Original Article

In *Vitro* Comparative Study on the Effect of Free Alcohol Mouth Wash Versus 10% Alcohol Based Mouth Wash on Oral Epithelial Cells Using Transformed Cell Line GMSM-K

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ABSTRACT

Introduction: Maintaining a good oral hygiene is very critical for the quality of life. The use of mechanical method alone to preserve the oral hygiene was insufficient activity. Therefore, various types of mouthwashes are available for use as daily oral hygiene measure. Several researches were examining the antibacterial effect of these agents. However, there are limited data about the cytotoxicity of these mouthwashes on epithelial cells. The target of the present study is to detect the toxic effect of zero alcohol mouth rinse with essential oils and sodium fluoride (EOF) compared with alcohol based mouthwash containing essential Oils (Eos) and chlorhexidine (CHX) mouthwash which used as positive control on GMSM-K cultured epithelial cells. Material and

Material and Methods: Zero alcohol mouth rinse with EOF, alcohol-based mouth rinse combined with EOs and CHX were applied to the epithelial cell line at 10%, 35% and 75% concentrations. The toxic effect of the mouthwashes was assessed by MTT assay to detect cell viability, flow cytometry using annexion V gene in order to detect cell apoptosis and necrosis and finally, by measuring the production of interleukin (IL)-6 by enzyme-linked immunosorbent assay.

Results: By analyzing the obtained data using ANOVA statistical test, it was found that the mouthwash with alcohol and Eos had the strongest toxic effect on epithelial cells in concentration dependent manner with statistically significant difference at 75% concentration followed by CHX and zero alcohol EOF mouthwashes respectively.

Conclusion: It was found that zero alcohol mouth rinse including EOF was the safest mouthwash on the epithelial cells.

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Key Words: Chlorhexidine; epithelial cell; essential oils; mouthwash containing alcohol; sodium fluoride.

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INTRODUCTION

Oral mucosa is lined by stratified squamous nonkeratinized epithelium and underlying loose connective tissue.[1] The epithelium is permeable to certain substances which can be used for therapeutic purpose, but also it is the most exposed to the toxic effects of different substances that can be found in the oral environment.[2] Various antimicrobial solutions are used as zero alcohol mouth rinse including Essential Oils and sodium fluoride (EOF), alcohol-based mouth rinse including Essential oils (Eos) and Chlorhexidine (CHX) in order to improve oral health and management of periodontal and gingival pathology.[3,4]

The three commercial mouthwashes examined in our study have well known mode of antimicrobial action. Essential oils mouthwash had a broad spectrum antibacterial activity, inhibit bacterial aggregation, retard bacterial multiplication, prevent plaque development and lowering mass of the plaque and pathogenicity.[5,6] Sodium fluoride is a chemical compound added to the mouthwashes to improve dental hygiene by formation of fluoroapatite.[7] In addition, it was reported that the existence of fluoride in mouthwash contributed to the inhibition of streptococcus mutants growth in plaque.[8] Chlorhexidine mouthwash binds to the surface of the microorganisms resulting in increasing the cell membrane permeability and loss of important contents of the cytoplasm. Other studies reported its role in cell death induction through dysfunction of the mitochondria.[9]

Alcohol is acting as vehicle in most mouthwashes for active essential oils to facilitate the plaque penetration and to give the patient a sensation of clean mouth.[10,11] The alcohol in the mouthwashes was having antimicrobial properties and facilitate the purpose of dissolving active ingredients[10] However, there are some contraindications for using mouthwashes containing alcohol, like the patients that have atrophied mucosa, pregnant women, alcohol addicts, and infants. There are also some unpleasant effects, like pain sensation in patients suffered from mucosal injuries, sensation of burning or soreness, or a sensation of mouth dryness.[12]

In order to avoid the contraindications of alcohol-based mouthwashes in certain conditions, scientific interest is becoming more widespread in introducing a mouthwash with powerful anti-plaque qualities, better biocompatibility to oral tissue and no alcoholic ingredients. One of these products is the zero alcohol essential oils with sodium fluoride mouthwash (EOF). Marchetti et al.[3] had been studied the effect of alcohol free EOF and Eos containing

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alcohol in inhibiting plaque formation and compared them with CHX mouthwash as positive control. They stated that alcohol free EOF showed the similar effect on inhibition of plaque formation as alcohol based Eos mouthwash without statistical significant difference. Thus, in our research we examined the biocompatibility of theses mouthwashes on epithelial cells. To the best of the authors` knowledge, this was the first study examined the toxic effect of zero alcohol EOF mouthwash on epithelial cells in *vitro*.

