the results of the study indicated that leukocytes and kidney cells are potential targets for primary DNA damage after oral exposure to CHX.[13]

To provide analgesic and anti-oedema effect, benzydamine hydrochloride was added to mouthwashes with CHX.[14] This indazole nonsteroidal anti-inflammatory drug provides analgesic, antipyretic and anti-oedema effect within the mouthwash which helps the patient to treat inflammation and its side effects.[15]

Use of effective mouthwashes is important in oral health but the availability and the toxicity of all the medicaments used for health are needed to be addressed as well. Therefore, the aim of the present study is to examine the cytotoxic effects of CHX including mouthwashes on L929 cell viability at different concentrations *in vitro*. L929 cells were used as a model cell line for determining *in vitro* biocompatibility for several studies and its fibroblastic nature can be a model for gingival and fibroblastic cells.[16]

MATERIAL AND METHODS

TEST MATERIALS, CHEMICALS, AND REAGENTS

L929 mouse connective tissue fibroblasts were obtained from HUKUK (Foot and Mouth Disease Institute, Animal Cell Culture Collection, Ankara, Turkey). Cell culture medium (RPMI 1640), L-glutamine, gentamicine, fetal bovine serum were purchased from Biochrom (Germany). Trypsin and MTT [3-(4.5-dimethyldiazol-2-yl)-2.5 diphenyl tetrazolium bromid] from Sigma (Germany) and Trypan blue and dimethylsulfoxide (DMSO) from Applichem (Germany) were used.

CHX containing four different mouthwashes and one root canal irrigation solution tested in this study. Mouthwashes named as A (0.2% CHX gluconate containing); B (0.12% CHX gluconate containing); C (0.2% CHX gluconate containing); D (0.2% CHX gluconate containing) and the root canal irrigation solution named as E (2% CHX gluconate containing). The authors declare that there is no conflict of interest between the manufacturers of the materials used in this study.

IN VITRO CYTOTOXICITY TEST

L929 mouse connective tissue fibroblasts were routinely cultivated in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C, and 5% CO_2. 6×10^3 cells were seeded into each well of a 96 well plate and incubated for 24 h at 37°C. After 24 h, the culture medium was replaced with fresh medium containing mouthwash solutions. The final chlorhexidine gluconate concentrations used were 0.2%, 0.02%, 0.002% for undiluted, ten fold diluted and one hundred fold diluted test materials respectively. After 1h, 3h and 24h incubation periods, exposure medium was discarded. Cell viability after exposures were determined using a MTT assay to determine the effects of mouthwashes on the mitochondrial function. MTT is a colorimetric assay which measures reduction of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide to a purple formazan product. Following discarding the exposure medium 0.5 mg/mL of MTT were added to each well and incubated at 37°C, and 5% CO_2 for 4 h. After that, 200 µL dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan salts. The absorbance was immediately determined at 570 nm using an UV-visible single beam spectrophotometer (VersaMax, UK). The survival rates of the cells after mouthwash treatment were calculated as a percentage of control values considered to be 100%. To determine the morphological differences of the cells, microscopic images were taken by inverted microscope (Olympus CK-40, Japan) at x20 magnification.

STATISTICAL ANALYSIS

All assays were repeated at least three times to ensure reproducibility and six replicates of each concentration were performed in each test. The significance of difference between the treatment periods, treatment concentrations and mouthwash types were analyzed by two way analysis of variance (ANOVA) followed by Bonferroni's post-hoc comparision by Prism 5.0 (Graphpad, USA). A p value less than 0.05 was considered to be statistically significant.

RESULTS

Cell viability rates after exposure to CHX were determined by MTT assay. For each CHX concentration and exposure time, the absorbances of the CHX treated cells were converted into percentages as CHX non-treated control cells absorbances were considered to be one hundred. The averages of cell viability percentages of replicates and standart deviations were listed (Table 1).

