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Assessment of Listerine Cool Mint mouthwash influence on possible DNA damage measured by buccal micronucleus cytome assay - preliminary results

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DOI: 10.31383/ga.vol3iss1pp24-35

Abstract

Listerine is a brand of mouthwash most used worldwide in oral hygiene maintenance. Due to its antimicrobial and antifungal characteristics, it can stop/diminish the development of plaque and gingivitis. Among different types of this mouthwash, all 5 ingredients of Listerine Cool Mint, 21.6% ethanol and 4 herbal extracts-thymol, menthol, eucalyptol and methyl salicylate, have shown capacity to cause cell damage and buccal epithelial cells are in direct contact. Buccal micronucleus cytome assay (BMN) measures changes in differentiation as the frequency of basal/differentiated, binuclear, and cells in different phases of cell death-apoptosis/necrosis (cells with condensed chromatin, karyorrhectic, pycnotic and karyolytic cells) and changes in genomic stability measured as micronuclei or nuclear buds/broken eggs frequency. Samples from 10 healthy individuals using Listerine Cool Mint mouthwash twice/day during two-weeks treatment were analyzed before and after the treatment. There was no significant influence on cell differentiation and genomic stability on the group level, although micronuclei frequency (MN) of entire group was higher after the treatment (1 vs. 1.5). We also found interindividual differences and showed that hard liquor consumers had higher MN frequency. Future studies should include more individuals, especially those that regularly consume alcohol for the analysis of possible synergistic influence and consequential increase in risk of changes in genomic stability. Genetic polymorphisms in enzymes responsible for metabolism of ethanol should also be considered, since they may drastically influence the duration of ethanol exposure and its metabolite acetaldehyde and also influence genomic instability and possible development of oral squamous cells cancer.

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Received

May, 2019

Accepted

May, 2019

Published

June, 2019

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Keywords

buccal cells, Listerine mouthwash, buccal micronucleus cytome assay, genome damage

Introduction

Mouthwashes are oral hygienic products used to maintain oral cavity hygiene (Croatian Chamber of Dental Medicine, 2013), usually divided in: alcoholic/aqueous solutions with different active substances such as chlorhexidine, triclosan, hexethidine, hydrogen peroxide, fluorine or essential oils (menthol, eucalyptus ...); or into cosmetic/therapeutic solutions and their combinations. Therapeutic mouthwashes include ingredients with antiseptic/anti-plaque characteristics that can prevent the onset or development of oral diseases, gingivitis and bad breath and inhibit organisms in the oral cavity that cause plaques (Fine et al., 2007) or caries development by preventing demineralization and stimulating the remineralisation of dental enamel and teeth strengthening (Boyle et al., 2014).

In the USA, a product that reduces the appearance of plaque and gingivitis should undergo two clinical trials to obtain a marketing authorization, and these studies must show 15% (estimated proportional reduction) or 20% (arithmetic mean of the estimated proportional reduction) reduction in plaque and gingivitis incidence in patients with mild gingivitis symptoms during testing for at least 4 weeks, taking into account the placebo group (American Dental Association ADA, 2011). In the European Union, mouthwashes are treated as a category of antibacterial products under the supervision of the European Medical Agency (EMA). If such a product is a blend of herbal preparations, it is expected that each individual ingredient must either increase clinical efficacy or alleviate side effects and does not exhibit toxic activity, which is tested in toxicological studies on each component of the blend separately and the blend itself (for more details see EMA webpages about herbals products and WHO, 1996). The most common brand name of mouthwash in the world is Listerine with proven anti-plaque (Johnson & Johnson, 2014a,b) and gingivitis suppression capabilities (Lamster et al., 1983; Gordon et al. 1985), together with strong antimicrobial and antifungal activity (Kubert et al., 1993; Yamanaka et al., 1994; Kasuga et al., 1997; Okuda et al., 1998). Listerine was originally a cosmetic herbal preparation consisting of 4 essential oils, peppermint, Eucalyptus, winter-green and

