Published online: 2019-09-27

Cytotoxicity of Mouthrinses on Epithelial Cells by Micronucleus Test

Ebru Olgun Erdemir[a], DDS, PhD
Abdulkadir Şengün[b], DDS, PhD
Mustafa Ülker[c], DDS, PhD

ABSTRACT

Objectives: To determine the cytotoxicity of three commercial mouthrinses Klorhex, Andorex and Tanflex on buccal epithelial cells using micronucleus (MN) test.

Materials and Methods: 28 patients with aged 16-24 undergone three mouthrinses' application were analyzed before and after one week exposure. Physiologic saline was used for the control group. The MN incidence was scored in the buccal epithelial of each participants. The difference in pre- and post-treatment after one week incidence of MN and plaque (PI) and gingival indices (GI) was compared by non-parametric statistical tests.

Results: The micronuclei incidence increased in Klorhex, Tanflex and Andorex groups after exposure to mouth rinses (P<.05). But when compared with the control group, there was not any difference between Andorex and control group (P>.05). In the other study groups, MN incidence was significantly increased after 7 days treatment (P<.05). GI scores of all groups were decreased significantly (P<.05). PI scores were decreased only in the Klorhex group (P<.05).

Conclusions: Our primary findings support the presence of possible cytotoxic effects of the mouthrinses on gingival epithelial cells. (Eur J Dent 2007;2:80-85)

Key Words: Mouthrinses; Cytotoxicity; Micronucleus test.

INTRODUCTION

Mouthrinses are a common adjunct to mechanical hygiene measures to facilitate the control of supragingival plaque, therefore dental caries and gingivitis.[1-3] Chlorhexidine (CHX) is a bisbiguanide antiseptic active against gram-positive and gram-negative bacteria, facultative anaerobes and aerobes, moulds, yeasts and viruses. Oral CHX mouthrinses have been effective in decreasing plaque formation and controlling gingivitis[4,5] and dental caries.[6,7] Its antibacterial activity arises from its positive charge at physiological pH, which produces nonspecific binding to the negatively-charged membrane phospholipids of bacteria; this causes an alteration in bacterial osmotic equilibrium, with potassium and phosphorus leakage. As the CHX concentration increases, cytoplasmic contents precipitate, triggering cell death. Several studies have shown that CHX has toxic effects on a variety of eukaryotic cells, with the presumed cytotoxicity mechanism being related to electrostat-

□ [a] Assistant Professor, Kirikkale University, Faculty of Dentistry, Department of Periodontology Kirikkale, Turkey.
[b] Associate Professor, Selcuk University, Faculty of Dentistry, Department of Conservative Dentistry, Konya, Turkey.
[c] Assistant Professor, Erciyes University, Faculty of Dentistry, Department of Conservative Dentistry, Kayseri, Turkey.

□ Corresponding author: Ebru Olgun Erdemir
Kırıkkale Universitesi, Diş Hekimliği Fakültesi, Periodontoloji AD., 71200, Kırıkkale, Turkey
E-mail: ebruerdemir@hotmail.com

ic resulting in inhibition of membrane-bound Na^+-K^+-ATPase. Release of lysosomal enzymes into the medium from rat peritoneal macrophages has also been described by Knuuttila and Söderling[8] and an increase in permeability to Ca^{2+} accompanied by leakage of LDH, from human gingival cells exposed to CHX by Babich et al.[9] This increased cell permeability due to the high affinity of CHX for negatively-charged organic radicals does not appear to be the only toxicity mechanism. It has been reported that protein synthesis is also affected in different degrees by CHX.[10,11] More recently CHX has been reported to inhibit the activities of two types of matrix metalloproteinases (gelatinases A and B) via a cation-chelating mechanism.[12]

It is a potent chemotherapeutic agent against Streptococcus mutans and dental caries. The effectiveness of chlorhexidine containing gels, mouthwashes, and toothpastes in caries prevention was confirmed by some researchers.[13-16]