TABLE 1: The averages of cell viability percentages of six replicates±SD.

Viability %	Exposure time	0.2%	0.02%	0.002%
A	1h	19.52±1.96	21.13±3.50	71.59±7.37
	3h	15.85±0.63	17.44±0.98	64.23±6.55
	24h	15.28±1.74	18.09±2.92	60.16±9.44
B*	1h	15.96±2.31	18.07±4.59	58.45±4.59
	3h	14.33±1.51	15.03±1.51	59.99±15.69
	24h	11.71±1.83	12.28±1.85	72.44±3.97
C	1h	16.25±2.89	16.93±2.94	66.99±12.96
	3h	13.39±0.96	13.87±1.08	55.06±5.35
	24h	11.56±1.81	13.21±1.66	61.77±7.85
D	1h	18.48±2.27	19.04±2.53	82.79±12.60
	3h	16.86±1.14	16.29±1.18	69.10±6.69
	24h	15.88±1.50	17.87±4.70	60.84±7.33
E	1h	15.00±0.06	24.69±1.02	57.65±8.23
	3h	18.42±0.66	20.03±1.96	52.81±9.61
	24h	15.00±0.66	16.20±2.52	49.76±5.64

*(CHX concentrations of B is 0.12%; 0.012%; 0.0012%).

There was no statistically significant difference among mouthwashes in 0.2%, ten fold diluted 0.02% CHX concentrations in any exposure period ($p>0.05$). Statistical findings revealed that there were statistically significant differences among mouthwashes at 0.002% CHX concentrations (Figure 1).

After exposure to the 0.2% and 0.02% CHX concentrations, cells were rounded up and progressively deattached from the culture plate, cell viability ratio was only around 20% (Figure 2). When we evaluate the 0.002% CHX concentration results, the cell viabilities are above 50%. The morphological features of the cells are elongated, flattened fibroblastic nature. But some of the cells were unable to perform the normal cytoplasmic spreading process (Figure 3).

Cell viability percentages of different mouthwashes at different exposure times were shown in Figure 3. At 0.2% and 0.02% CHX concentrations, cell viabilities are lower than 20% for 1, 3, 24 hour exposure periods. At 0.002% CHX concentrations, cell viabilities are higher than 20% for all the exposure periods. At 0.002% CHX concentration, cell viabilities are between 55-65% for all the mouthwash types at 1, 3 and 24 h exposure periods. Briefly, in our study, when comparing the cell viabilities between non-treated control cells and CHX exposed cells, CHX reduces cell viability rate between 80% and 40% at all concentrations. Although B has CHX concentrations were lower then the others (0.12%, 0.012% and 0.0012%), there was not any statistically significant cell via-

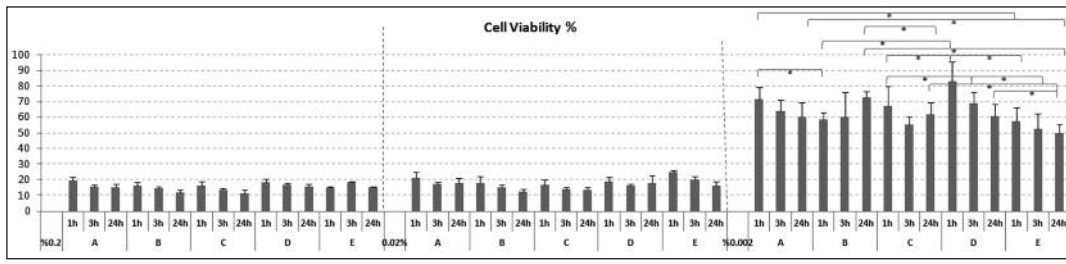

FIGURE 1: Cell viability ratio of different mouthwashes at different exposure times, *indicates the significant differences ($p<0.05$) between the groups compared to each other, (CHX concentrations of B is 0.12%; 0.012%; 0.0012%).