thyme oil (Lambert Pharm Company, 1912; Vlachojanis 2015, 2016). Its composition has changed over the centuries and finally extracted oil mixture consists of methanol (0.042%), eucalyptol (0.092%), methyl salicylate (0.06%) and thymol (0.064%) in a 27% ethanol solvent (21.6-26.9%) (FDA, 2003; Vlachojanis, 2015, 2016). Today methanol and methyl salicylate are synthesized, so they are no longer natural herbal extracts. Listerine also contains inactive ingredients: water, ethanol (21.6%), sorbitol, flavor, poloxamer 407 (polymeric stabilizer that increases the solubility of poorly water-soluble compounds), benzoic acid, sodium saccharin, sodium benzoate and a protected compound of Listerine brand FD & C Green No. 3. Since Listerine contains 21.6% of alcohol, which is known to cause oxidative damage and is already metabolised in the mouth, which of itself can cause an increased amount of DNA damage, the hypothesis of this paper was that Listerine Cool Mint stimulates apoptosis in the buccal cells and that due to the potential genotoxic effect on DNA it may have a lasting effect in changes of cells genomic stability.

Long-term exposure to high alcohol content should also favor genotoxic effect and stimulate programmed cell death due to increased membrane permeability and cell dehydration (Manzo-Avalos & Saavedra-Molina, 2010). The two general aims of the study were: (a) to determine whether two-week treatment with a specific mouthwash containing 21.6% ethanol causes changes in the rate and frequency of buccal cell differentiation, and whether it induces apoptosis (fused chromatin, karyocytic cells, pycnotic and kariolytic cells) and (b) to determine the effect on the amount and form of genomic defects measured as differentiated cells with micronucleus, and with the nuclear bud/broken egg structure.

Materials and methods

Volunteers and questionnaire

Ten healthy subjects, 5 male and 5 female, from the Zagreb area participated in the study. All respondents were less than 60 years old due to the fact that the parameters for micronucleus test on buccal cells change for the age group over 60 years.

After the interviewers had explained the purpose of the research and the manner of conducting the treatment, volunteers completed questionnaires and gave written consent. The questionnaires included questions about the lifestyle, general diet and habits and a detailed food and drink description consumed

Sampling, slide preparation and scoring

Unless otherwise stated, the reagents were purchased from Sigma Aldrich, USA, plastics from Eppendorf, Germany and the glass slides and coverslips from Biognost, Croatia. The samples were taken before

Table 1. Answers from questionnaire on lifestyle and habits of respondents.

QUESTIONS	ANSWERS (N)			
SEX	Male 5	Female 5		
AGE	Mean 32	Standard deviation 10.64	Range 18-51	
EXPOSURE TO TOXIC AGENTS	Pesticides, herbicides, fungicides 0	Paints, varnishes, adhesives 0	Processing of wood, metal 0	Cytotoxic drugs, organic solvents 3
ALCOHOL CONSUMPTION	Few times/week 3	Few times/month 6	Never 1	
SMOKING	Yes 5	No 5		
DIAGNOSTIC/THERAPEUTIC TREATMENTS IN HOSPITAL	Ionizing radiation 3	Surgery 0		
DISEASES	Chronic 2	Tumour 0		
THE USE OF DRUGS	Antibiotics 2	Analgetics 0	The rest 0	
THE USE OF SUPPLEMENTS IN DIET	Vitamins 3	Minerals 2	Fish Oil 0	

24h before sampling. If the volunteers used Listerine or other mouthwashes, they were not supposed to take them at least three months before the study started. The summary of the main questions with answers is given in Table 1.

Ethics Committee

This research was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb.

Treatment of subjects

The subjects used Listerine Cool Mint mouthwash twice a day, in the morning and evening. Each time 20 mL of solution was swished in the mouth during 30 seconds without rinsing, as suggested by the producer for regular use.