MATERIAL AND METHODS

Laboratory procedures

This study was done on GMSM-K epithelial cell line (ATCC, No. ACS-4005, USA) (Figure 1) in (Vacsera Co., Egypt) following a previous protocol.[12] In brief, Cells were cultured using DMEM (In*vitro*gen/Life Technologies) supplemented with 10% FBS (Hyclone,), 10 ug/ml of insulin (Sigma), and 1% penicillin-streptomycin. Cells were sub-cultured into 96-well plates (ATCC, USA). Commercial mouthwashes were diluted to 10%, 35% and 75% using distilled water. Mouthwashes used in the research were consists of:

- Alcohol based EOs mouthwash: consisting of thymol (0.0060%), eucalyptol (0.09%), mentol (0.042%) and methyl-salicylate (0.064%) in a 26.9% hydroalcoholic vehicle.
- CHX mouthwash: formed of 0.12% chlorhexidine hydrochloric acid. Used as positive control.
- Zero alcohol EOF mouthwash: formed of thymol (0.0060%), eucalyptol (0.09%), mentol (0.042%) and methyl-salicylate (0.064%), zero alcohol and 0.02% sodium fluoride.

Cells were incubated with mouthwashes for 1, 5 and10 minutes in the 96-well plates at 37◦C.

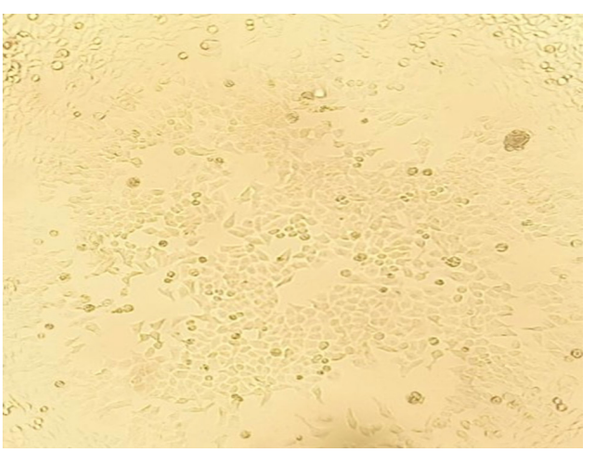

Fig. 1: Microscopic examination of cultured GMSM-K epithelial cell line (unstainedx200).

Assessment of the cellular viability

The viability of the cells was assessed using 3- (4, 5-dimethyl-thiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) colorimetric assay. Spectrophotometrically measure absorbance at 570 nm wavelength.

Assessment of cell death using Flow Cytometry analysis of Annexin V-FITC (Biovision Catalog #: K101-25, -100, -400)

1. Collect 1-5 x 105 cells by centrifugation after application of the three mouthwashes at 75%.
2. Resuspend cells in 500 µl of 1X (Annexin V) Binding Buffer.
3. Add 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI 50mg/ml.) in order to detect necrosis of the cells.
4. Incubate for 5 min at room temperature in the dark.
5. Analyze Annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 530 nm) using FITC signal detector and PI staining by the phycoerythrin emission signal detector .

Measurement of IL-6 production by enzyme-linked immunosorbent assay (ELISA)

1. Epithelial cells (1×10^6 cells mL−1) were divided into 24-well flat-bottomed plates (TPP TechnoPlastic Products AG, Trasadingen, Switzerland) and incubated with Eos, CHX and EOF mouthwashes at 75% concentration at 37◦Cand 5% CO2.
2. IL-6 had been measured by ELISA, following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, a 96-well flat bottom Maxisorp (Thermo Fisher Scientific, Waltham, MA, USA) was coated with IL6 antibody. Then, we washed the plate and blocked before 100 µL of the supernatants and diluted specific standards were added to the respective wells serially.
3. After washing several times, the IL6 had been detected by using the specific antibody. The reagent was added into each well and, after development of the color, the plate was read at 450 nm by ELISA plate reader.

Statistical analysis

Data was described using IBM statistical package for social sciences (SPSS) advanced statistics, version 21 (SPSS Inc., Chicago, IL). Comparing the three groups was carried out by one-way ANOVA, then Post-hoc Tukey test to compare between groups. *P-value*≤0.05 considered a significant difference.