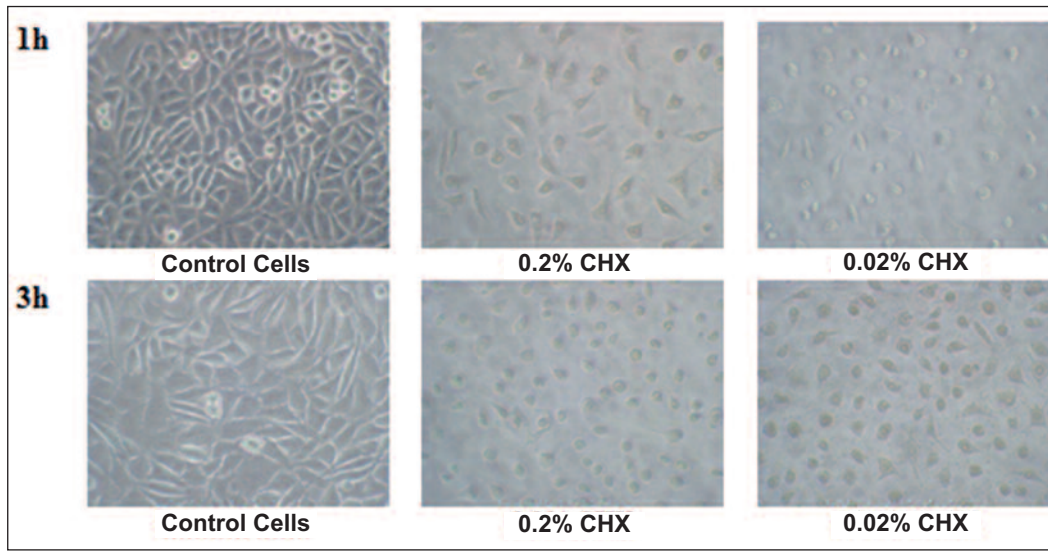

FIGURE 2: Representative microscopic photographs of the cells after exposure to the %0.2 and %0.02 CHX concentrations of E.
(See for colored form http://dishekimligi.turkiyeklinikleri.com/)

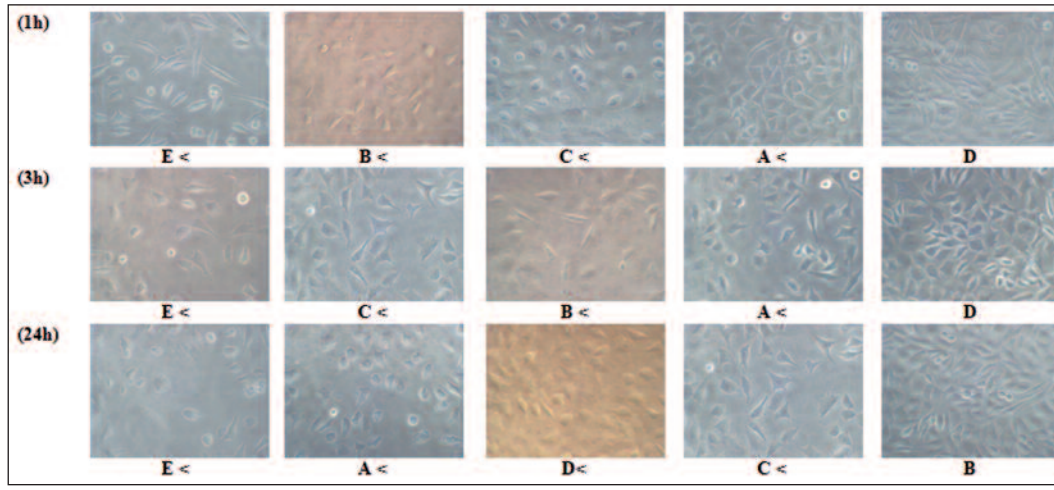

FIGURE 3: Representative microscopic photographs of the cells after exposure of 0.002% CHX of the test materials in ascending order according to cell viability. (See for colored form http://dishekimligi.turkiyeklinikleri.com/)

bility difference. This may attribute to its benzydamine-HCl (0.15%, 0.015% and 0.0015%) content.