The treatment lasted for 2 weeks. After the treatment examinees were asked for a subjective impression and they all noted the burning sensation while swishing the solution in the mouth.

and after the two weeks of Listerine use. All respondents gave their samples after washing the mouth three times with water to remove bacteria and dead cells. The samples were collected with a sterile hard tooth brush with a small head so that the subjects made 10 circles on the inside of each cheek, after which the brush was immersed in a conical polypropylene centrifuge tube (50 mL) containing 20 mL buffer for buccal cells. All the solutions were prepared and samples processed according to the protocol by Thomas et al. (2009) with few modifications (Milić et al., 2018; Pastorino et al., 2018). The samples were processed on the day of collection. After centrifugation, supernatant was removed and the cells resuspended in 20 mL buccal buffer and aspirated with 18G needle. The procedure was repeated three times. The step involving homogenization was omitted because it caused excessive cell loss. In order to increase the number of clearly separated cells, after the third centrifugation of the suspension, the cells were aspirated and expelled 6 times through 18G needle.

The suspension was then filtered through a 100 µm nylon filter. The nylon filter was placed in the filter holder along with the rubber seal to prevent fluid leakage near the filter. The cell filtrate was collected in 15 mL centrifuge tubes. The sample was centrifuged for 10 minutes at 1600 rpm. Upon completion of centrifugation the supernatant was removed and the cells resuspended in 1 mL of buffer. The average number of cells in suspension was determined using the Burcker-Turk Cell Counting Chamber. Depending on the result obtained, the cell suspension was diluted with the buccal cell buffer to a desired concentration of 80,000 cells/mL. Since the cells were already well separated, it was not necessary to add dimethylsulfoxide to the pellet (used when the cells are aggregated, Thomas et al., 2009). Microscope slides pre-cleaned with ethanol and cytocentrifuge sample funnels were prepared. For each volunteer, 4 slides were prepared. Sixty µL of the buffer was added to each the centrifuge funnel and centrifuged for 3 minutes at 600 rpm. Upon completion of centrifugation, 120 µL of cell suspension was added to the same funnel and centrifuged for 6 minutes at 600 rpm. The prepared slides with samples were detached from the funnel and were left to be air-dried at room temperature. The dry slides were fixed for 10 minutes at 4°C in a Coplin vessel filled with 200 mL of the fixation solution (cold glacial acetic acid: cold methanol, 1:3, Kemika, Croatia) and left to dry at room temperature.

Afterwards, the slides were kept for 1 minute in 50% ethanol and then in 20% ethanol and allowed to dry. The dried slides were immersed in freshly prepared 5 M HCl (Kemika, Croatia) for 30 minutes and washed in water for 3 minutes thereafter.

After drying, the slides were placed in a Coplin's container with Schiff's reagent for 1.5 hour, at room temperature, protected from the light. After washing in water for 5 minutes, the slides were stained by immersion in a 50% water solution of Fast Green for 2 seconds and thoroughly washed with distilled water (Yasenka Vukovar, Croatia). When the slides were dried, 1-2 drops of DePex adhesive was applied to the sample area by means of which a coverslip was mounted, ensuring that the adhesive was evenly distributed so that no bubbles remain. The slides were left overnight in the hood to dry and

stored in a slide box at room temperature. The cells were counted on a fluorescence microscope at a 400-fold magnification with oil immersion. 1000 cells were counted to determine the frequency of each cell type: basal, differentiated mononuclear, binuclear, cells with condensed chromatin, karyorrhectic, picnotic and karyolytic cells. Thereafter, 2000 differentiated mononuclear cells were screened to determine the presence of micronuclei, nuclear buds and broken eggs (Thomas et al., 2009; Tolbert et al., 1992). The data were processed using STATISTICA 13 (StatSoft, Dell) software. The data from the questionnaire itself and the data analyzed by the buccal micronucleus cytome assay were analyzed by descriptive statistics. Mann Whitney's U-test was used to compare the groups before and after the treatment. Spearman rank correlation analysis was used to correlate the damage in the whole group and in each individual, before and after the treatment. ANOVA variance analysis was used to check variation within the group. Each of the examined categories in the micronucleus test was analyzed by a chi square for each individual comparing the results before and after the treatment. Nuclear buds and broken eggs categories were merged into one variable.

As for the micronuclei, they were analysed in 2000 differentiated cells but they were expressed as MN frequency per 1000 differentiated cells. Statistical significance was set at ≤ 0.05 .