RESULTS

Statistical analysis of the results showed that alcohol-based Eos, CHX and zero alcohol EOF mouthwashes

resulted in progressive decrease of the viability of epithelial cells by increasing the concentration of the mouthwashes (Table 1, Figure 1). After application of 10% alcohol based Eos the viability of epithelial cells was reduced to 92.8% while after 75% the viability was decreased to 86% with statistical significant difference when compared to each other ($p \leq 0.05$)(table 1). While CHX mouthwash showed gradual decrease of the viability of the cell by increasing the concentration but without significant difference ($p \geq 0.05$) and less toxicity than alcohol-based Eos mouthwash which was unpredictable. However, zero alcohol EOF showed mildest decrease in cell viability by increasing the concentration when compared to other mouthwashes with statistically significant difference ($p \leq 0.05$) (Table 1, Figures 2,3).

By using flow cytometry test to detect the amount of cell death at 75% of the mouthwashes, it was observed that the zero alcohol EOF mouthwash showed the least apoptotic cells (14.6196) and necrotic cells (10.0196) followed by CHX with apoptotic cells (19.7061) and necrotic cells (13.6303).The most toxic mouthwash was alcohol based Eos causing apoptosis of the cells (20.4703) and necrosis (14.2161) (Table 2, Figures 4,5).

Regarding IL-6 production, alcohol-based Eos mouthwash promote IL-6 production (12.7) more than CHX mouthwash (12.2) and zero alcohol EOF mouthwash (9.3) without any statistical significant difference ($p \geq 0.05$) (Table 3, Figure 6).

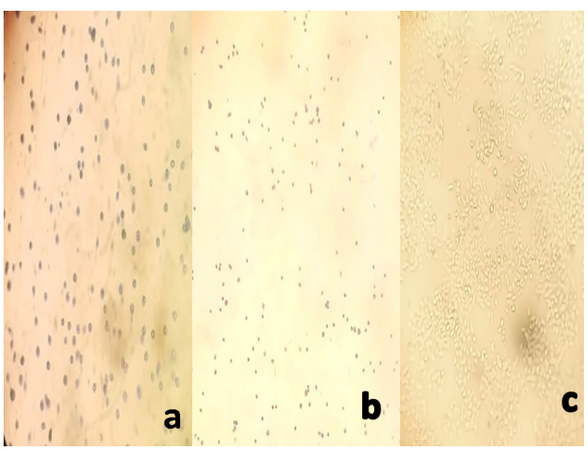

Fig. 2: Microscopic examination of cultured epithelial cell after application of 75% Eos mouthwash showing marked cell death (a), 75% of CHX mouthwash showing moderate cell death (b) and 75% of EOF showing minimal cell death(c) (unstained x200).

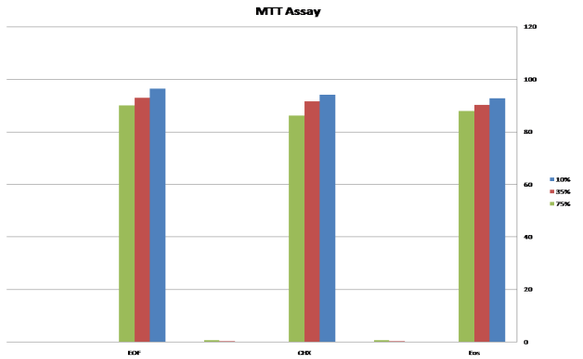

Fig. 3: Viability of epithelial cells at different concentrations of the mouthwashes using ANOVA and Post hoc Tukey. Eos and EOF showing statistical significant difference.

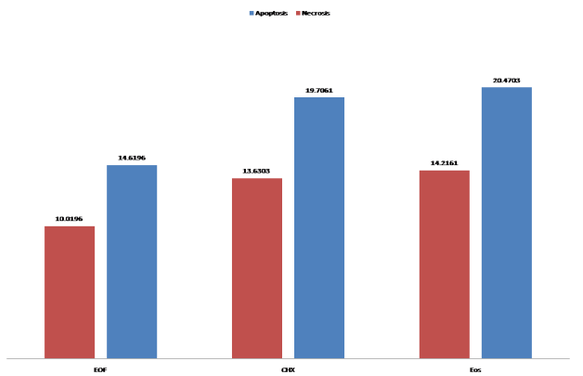

Fig. 4: Flow cytometry assessment of epithelial cells death after application of different mouthwashes at 75% concentration.