Benzydamine HCl is a unique inflammatory analgesic agent structurally unrelated to steroid group, but also differs chemically from other nonsteroidal anti-inflammatory agents in that it is a base rather than an acid. Similar to corticosteroids, it stabilizes the cell membrane preventing the release of arachidonic acid, which initiates the inflammatory process. In common with NSAID's, Benzydamine inhibits cyclo-oxygenase, reducing synthesis of prostaglandin and related substances.[17]

Biomarkers of genotoxicity can be used as indicators of environmental carcinogen exposure. Micronucleus (MN) formation has been shown to be a reliable and sensitive biomarker for cytogenetic damage due to potential environmental mutagens. Briefly, micronuclei are acentric chromosome fragments or whole chromosomes left behind during mitotic cellular division and appear in the cytoplasm of interphase cells as small additional nuclei.[18,19] In light of the fact that over 90% of cancers are of epithelial origin[20] the MN assay has also been used in many epidemiological studies as an effective indicator of chromosome damage in exfoliated epithelial cells from lung, bladder, nasal and buccal cavity and cervix.[21-25]

In the present study, our aim was to determine the cytotoxicity of the mouthrinses Klorhex (0.2% Chlorhexidine Gluconate), Andorex (0.15% Benzydamine HCL and 0.12% Chlorhexidine Gluconate) and Tanflex (0.15% Benzydamine HCL) on buccal epithelial cells by MN test.

MATERIALS AND METHODS

The study population included 28 patients, 13 males and 15 females with aged 16-24 undergone three mouthrinses' application were analyzed before and after one week exposure. DMFT (The proportion of the number of teeth Decayed, Missing/extracted or Filled) scores of patients were zero. The patients had gingivitis as evidenced by multiple sites with a probing depth of 3 mm or less and without bone loss by radiographs. All participants had not previously received non-surgical and surgical periodontal therapy and were drawn from the patients with gingivitis at the Department of Periodontology. Subjects were medically healthy with no relevant medical or pharmacotherapy history that might influence the conduct of the study past 6 months and all of them were non-smokers and were not alcohol consumers. It was important that they had no caries or dental restorations; because it is known that some dental materials increase the frequency of micronuclei.[26] Therefore, the patients were chosen carefully among whose DMFT scores were zero. The criteria of patient selection were mentioned above and they were applied for all patients in this study.

The informed consent was taken from each patient. The patients were classified as three equal groups according to mouthrinses they used and the age differences among groups were not statistically significant (P>.05).

All participants received primary phase of nonsurgical treatment including oral hygiene instruction and scaling. After the treatment, mouthrinses were prescribed and the rinses were selected randomly. During one week, the patients used the prescribed mouthrinses rinsing twice a day. The rinses were Klorhex (0.2% Chlorhexidine Gluconate) (Drogsan, Turkey), Andorex (0.15% Benzydamine HCL and 0.12% Chlorhexidine Gluconate) (Delta Vital, Turkey) and Tanflex (0.15% Benzydamine HCL) (Abdi Ibrahim, Turkey). Physiologic saline was used for the control group. At baseline and after one week plaque index[27] (PI) (0=No plaque in the gingival area, 1=A film of plaque adhering to the free gingival margin, and adjacent area of the tooth. The plaque may be recognized only by running a probe across the tooth surface, 2=Moderate

accumulation of soft deposits within the gingival pocket and on the gingival margin and/or adjacent tooth surface that can be seen by the naked eye, 3=Abundance of soft matter within the gingival margin and adjacent tooth surface) and gingival index[28] (GI) (0=Normal gingiva, 1=Mild inflammation, slight change in color, slight edema; no bleeding on palpation, 2=Moderate inflammation, redness, edema, and glazing; bleeding on probing, 3=Severe inflammation, marked redness and edema, ulcerations; tendency to spontaneous bleeding) and MN incidence were recorded. Plaque and gingival indices were scored from buccal, lingual, mesial and distal points of each tooth.

