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RESEARCH ARTICLE

Apoptosis inducing effects of chlorhexidine and essential oil mouthwashes on BHK-21 fibroblast cell line: An *in vitro* study

[version 1; peer review: 1 approved, 1 not approved]

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Abstract

Background: The maintenance of oral health can be achieved mainly by mechanical and chemical means. Among chemical agents, mouthwashes are widely used for personal oral hygiene because of their ability to inhibit dental plaque. The antibacterial effects of essential oils (EOs) and chlorhexidine (CHX) are well documented; however, the reaction of host tissue to these substances has a poor documentation. Until now studies have not examined the effect of EOs with sodium fluoride (EOF) on fibroblast cell lines. The aim of this study was to examine the effect of mouth rinse EOs, EOF and CHX on the apoptosis of fibroblast cell line.

Methods: BHK-21 fibroblast cell line was cultured and incubated in Eagle's Minimum Essential Medium containing EOs, EOF and CHX mouthwashes with different doses (15% or 25%) and various exposure times. Cell apoptosis was assayed using RT-PCR.

Results: EOs, EOF and CHX induce apoptotic effects on fibroblasts in a dose and time dependent manner.

Conclusion: CHX is the most cytotoxic mouthwash to fibroblasts as compared to mouthwashes containing EOs and EOF.

Keywords

fibroblast, chlorhexidine, essential oils, essential oils with sodium fluoride, mouthwash.

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Approval Status

	1	2
version 1
26 Oct 2018	view	view

1. **Marwa Mokbel ElShafei**, Misr International University, Cairo, Egypt
2. **Thomas E. Lallier**, Louisiana State University Health Science Center, New Orleans, USA

Any reports and responses or comments on the article can be found at the end of the article.

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Introduction

Fibroblasts are the common cell type in connective tissue and plays a major role in normal function and in pathologic changes of oral tissues^{1,2}. Various antiseptic agents are available in the form of mouthwashes, among them are essential oils (EOs), EOs with sodium fluoride (EOF) and chlorhexidine (CHX). The antibacterial action of these agents had been widely examined^{1,3,4}.

In fibroblasts, EOs can induce depolarization of the mitochondrial membranes by decreasing the membrane potential, affecting ionic Ca^{++} cycling^{5,6}. They change the permeability of membranes resulting in liberation of cytochrome, leading to apoptosis⁷. Fluoride inhibits protein synthesis and thus it influences certain signalling pathways included in apoptosis such as the P53 pathway^{8,9}.

CHX inhibits protein and DNA synthesis, resulting in cell apoptosis^{3,10}. Moreover, CHX can damage mitochondrial membranes resulting in leakage of apoptosis-inducing proteins^{11,12}.

Many previous studies have compared the effect of both CHX and EOs mouthwashes on cultured fibroblasts^{3,5}. However, to the best of our knowledge, the combined effect of EOs and EOF hasn't been studied yet. In this study, we investigated the effect of different doses and different exposure periods of mouthwashes containing EOs, EOF and CHX on a fibroblast cell line.

Methods

Laboratory procedures

This study was performed on BHK-21 cell line in (Vacsera Co., Egypt). It was cultured according to a previous protocol³. Briefly, BHK-21 cells were cultured in Eagle's Minimum Essential Medium (ATCC, Manassas, VA, USA). They were sub-cultured into 96-well plates (ATCC, USA).

Commercial mouthwashes (3 total) were diluted to 15% and 25% using distilled water. Mouthwashes used were composed as followed:

- EO mouthwash: containing thymol (0.0060%), eucalyptol (0.09%), mentol (0.042%) and methyl-salicylate (0.064%) in a 26.9% hydroalcoholic vehicle;
- EOF mouthwash: containing EOs as before and 0.2% sodium fluoride;
- CHX mouthwash: containing 0.12% chlorhexidine hydrochloric acid.

Cells were incubated with mouthwashes for 0, 1, 2, 3, 5, 10 minutes in the 96-well plates at 37°C.