Results and Discussion

Buccal micronucleus cytome assay, unlike the micronucleus assay on lymphocytes, is a non-invasive and simple technique, and, requiring no cell culture establishment, it can give the results on the day of sampling. Since the cells, after differentiation from the basal cells, do not have active repair mechanisms and they are sufficiently large, any morphological changes after the exposure to a harmful agent can be easily recognized with little doubt. The technique comprises measuring the incidence of micronuclei, small circular retentions in cell cytoplasm after initial cell division (basal cells) which is a sign of the loss of an entire chromosome or its part, the nuclear bud as an indicator of the future micronucleus not yet separated from the

nucleus or amplification of genes, and structures called broken eggs that were merged with nuclear buds, although it is still uncertain whether buds and broken eggs are of the same origin. The technique can also measure changes in the frequency of apoptotic/necrotic events and changes in cytokinesis. Since buccal cells are constantly distributed and regenerated, if they are exposed to an agent such as mouthwash, in two to three weeks the damage to cells in the form of micronuclei or nuclear buds/broken eggs can be detected (Paetau et al., 1999; Gillespie 1969).

For this reason, the selected treatment of 2 weeks of exposure may show the changes measured by this test. In this preliminary study we wanted to examine the effect of a two week treatment of oral cavity with Listerine Cool Mint on the proliferation and differentiation cycle of the buccal epithelial cell and their genomic stability in a group of ten healthy randomly selected individuals.

The results of the buccal micronucleus test on the whole group were compared before and after the treatment (Table 2).

Table 2. Descriptive analysis of entire group before and after the treatment

Variable	Mean	Median	Min	Max	SD	SE	
BC	2.80	1.50	0.00	9.00	3.12	0.99	BEFORE
DF	700.60	789.50	298.00	887.00	198.75	62.85	
CC	55.80	56.50	27.00	87.00	19.98	6.32	
KARRC	21.40	14.50	8.00	75.00	19.85	6.28	
PC	5.70	4.50	2.00	16.00	4.45	1.41	
KYC	207.60	134.00	45.00	583.00	184.16	58.24	
BN	6.10	2.50	0.00	34.00	10.16	3.21	
MN	2.00	2.00	0.00	5.00	1.56	0.49	
BE	2.70	0.50	0.00	10.00	3.86	1.22	
NB	1.30	0.50	0.00	6.00	2.06	0.65	
MN freq	1.00	1.00	0.00	2.50	0.78	0.25	
BE+NB total	4.00	2.50	0.00	16.00	5.23	1.65	
BE+NB freq	2.00	1.25	0.00	8.00	2.61	0.83	
BC	3.80	3.00	0.00	11.00	3.68	1.16	AFTER
DF	771.00	789.00	678.00	858.00	71.84	22.72	
CC	65.40	62.00	24.00	121.00	32.61	10.31	
KARRC	18.90	15.50	0.00	69.00	20.10	6.36	
PC	4.60	3.50	0.00	15.00	4.48	1.42	
KYC	130.40	114.50	58.00	260.00	69.74	22.05	
BN	5.90	3.00	1.00	21.00	6.69	2.12	
MN	3.00	2.50	0.00	7.00	2.40	0.76	
BE	2.90	2.50	0.00	10.00	2.88	0.91	
NB	1.80	0.50	0.00	9.00	2.90	0.92	
MN freq	1.50	1.25	0.00	3.50	1.20	0.38	
BE+NB total	4.70	3.50	1.00	10.00	3.33	1.05	
BE+NB freq	2.35	1.75	0.50	5.00	1.67	0.53	

B-basal cells, DF-differentiated cells, CC-cells with condensed chromatin, KARRC-karriorhectic cells, PC-pycnotic cells, KYC-karyolitic cells, BN-binucleated cells; counted in 1000 cells; MN-micronucleus, BE-broken egg, NB-nuclear bud; counted in 2000 DF, frequency at 1000; Min- Minimum, Max- maximum, SD-standard deviation, SE-standard error, freq-frequency

Mann Whitney-U test for the entire group did not show statistically significant differences before and after the treatment. Cell differentiation results were similar to Thomas et al. (2009) for the younger population. Spearman correlation demonstrated that the incidence of differentiated cells was in negative correlation with the occurrence of karyolytic cells ($R=-0.732682$) and karyorrhectic cells ($R=-0.574289$), indicating that two weeks treatment causes higher percentage of cell departure in later phases of apoptosis.