Micronucleus test

The MN incidence was analyzed before and after one week three mouthrinses' application. The MN incidence was obtained in the buccal epithelial cells of each participant. Epithelial cells collected from oral mucosa were smeared on to clean microscope glass slides. The cells were fixed with cold 100% methanol. The slides were aged at 37^{0}C overnight and then stained with Giemsa and screened and 3000 nucleated cells were analyzed for the presence of MN at a final 100x magnification for each participant. The cells harboring micronucleus were recorded. Micronuclei were identified as DNA-containing structures in the cytoplasm, separated form the main nucleus, and of an area less than 1/3 of the area of the main nucleus, non-refractivity, not touching and same the color as the nucleus or lighter.[29]

Statistical analysis

The difference in the pre- and post-treatment incidence of PI, GI and MN was compared by Wilcoxon Signed Ranks Test. Changing of MN incidence was calculated as percentage for each group and compared with control by Oneway ANOVA and Tukey HSD tests. Statistical significance was determined at P< .05.

RESULTS

The difference in the pre- and post-treatment incidence of the MN, GI and PI values of this study at initial and the1st week are shown in Table 1. In groups of mouthrinses, incidence of the MN was increased. GI scores were decreased significantly in all groups (P<.05). There was no difference between pre- and post-treatment period in PI scores in Andorex and Tanflex groups (P>.05). In the third group (Klorhex), PI scores were decreased significantly (P<.05).

The changing of MN incidence as percentage for each group and comparing with control are shown in Table 2. There was not any difference between Andorex and control group (P>.05). In the other study groups, MN incidence was significantly increased after 7 days treatment of the mouthrinses (P<.05).

DISCUSSION

Increased frequency of micronucleated cells is

Table 1. The MN, GI and PI values (Mean±SD and WSR) of this study at initial and the 1st week (n=7).

Parameters	Mouthrinses	Pre-application Mean±SD	Post-application Mean±SD	WSR P
MN	Tanflex	2.29±0.49	7.14±1.86	0.018
	Andorex	5.71±6.32	9.00±8.35	0.018
	Klorhex	5.57±5.00	11.86±8.51	0.018
	Control	1.50±1.2	2.00±1.85	NS
GI	Tanflex	1.69±0.23	1.41±0.32	0.027
	Andorex	1.59±0.32	1.10±0.15	0.018
	Klorhex	1.66±0.13	1.17±0.17	0.018
	Control	1.56±0.23	1.43±0.32	NS
PI	Tanflex	1.13±0.24	1.04±0.11	NS
	Andorex	1.41±0.39	1.17±0.37	NS
	Klorhex	1.41±0.30	0.91±0.43	0.028
	Control	1.15±0.23	1.11±0.04	NS

WSR: The statistical significance according to Wilcoxon Signed Ranks Test
NS: Not significant (P>.05)

a biomarker of genotoxic effects that can reflect exposure to agents with clastogenic and aneugenic modes of action.[21] The micronucleus assay was applied to verify the effects of the three mouthrinses' treatment. The results of this study showed that although the micronuclei incidence increased in Klorhex, Tanflex and Andorex groups after exposure to mouthrinses, the micronuclei incidence of Klorhex and Tanflex groups increased, except Andorex, when compared with the control group.

CHX is a chemical agent currently used as a local antiseptic in daily clinical practice. The cytotoxicity of CHX has been assayed using cell lines or primary cultures of mammalian cells.[8,11] Eren et al[30] evaluated the CHX with comet assay (single cell gel electrophoresis, or SCGE) and suggested that a statistical increase was observed in the damaged buccal and blood cells after the CHX application. They mentioned that detected DNA damage after CHX use might be the indication of an earlier effect, before DNA repair begins, and could be reversible. Wilken et al[31] determined the in vitro cytotoxic effect of 0.2% chlorhexidine gluconate and 0.15% benzydamine-HCL and revealed that all the human gingival fibroblasts exposed to chlorhexidine gluconate and benzydamine-HCL were immediately fixated onto the tissue culture surfaces. They concluded that it should be ascribed to the activity of the active ingredients in the mouthrinses and the relative cytotoxicity of the active ingredients of all mouthrinses is very high for human gingival fibroblasts. Various organisms and genetic endpoints, including the gene mutations as well as chromosomal damage in mammalian cells, comprise a test battery for analyzing the mutagenic activity of a chemical.[32] Among these assays, the micronucleus test in vitro is a multiple-endpoint test to indicate chromosomal aberrations.[33,34] However, in literature there is not a study that evaluates the cytotoxicity of neither CHX nor benzydamine-HCL by MN test.