Assessment of apoptosis

After cell culturing, RNA extraction and quantitative real time polymerase chain reaction (PCR) was performed as follows¹:

1. Total RNA was isolated using the RNeasy Micro Kit (Qiagen, Germany) and RNA concentration was measured spectrophotometrically using Nanodrop ND-1000.

2. Reverse transcription was performed on 600 ng of total RNA using oligo dT primers and MMLV Reverse Transcriptase in a final volume of 20 mL (Invitrogen, CA, USA) for 5 minutes at 65°C, followed by one hour at 37°C.
3. Samples were subsequently heated for 15 minutes at 70°C to terminate the reverse transcription reaction.
4. Real-time quantitative PCR was performed on the cDNA samples using an Applied Biosystem Real-Time Detection System. Annexin V was the target gene for apoptosis. Primer sequences for Annexin V: sense primer, 5' CAGTCTAGGTGCAGCTGCCG 3' and antisense primer, 5' GGTGAAGCAGGACCAGTGT3'. The following primer sequences were used for GAPDH (housekeeping gene): sense primer, 5' ATG GCC TTC CGT GTT CCT AC 3' and antisense primer, 5' GCC TGC TTC ACC AC C TTC TT 3'.
5. Real-time PCR was conducted by amplifying the cDNA with iQ SYBR Green Universal Master Mix (Applied Biosystems, CA, USA).
6. Melting curve analysis of amplification products was performed at the end of the PCR reaction to confirm that a single PCR product was detected. For every PCR reaction, GAPDH was used as the internal control.
7. A relative quantification method $2^{-\Delta\Delta CT}$ method was used¹³. The relative quantitation value of target was normalized to the internal control (GAPDH).

Statistical methods

Data was analysed using IBM Statistical Package for Social Sciences (SPSS), version 21 (SPSS Inc., IL, USA). Numerical data was described as mean and standard deviation and comparisons between these were performed using ANOVA and a post-hoc Tukey test.

Results

Microscopic examination

Microscopic examination of fibroblast cell line after application of different mouthwashes at different concentrations and for different time durations is shown in [Figure 1](#).

Effect of concentration of EOs, EOF and CHX mouthwashes on apoptosis induction in fibroblast cell line

In all mouthwashes 25% concentration showed a statistically significant increase in apoptosis compared with 15% and the untreated control ([Table 1](#) and [Figure 2](#)).

Effect of duration of application of EOs, EOF and CHX mouthwashes on apoptosis induction in fibroblast cell line

In the EO mouthwash, values for apoptosis continued to significantly increase after 2, 3, 5 and 10 minutes for the 25% concentration ([Table 2](#) and [Figure 3](#)).

In the EOF mouthwash, at 25% concentration, a significant increase in apoptosis values was detected after 5 and 10 minutes ([Table 3](#) and [Figure 4](#)).

Figure 1. Microscopic examination of fibroblast BHK-21 cells treated with various mouthwashes at different concentrations and for different durations. From L-R (A) Untreated fibroblast cell line demonstrated confluent growth of elongated cells. Some cells appeared bipolar and some were multipolar. (B–E) Essential oil mouthwash: (B) 15% for 1 minute showed many viable spindle shaped fibroblasts; (C) 15% for 10 minutes showed decreased spindle shaped fibroblasts; (D) 25% for 1 minute showed few viable spindle shaped fibroblasts; (E) 25% for 10 minutes showed obvious cell free areas. (F–I) Sodium fluoride mouthwash: (F) 15% for 1 minute showed many viable spindle shaped fibroblasts and few apoptotic cells; (G) 15% for 10 minutes showed destroyed fibroblasts; (H) 25% for 1 minute showed large cell free areas; (I) 25% for 10 minutes showed more obvious large cell free areas. (J–M) Chlorhexidine mouthwash: (J) 15% for 1 and (K) 10 minutes many viable fibroblasts were detected; (L) 25% for 1 minute showed massive reduction in cell viability, many dead or destroyed fibroblasts surrounded by large cell free areas; (M) 25% for 10 minutes, only few remnants of dead fibroblasts were obvious.