Although three studies conducted *in vivo* with the intention of studying the cytotoxicity of Listerine demonstrated no statistical significance in buccal cells, Tsourounakis et al. (2013) demonstrated that the use of Listerine Hydroxide induced apoptosis of almost the entire population of human gingival fibroblasts and periodontal ligament fibroblast 24 hours after treatment for 60 s. Ros-Llor and Lopez-Jornet (2014) in a similar two-week treatment of 80 people divided into 4 groups of 20 showed that there was no significant difference in nuclear rupture between different mouthwash groups. They used chlorhexidine (no alcohol), triclosan, mouthrinse with oil extracts in ethanolic solution, with control group receiving the placebo mouthwash-physiological saline. The problem of the Listerine basic formula is that it consists of: 21.6% ethanol, a substance that is associated with the risk of developing oral tumors (Wight & Ogden, 1998; Fioretti et al., 1999; Schlecht et al., 1999; IARC Monographs, 2012). Then there is 2.69 mM menthol that showed a cytotoxic activity on cell line A-375 at a concentration of 0.012 mM with 50% survival of cells (LC50) (Kijpornyongpan et al., 2014), and that acts on the lipid phase of plasma membrane (Kupisz et al. 2015). Listerine thymol concentration is 4.3 mM, and besides LC50 at 0.7 mM (Stammati et al., 1999), thymol demonstrated in those lower concentrations its antibacterial capabilities (Shapiro et al., 1994; Didry et al., 1994; Botelho et al., 2007; Karpanen et al. 2008) in the Hep-2 cell line (Pemmaraju et al., 2013; de Vasconcelos et al., 2014). It also demonstrated that it affects cell membrane damage and hence the release of intracellular substances and changes in transmembrane potential (Shapiro and Guggenheim, 1995), probably not acting on calcium TRP channel

receptors but through mitochondrial damage and stimulation on apoptosis. Then there is 3.94mM methyl salicylate for which a pilot document for the determination of the initial *in vitro* dose for acute toxicity testing has been found to have an LC50 value of 1.7mM (Website, 2001; Vlachojannis, 2015). Eucalyptol in Listerine has the least toxic effect (Ribeiro et al., 2006; Wang et al., 2012), but it has also been shown by SEM microscopy it can cause damage the cells (Dörsam et al., 2014; Zengin & Baysal, 2014).

The frequency of micronuclei after treatment was slightly higher (1.5 vs. 1 before treatment), but still within the limits of normal values recommended by Bonassi et al. (2011) (upper limit of 1.7 micronuclei per 1000 differentiated cells).

Due to statistical analysis, we have combined the category of nuclear buds and broken eggs into one. Spearman correlation showed that the occurrence of micronuclei after the treatment was in a positive correlation with the frequency of binuclear cells ($R=0.693673$), condensed and karyorrhectic cells ($R=0.748022$). This means that individuals with higher DNA damage also had higher percentage of cells moving into early and late apoptosis.

Although the whole group after treatment did not differ significantly from the results before the treatment considering the genomic stability parameters, in some individuals the treatment caused greater number of micronuclei and with them a greater number of binuclear cells, and a decrease in the number of differentiated with the increase in the number of cells in the late phase of apoptosis. Such results show the existence of interindividual differences in the group and the presence of individuals that are more sensitive to Listerine exposure.