Table 2. Changing of MN incidence as percentage for each group and comparing with control group.

	Mean	SD
Tanflex	214.3	69.0[c]
Andorex	91.1	75.6[a,b]
Klorhex	164.4	120.1[b,c]
Control	31.3	53.0[a]

[a]: P>.05 according to Oneway ANOVA and Tukey HSD tests.
[b]: P<.05 according to Oneway ANOVA and Tukey HSD tests.
[c]: P<.05 according to Oneway ANOVA and Tukey HSD tests.

Although our main aim was not to compare the measurements of plaque and/or gingivitis between the mouthrinses, the approach clearly revealed the expected result that CHX was significantly more effective at preventing the development of plaque than the other two rinses. However, it might be doubtful whether CHX is still the golden standard as mouthrinse for the prevention of plaque formation and development of gingivitis[35-37] when considering its cytotoxicity on human cells as shown in the present study.

CHX mouthrinses have been effective in decreasing plaque formation and controlling both gingivitis[4,5] and dental caries.[6,7] But, in this study the patients were chosen carefully among whose DMFT scores were zero, because it is known that some dental materials increase the frequency of micronuclei,[26] and this could affect the results of the study. Therefore, patient selection was one of the most important parts of this study.

MN test that we have applied to buccal epithelial cells of patients detects the possible cytotoxic and/or mutagenic effects of some mouthrinses in this tissue. This study suggests that the amount of absorbed mouthrinses may have been sufficient to induce MN frequencies in buccal epithelial cells of patients even in the range of the biological tolerance levels of these mouthrinses. But it was strange that although there were differences between both Klorhex and the control groups and Tanflex and the control groups, there were not any differences between Andorex and the control groups in our study. Hidalgo & Dominguez[38] showed that cytotoxicity mechanism could be produced in a time- and CHX concentration- dependent manner. This reason can be contributed to this result, as lowered concentration of CHX exists in Andorex. In another point of view, the combinations of CHX and Benzydamine HCL can lessen the cytotoxicity of this mouthrinse. Although there is not any study about the effects of this combination (Andorex) on cytotoxicity, Waaler et al[39] and Barkvoll & Rolla[40] noted that triclosan reduced the toxicity of sodium lauryl sulphate while used in combinations.

The exposure to cytotoxic and/or mutagenic

factors, host factors, methods and scoring data explain the 75% of total variance, with the largest contribution attributable to laboratory methods.[41] Regarding the host factors, Bonassi et al[41] suggested that the large majority data showed a higher MN frequency in females and a uniform increase in MN frequencies with age in both genders, an increase that is especially steep after 40 years of age. In our study, we could not find any differences in genders and all study subjects were young people, therefore we could not find also any differences in age.

It is common in Turkey to prescribe mouthrinses to the patients who suffer from periodontal diseases by the clinicians. Mouthrinses are widely utilized in daily oral and dental hygiene to control plaque.[4,7] Patients should be alerted that mechanical plaque control is more important than chemical plaque control and warned about improper use of these products. The findings support only the prescription of agents or products with the highest proven clinical indication.

The short-term effects of mouthrinses were evaluated in this study. More detailed further studies of the long-term effects of mouthrinses are needed.

CONCLUSIONS

In conclusion, our findings with the micronucleus test to indicate chromosomal mutations add valuable information to complete the overall elucidation of the cytotoxic activity of some mouthrinses. The formation of micronuclei to various extents by these chemicals was occurred in the present investigation.