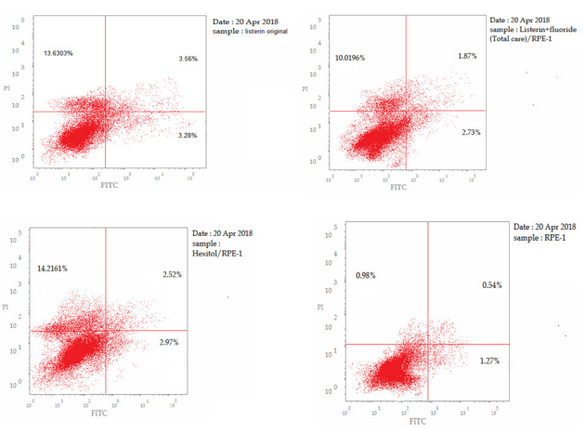

Fig. 5: Flow cytometry curves of epithelial cell death after addition of three different mouthwashes at 75% concentration

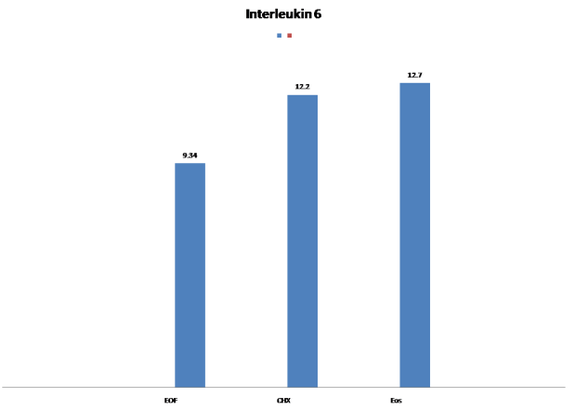

Fig. 6: Significant production of IL-6 from epithelial cells after application of mouthwashes at 75% concentrations using ANOVA and Post hoc Tukey.

Table 1: Showing cell viability in different groups.

Groups / Conc.	1o%	35%	75%	*P-value* (ANOVA)
EOs	92.8±3.3[a]	90.3±3.1[a]	86±3.7[b]	$P \leq 0.05$
CHX	94.1±4[a]	91.6±4.6[a]	88.1±6.2[a]	$P \geq 0.05$
EOF	96.5±1.4[a]	93±1.36[b]	90±2.2[c]	$P \leq 0.05$

Table 2: Flow Cytometry assessment of epithelial cells death after application of the mouthwashes at 75% concentration.

Groups / test	necrosis	apoptosis
EOs	20.4703	14.2161
CHX	19.7061	13.6303
EOF	14.6196	10.0196

Table 3: Epithelial cell Production of IL-6 after application of the mouthwashes at 75% concentration using one way ANOVA and Post hoc Tukey showing statistical significance.

Groups	75%
EOs	12.7±1.96[a]
CHX	12.2±1.25[a]
EOF	9.34±1.90[a]
P-value	$p > 0.05$

*Groups with different letters are statistically significantly different

DISCUSSION

The aim of our study was to detect the toxic effects of zero alcohol- essential oils with sodium fluoride mouthwash (EOF) and an alcohol-based essential oils mouthwash (EOs) compared with a positive control (CHX 0.12%) on epithelial cell line using MTT assay, flow cytometry and measuring production of IL6 by ELISA. The results stated that the zero alcohol EOF mouthwash was the most biocompatible mouthwash and has the least toxic effect on the epithelial cell line when compared with other mouthwashes. Chlorhexidine mouthwash is used as positive control because of its documented cytotoxicty to various cell types including epithelial cells.[12-14]

Essential oils containing alcohol and CHX mouthwashes were used for long time in conjugation with tooth brushing in order to improve oral hygiene and treatment of gingival and periodontal pathology, so their cytotoxic effect on various oral cells was well documented.[4,13,14] However, little is known about the recent forms of mouthwash which is formed of zero alcohol essential oils containing sodium fluoride EOF, used in the current study.

As observed in this study, alcohol-based Eos mouthwash has the most destructive effect leading to a significant drop in viability of epithelial cell line at 75% followed by CHX and EOF which leading to drop of cell viability to 88% and 90% respectively. In addition, these results were confirmed by detection of cell death using flow cytometry, it had been found that alcohol-based Eos mouthwash was the most superior mouthwash in apoptosis and necrosis induction followed by CHX and zero alcohol EOF mouthwashes respectively. Moreover, it had been found in our study that alcohol-based Eos induce the production of pro-inflammatory cytokine IL-6 more than CHX and zero alcohol EOF mouthwashes respectively.