CHX is a local medicament used in daily clinical practice in dentistry for a long period of time.[1] Its proven effect on decreasing plaque formation and controlling both gingivitis and dental caries and the antimicrobial effect on root canal microbiota increase its use in variety of dental materials.[2,17] However, there is a growing interest and suspicion on its cytotoxic properties and researchers are still working on to find out the intrinsic mechanism of CHX-induced cytotoxicity in eukaryotic cells.[11] Giannelli et al. reported that CHX has pro-apoptotic and pro-necrotic cell death effects *in vitro* which must be taken into consideration in the use of CHX for the treatment of periodontal and peri-implant diseases for regenerative therapy.[10]

Faria et al. shed a light on CHX induced cytotoxicity, they are reported that CHX elicits accumulation of proteins in the endoplasmic reticulum, which causes ER overload, resulting in ER stress and cell death either by necrosis or apoptosis.[11] CHX has serious toxic effects on several cells like gingival fibroblasts, endothelial cells, alveolar osteoblast cells and odontoblast-like cells *in vitro*.[10,18,19] Grassi et al. reported that CHX has cytotoxic effects on rat blood and kidney cells in vivo.[13]

Studies showed that the cytotoxic effects of CHX are dose and time dependant.[6,7,8,10,12,20,21] High concentration of the CHX causes immediate cell death, detachment of the cells from the culture plate, shrinking of the cells and induces irreversible cell damage.[8] Giannelli et al. showed that Saos-2 cells were sensitive to the CHX, after exposure to 0.01% concentration of CHX for 1 min the cell viability of the Saos-2 cells were reduced approximately by 57.5%.[10]

CHX also effects protein synthesis of human periodontal ligament cells. Chang et al. reported that at the CHX concentration of 0.01% the protein synthesis is inhibited by 80%.[6] Similar to the findings of the above mentioned studies, even the low concentrations of the CHX tested in our study affected cell death significantly. In this study, L929 cells were highly affected from the CHX exposure, since their viability was reduced approximately 80% in comparison with the unexposed control cells at 0.2% and 0.02% CHX concentrations for 1h, 3h and 24h time periods. In our study, even 0.002% CHX concentrations caused viability reduction effect in the range of 50% and 80%. Cytotoxicity of the mouthwashes may be affected by the other active ingredients such as alcohol or benzydamine HCl that is included. Likewise, although the CHX concentration was lower then the others in the mouthwash B containing benzydamine HCl in the

present study, findings revealed similar toxic effect *in vitro*.

In vitro cell viabilities may show some differences rather then in vivo cell viability. Similarly, Ergun et al. stated that not every compound which is cytotoxic *in vitro* necessarily be cytotoxic in vivo.[22] There are studies which are showed using CHX before and after oral surgery results in better healing conditions and less complication rates.[23,24]

Thus the concentrations of the CHX in the mouthwashes available in the market prove to be effective as an antimicrobial however at high concentrations the cytotoxicity of CHX remains as a problem.

In this study, the cytotoxicities of mouthwashes including CHX were compared to each other. According to the results, it can be concluded that not the type of the mouthwash but the CHX concentrations and exposure times are far more important in the cytotoxicity of the mouthwashes. Therefore, it is important to be precautious about such materials and due to the findings of an *in vitro* cyctotoxicity study. In addition, further studies must be done to understand the mechanism of CHX induced cytotoxicity in vivo. Also for clinical use in periodontology and endodontics, there must be caution sentences on the mouthwashes for not being swallowed and not used for a longer time then prescription.

Acknowledgement

This study is partially funded by 'Ege University, Directorate of Administrative and Financial Affairs by a grant number 2006-DIS-015.

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