REFERENCES

1. Mandel ID. Chemotherapeutic agents for controlling plaque and gingivitis. *J Clin Periodontol* 1988;15:488–498.
2. Brecx M. Strategies and agents in supragingival chemical plaque control. *Periodontol* 2000 1997;15:100–108.
3. Hickel R. Wirkstoffe gegen Plaque und Bakterien. *Quint Bonusausgabe* 1997;1:45–57.
4. Rosin M, Welk A, Kocher T, Majic-Todt A, Kramer A, Pitten FA. The effect of a polyhexamethylene biguanide mouthrinse compared to an essential oil rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque regrowth. *J Clin Periodontol* 2002;29:392-399.
5. Sreenivasan P, Gaffar A. Antiplaque biocides and bacterial resistance: a review. *J Clin Periodontol* 2002;29:965-974.
6. Guggenheim B, Regolati B, Schmid R. & Mühlemann HR. Effects of the topical application of mutanase on rat caries. *Caries Res* 1980;14:128–135.
7. Lindquist B, Edward S, Torell P, Krasse B. Effect of different caries preventive measures in children highly infected with mutans streptococci. *Scand J Dent Res* 1989;97:330-337.
8. Knuuttila M, Söderling E. Effect of chlorhexidine on the release of lysosomal enzymes from cultured macrophages. *Acta Odont Scand* 1981;39:285–289.
9. Babich H, Wurzburger BJ, Rubin YL, Sinensky MC, Blau L. An in vitro study on the cytotoxicity of chlorhexidine digluconate to human gingival cells. *Cell Biol Toxicol* 1995;11:79–88.
10. Goldschmidt P, Cogen R, Taubman, S. Cytopathologic effects of chlorhexidine on human cells. *J Periodontol* 1977;48:212–215.
11. Purcher JJ, Daniel JC. The effects of chlorhexidine digluconate on human fibroblasts in vitro. *J Periodontol* 1992;63:526–532.
12. Gendron R, Grenier D, Sorsa T, Mayrand D. Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine. *Clin Diagnos Lab Immunol* 1999;6:437–439.
13. Kidd EAM . Role of chlorhexidine in the management of dental caries. *Int Dent J* 1991;41:279–286.
14. Emilson CG. Potential efficacy of chlorhexidine against mutans streptococci and human dental caries. *J Dent Res* 1994;73:682–691.
15. Van Rijkom HM, Truin GJ, Van't Hof MA. A meta analysis of clinical studies on the caries-inhibiting effect of chlorhexidine treatment. *J Dent Res* 1996;75:790–795.
16. Baca P, Munoz MJ, Bravo M, Junco P, Baca AP. Effectiveness of chlorhexidine–thymol varnish (Cervitec) for caries reduction in permanent first molars of 6–7-year-old children. 24-month clinical trial. *Commune Dent Oral Epidemiol* 2002;30:363–368.
17. Matthews RW, Scully CM, Levers BG, Hislop WS. Clinical evaluation of benzydamine, chlorhexidine, and placebo mouthwashes in the management of recurrent aphthous stomatitis.*Oral Surg Oral Med Oral Pathol* 1987;63:189-191.
18. Heddle JA, Hite M, Kirkhart B, Mavourinin K, Gregor JTM. Newell GW, Salamone MF. The indication of micronuclei as a measure of genotoxicity. *Mutat Res* 1983;123:61–118.
19. Albertini RJ, Anderson GR, Douglas L, Hagmar K, Hemminki F, Merlo AT, Natarajan H, Norppa DEG, Shuker R, Tice MD, Waters, A. Aitio. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutat Res* 2000;463:111–172.
20. Cairns J. Mutation selection and the natural history of cancer. *Nature* 1975;255:197–200.