These results could be explained by previous scientists who documented that alcohol in mouthwashes acts as an antiseptic, preservative and solvent. It causes denaturation of the protein and dissolution of lipid, so it has antibacterial and antiviral. It had been found that mouthwashes containing alcohol above 20% may have adverse effects in oral mucosa such as mucosal ulceration, detachment of epithelium, gingivitis, keratosis, petechiae and pain.[5] Moreover, Eos mouthwash had an adverse effect on eukaryotic cells including epithelial cells. Essential oils mouthwash can induce mitochondrial membranes depolarization by reducing the potentiality of the membrane, affecting Ca++ cycling and decreasing the gradient of pH, affecting the ATP pool resulting in cell death.[14]

It had been reported that CHX mouthwash resulting in rupture of mitochondrial membrane and leakage of cytochrome c protein inducing apoptosis.[15] Tsutsui et al.[16] showed that the viability of cultured gingival keratinocytes reduced progressively with increasing concentrations of CHX. In addition, Kanjevac et al.[13] reported that the CHX mouthwash have cytotoxic effect on epithelial cells of buccal mucosa in time dependent manner. Flemingson et al.[13] and Tsourounakis et al.[4] had been reported a similar effect of CHX, but on fibroblasts in culture. Because of the different structure and biological behaviour, this coincidence of the results should be taken with a grain of salt.

It had been documented that fluoride inhibits secretion and synthesis of proteins that affects certain signalling pathway involved in apoptosis such as mitogen-activated protein kinase, and pathways of nuclear factor kappa B causing cell death.[17,18] However, the addition of fluoride in mouthwashes was in low concentrations (0.02%) thus was nontoxic to various oral cells including epithelial cells.

CONCLUSION

Finally, it was found that zero alcohol EOF mouthwash was the best biocompatible mouthwash to epithelial cells as compared to Eos and CHX mouthwash. In our study EOF without alcohol mouthwash was showed the mildest cytotoxic effect on epithelial cell line and thus could be one of the best oral antiseptics. The presented results are interesting, but to confirm the clinical significance, further clinical studies are needed.

ABBREVIATIONS

EOs: Essential oils mouthwash, **EOF:** Essential oils with sodium fluoride mouthwash, **CHX:** Chlorhexidine mouthwash, **IL:** Interleukin, **DMEM:** Dulbecco`s Modified Eagle Medium, **FBS:** Fetal Bovine Serum, **ELISA:** enzyme-linked immunosorbent assay.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة مقارنة في المختبر حول تأثير غسول الفم الخالي من الكحول مقابل غسول الفم المعتمد على الكحول بنسبة ١٠ ٪ على الخلايا الظهارية للفم باستخدام خط الخلايا المحول GMSM-K

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المقدمة: الحفاظ على نظافة الفم أمر بالغ الأهمية لنوعية الحياة. كان استخدام الطريقة الميكانيكية وحدها للحفاظ على نظافة الفم نشاطًا غير كافٍ. لذلك ، تتوفر أنواع مختلفة من غسول الفم للاستخدام كإجراء يومي لنظافة الفم. تم إجراء العديد من الأبحاث حول التأثير المضاد للبكتيريا لهذه العوامل. ومع ذلك ، هناك بيانات محدودة حول السمية الخلوية لغسول الفم على الخلايا الظهارية.

الهدف من البحث: هو الكشف عن التأثير السام لغسول الفم الخالي من الكحول بالزيوت الأساسية وفلوريد الصوديوم (EOF) مقارنة بغسول الفم الذي يحتوي على الكحول والذي يحتوي على زيوت أساسية (Eos) وغسول الفم الكلورهيكسيدين (CHX) والذي يستخدم كعنصر تحكم إيجابي على الخلايا الظهارية المستزرعة GMSM-K.

مواد واساليب العلاج: شطف الفم الخالي من الكحول باستخدام EOF ، شطف الفم بالكحول مع EOs و CHX تم تطبيقهما على خط الخلايا الظهارية بتركيزات ١٠٪ و ٣٥٪ و ٧٥٪. تم تقييم التأثير السام لغسول الفم عن طريق مقايسة MTT للكشف عن حيوية الخلية ، وقياس التدفق الخلوي باستخدام جين الملحق V من أجل الكشف عن موت الخلايا المبرمج والنخر ، وأخيراً عن طريق قياس إنتاج الإنترلوكين (IL) -٦ عن طريق مقايسة الممتز المناعي المرتبط بالإنزيم.

النتائج: من خلال تحليل البيانات التي تم الحصول عليها باستخدام اختبار ANOVA الإحصائي ، وجد أن غسول الفم بالكحول و Eos كان له التأثير السمي الأقوى على الخلايا الظهارية بطريقة تعتمد على التركيز مع وجود فرق معتد به إحصائيًا عند تركيز ٧٥ ٪ يليه CHX وغسولات EOF الخالية من الكحول على التوالي.

الاستنتاج: وجد أن غسول الفم الخالي من الكحول بما في ذلك EOF كان أكثر غسول الفم أمانًا على الخلايا الظهارية.