Table 1. Apoptosis induction in BHK-21 fibroblast cell line by EO, EOF and CHX mouthwashes compared to an untreated group (n=3).

Mouthwash	Untreated	15% mouthwash dilution	25% mouthwash dilution	P-value**
EO	1.01±0.2 ^a	1.34±0.470 ^a	5.97±1.90 ^b	0.0000
EOF	1.01±0.2 ^a	1.77±0.710 ^a	5.30±1.93 ^b	0.0000
CHX	1.01±0.2 ^a	1.97±0.52 ^a	10.3±2.61 ^c	0.0000

*Groups with different letters are statistically significantly different. **ANOVA. EO, essential oil; EOF, sodium fluoride; CHX, chlorhexidine.

Figure 2. Apoptosis induction in fibroblast BHK-21 cells after incubation with various mouthwashes at different concentrations. EO, essential oil; EOF, sodium fluoride; CHX, chlorhexidine (n=3).

Table 2. Effect of duration of application and percentage of an essential oil mouthwash on apoptosis induction in BHK-21 fibroblast cell line.

Mouthwash	Time						P-value**
	0	1	2	3	5	10	
15%	1.01±1.5 ^a	1.15±0.18 ^a	1.17±0.21 ^a	1.22±0.46 ^a	1.5±0.502 ^a	1.74±0.65 ^a	0.6448
20%	1.01±1.5 ^a	4.66±1.28 ^b	5.26±1.58 ^b	5.99±1.64 ^b	6.57±1.65 ^c	6.78±2.3 ^c	0.0001

*Groups with different letters are statistically significantly different. **ANOVA

In the CHX mouthwash, at 25% concentration, a significant increase in apoptosis values started after 1 minute and continued to increase by time (Table 4 and Figure 5).

Dataset 1. File containing: Part A, Raw numerical data of RNA concentration at each concentration and duration of application of the mouthwashes; part B, raw phase contrast photomicrograph of fibroblast cell line after application of mouthwashes at different concentrations and durations

<https://doi.org/10.5256/f1000research.16337.d220684>

Discussion

The effectiveness of CHX and EOs mouthwashes in controlling the formation of plaque and gingivitis has been demonstrated¹⁴. However, there are concerns that these products are harmful to oral cells¹⁵⁻¹⁷.

CHX is toxic, even in low concentrations, for different cell types including fibroblasts in culture^{18,19}. Topical application of CHX can result in its penetration through the epithelial barrier leading to tissue damage²⁰. In our study, and increase in concentration for EOs, EOF and CHX mouthwashes resulted

Figure 3. Apoptosis induction in BHK-21 fibroblast cell line after application of different concentrations of essential oil containing mouthwash for different time durations (n=3).

Table 3. Effect of duration of application and percentage of a sodium fluoride mouthwash on apoptosis induction in BHK-21 fibroblast cell line.

Mouthwash	Time						P-value**
	0	1	2	3	5	10	
15%	1.01±1.5 ^a	1.19±0.15 ^a	1.24±0.18 ^a	1.42±0.34 ^a	2.13±0.31 ^a	2.74±0.25 ^a	0.0019
20%	1.01±1.5 ^a	4.82±2.06 ^a	4.99±0.95 ^a	5.38±1.36 ^a	6.02±2.7 ^b	7±1.96 ^b	0.0008

*Groups with different letters are statistically significantly different. **ANOVA

in an increase in apoptosis. This was also observed by Faria *et al.*¹ who reported that CHX induced apoptosis of cultured fibroblasts in a concentration dependent manner. Values for apoptosis at 15% and 25% concentrations of EOF were slightly higher than EOs alone in our study, demonstrating the ability of fluoride to enhance apoptosis induction, as previously described⁹.

A significant increase in apoptosis induction was seen at 15% concentration CHX and at 25% concentration EOs and EOF. This suggests that CHX is more effective than EOs and EOF in apoptosis induction. This result was in agreement with Tsourounakis *et al.*⁵ who reported that there was a significant reduction in cell survival that occurred at concentrations of 15% CHX and 25% EOs²¹.