21. Burgaz S, Iscan A, Büyükbingöl ZK, Bozkurt A, Karakaya AE. Evaluation of micronuclei in exfoliated urothelial cells and urinary thioether excretion of smokers. *Mutat Res* 1995;335:163–169.
22. Lehucher-Michel MP, Favre-Eloff G, Devictor B, Duffaud F, Botta A. Effect of smoking on micronucleated epithelial cells in smears from the uterine cervix. *Carcinogen* 1997;18:749–753.
23. Valverde M, del Carmen Lopez, I Lopez, I Sanchez TI, Fortoul P, Ostrosky-Wegman E, Rojas. DNA damage in leukocytes and buccal and nasal epithelial cells of individual exposed to air pollution in Mexico City. *Environ Mol Mutagen* 1997;30:147–152.
24. Salama SA, Serrana M, Au WW. Biomonitoring using accessible human cells for exposure and health risk assessment. *Mutat Res* 1999;436:99–112.
25. Burgaz S, Çakmak G, Erdem O, Yilmaz M, Karakaya AE. Micronuclei frequencies in exfoliated nasal mucosa cells from pathology and anatomy laboratory workers exposed to formaldehyde. *Neoplasma* 2001;48:144–147.
26. Burgaz S, Demircigil GC, Yilmazer M, Ertas N, Kemaloglu Y, Burgaz Y. Assessment of cytogenetic damage in lymphocytes and in exfoliated nasal cells of dental laboratory technicians exposed to chromium, cobalt and nickel. *Mutat Res* 2002;521:47–56.
27. Silness J, Löe H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. *Acta Odontol Scand* 1964;22:121-135.
28. Löe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand 1963;21:533-551.
29. Countryman PI, Heddle JA. The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutat Res* 1976;41:321-332.
30. Eren K, Ozmeric N, Sardas S. Monitoring of buccal epithelial cells by alkaline comet assay (single cell gel electrophoresis technique) in cytogenetic evaluation of chlorhexidine. *Clin Oral Invest* 2002;6:150-154.
31. Wilken R, Botha SJ, Grobler A, Germishuys PJ. In vitro cytotoxicity of chlorhexidine gluconate, benzydamine-HCl and povidone iodine mouthrinses on human gingival fibroblasts. *SADJ* 2001;56:455-460.
32. Zeiger E. Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: premises, promises and performance. *Regul Toxicol Pharmacol* 1998;28:85-95.
33. Kirsch-Volders M, Elhajouji A, Cundari E, Van Hummelen JM. The in vitro micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction. *Mutat Res* 1997;392:19-30.
34. Miller B, Pötter-Locher F, Seelbach A, Stoper H, Utesch D, Madle S. Evaluation of in vitro micronucleus test as an alternative to the in vitro chromosomal aberration assay: position of the GUM Working Group on the in vitro micronucleus test. *Mutat Res* 1998;410:81-116.
35. Addy M, Moran J, Wade W. Chemical plaque control in the prevention of gingivitis and periodontitis. In: Lang NP, Karring T, eds. Proceedings of the 1st European Workshop on Periodontology London: *Quintessence* 1994:244–257.
36. Bollen CML, Quirynen M. Microbiological response to mechanical treatment in combination with adjunctive therapy: a review of the literature. *J Periodontol* 1996;67:1143–1158.
37. Quirynen M, Avondroot P, Peeters W, Pauwels, M, Coucke W, Van Steenberghe D. Effect of different chlorhexidine formulations in mouthrinses on de novo plaque formation. *J Clin Periodontol* 2001;28:1127-1136.
38. Hidalgo E, Dominguez C. Mechanisms underlying chlorhexidine induced cytotoxicity. Toxicol in Vitro 2001;15:271-276.
39. Waaler SM, Rolla G, Skorland KK, Ogaard B. Effects of oral rinsing with triclosan and sodium lauryl sulphate on dental plaque formation: a pilot study. *Scand J Dent Res* 1993;101:192-195.
40. Barkvoll P, Rolla G. Triclosan protects the skin against dermatitis caused by sodium lauryl sulphate exposure. *J Clin Periodontol* 1994;21:717-719.
41. Bonassi M, Fenech C, Lando Y, et al. Human micronucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes.1. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environ Mol Mutagen* 2001;37:31–45.