Figure 4. Apoptosis induction in BHK-21 fibroblast cell line after application of different concentrations of sodium fluoride containing mouthwash for different time durations (n=3).

Table 4. Effect of duration of application and percentage of a chlorhexidine mouthwash on apoptosis induction in BHK-21 fibroblast cell line.

Mouthwash	Time						P-value**
	0	1	2	3	5	10	
15%	1.01±1.5 ^a	1.86±0.71 ^a	1.87±0.36 ^a	1.94±0.69 ^a	2.06±0.25 ^a	2.38±0.45 ^a	0.1606
20%	1.01±1.5 ^a	8.34±1.9 ^c	10.6±3.4 ^b	11.1±1.96 ^d	11.3±0.83 ^b	11.4±1.5 ^b	0.0000

*Groups with different letters are statistically significantly different. **ANOVA

The increase in duration of application of EOs, EOF and CHX didn't significantly increase apoptosis at the low concentration of 15% in the present study. However, at 25% CHX, increase in the duration of treatment significantly enhanced apoptosis, which was significant obvious after 1 minute. This means that

CHX is more efficient than EOs and EOF in apoptosis induction at lower concentrations. This was in accordance with Flemingson *et al.*³ who stated that the apoptotic effect of CHX on fibroblasts occurs early, after 1 minute exposure, and added that CHX had the maximum cytotoxicity followed by EOs.

Figure 5. Apoptosis induction in BHK-21 fibroblast cell line after application of different concentrations of chlorhexidine containing mouthwash for different time durations (n=3).

Conclusion

CHX mouthwash is the most cytotoxic to fibroblasts compared to EOs and EOF containing mouthwashes. Adding fluoride to EOs in low concentrations didn't worsen the adverse effects of Eos as shown by the combination mouthwash containing EOs and fluoride.

Data availability

F1000Research: Dataset 1. File containing: Part A, Raw numerical data of RNA concentration at each concentration and

duration of application of the mouthwashes; part B, raw phase contrast photomicrograph of fibroblast cell line after application of mouthwashes at different concentrations and durations., [10.5256/f1000research.16337.d220684](https://doi.org/10.5256/f1000research.16337.d220684)²².

Grant information

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Thomas E. Lallier

Department of Oral Biology, Louisiana State University Health Science Center, New Orleans, LA, USA

The authors demonstrate that chlorohexidine (CHX)- and essential oil (EO)-containing mouth rinses induce cell death in fibroblasts in culture. They attempt to prove that this is through apoptosis, using qPCR to quantify the expression of Annexin V (Annexin-A5) in these cells. Unfortunately, this assay is flawed. Annexin V is a normally-expressed protein that happens to bind to phosphatidylserine, a phospholipid normally on the cytoplasmic leaflet of the plasma membrane. During apoptosis, this inner leaflet is exposed to the extracellular environment. Thus, Annexin V can be used to identify apoptotic cells fluorometrically. An increase in Annexin V transcript expression has not been linked with apoptosis. Furthermore, apoptosis is a post-translational event involving cytochromes, caspases and other proteins (e.g. BCL2 and BAX), and thus cannot be detected using a transcriptional assay like qPCR.

Thus, the only finding of this manuscript is that these mouth rinses lead to cell death, which is neither novel nor worthy of independent indexing.

The manuscript also contains numerous other errors (e.g. the photomicrographs in Figure 1 are of too little contrast to demonstrate anything, and are not even properly identified), and this is not yet ready for indexing.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cell biology, including cell survival, cell attachment, cell migration assays, as well as qPCR, ELISA, and immunocytochemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 09 November 2018

<https://doi.org/10.5256/f1000research.17846.r39910>

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Marwa Mokbel ElShafei

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In my opinion normality tests should be performed before statistical tests and considering the small sample size, non-parametric tests could have been used. The article is approved, though a recommendation can be added after the conclusion in order to recommend further studies to evaluate the amount and percentage of mouth wash absorption to the connective tissue and hence reaching fibroblasts as the direct effect on the cell line doesn't mimic the real life effect.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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