ANOVA analysis has shown that there are differences in the incidence of different types of differentiated cells and the frequency of genomic damage within groups, and therefore each individual was analyzed separately by means of hi-squares. Although the group was small, the three people who stated that they consumed larger amounts of strong alcoholic beverages had higher frequency of genomic damage (micronuclei and nuclear buds) after the treatment (Table 3). Smokers also showed higher incidence of micronuclei, but as the two

Table 3. Results of the buccal micronucleus cytome assay for each individual before and after the treatment

samples	Cell differentiation changes										Genomic instability freq.			
	B	DC	CC	KARRC	PC	KYC	BN	MN	BE	NB				
I before	0	754	87	8	5	139	7	3	0	0				
I after	1	829	71	12	4	79	4	2	1	0				
hi-square, p	1, P=0.3173	17.04, P=<.0001	1.76, P=0.1846	0.81, P=0.3681	0.11, P=0.7401	18.53, P=<.0001	0.82, P=0.3652	0.2, P=0.6547	1, P=0.3173	1, P=0.3173				
II before	0	298	70	29	16	583	4	3	0	4				
II after	0	685	121	20	2	157	15	2	3	0				
hi-square, p	0, P=1	299.62, P=<.0001	15.06, P=0.0001	1.69, P=0.1936	10.99, P=0.0009	389.27, P=<.0001	6.43, P=0.0112	0.2, P=0.6547	0.14, P=0.7083	0.14, P=0.7083				
III before	1	831	72	11	2	49	34	1	0	0				
III after	1	840	57	8	0	73	21	7	0	1				
hi-square, p	0, P=1	0.29, P=0.5902	1.86, P=0.1726	0.48, P=0.4884	2, P=0.1573	5.03, P=0.0249	3.16, P=0.0755	4.52, P=0.0335	1, P=0.3173	1, P=0.3173				
IV before	2	887	38	20	7	45	1	3	3	1				
IV after	6	691	24	2	15	260	2	1	4	4				
hi-square, p	2.01, P=0.1563	115.38, P=<.0001	3.26, P=0.071	14.89, P=0.0001	2.94, P=0.0864	178.83, P=<.0001	0.33, P=0.5657	1, P=0.3173	1.34, P=0.247	1.34, P=0.247				
V before	9	460	69	75	4	375	8	5	4	0				
V after	5	762	113	20	6	92	2	3	0	9				
hi-square, p	1.15, P=0.2835	191.86, P=<.0001	11.7, P=0.0006	33.43, P=<.0001	0.4, P=0.5271	223.74, P=<.0001	3.62, P=0.0571	0.5, P=0.4795	1.93, P=0.1648	1.93, P=0.1648				
VI before	7	793	55	13	2	129	1	1	9	1				
VI after	3	678	79	19	3	216	2	3	4	0				
hi-square, p	1.61, P=0.2045	33.99, P=<.0001	4.61, P=0.0318	1.14, P=0.2857	0.2, P=0.6547	26.51, P=<.0001	0.33, P=0.5657	1, P=0.3173	2.58, P=0.1082	2.58, P=0.1082				
VII before	5	852	27	9	10	97	0	0	0	1				
VII after	0	858	62	7	9	62	2	1	2	1				
hi-square, p	5.01, P=0.0252	0.15, P=0.6985	14.41, P=0.0001	0.25, P=0.6171	0.05, P=0.8231	8.37, P=0.0038	2, P=0.1573	1, P=0.3173	1, P=0.3173	1, P=0.3173				
VIII before	1	786	55	13	3	141	1	2	1	0				
VIII after	8	831	29	69	1	58	4	4	10	0				
hi-square, p	5.47, P=0.0193	6.54, P=0.0105	8.4, P=0.0038	39.88, P=<.0001	1, P=0.3173	38.44, P=<.0001	1.8, P=0.1797	0.67, P=0.4131	7.38, P=0.0066	7.38, P=0.0066				
IX before	2	530	27	16	2	420	3	2	10	6				
IX after	3	816	36	0	2	137	6	0	3	3				
hi-square, p	0.2, P=0.6547	185.84, P=<.0001	1.33, P=0.2488	16.13, P=<.0001	0, P=1	199.29, P=<.0001	1, P=0.3173	2, P=0.1573	4.57, P=0.0325	4.57, P=0.0325				
X before	1	815	58	20	6	98	2	0	0	0				
X after	11	720	62	32	4	170	1	7	2	0				
hi-square, p	8.38, P=0.0038	25.29, P=<.0001	0.14, P=0.7083	2.84, P=0.0919	0.4, P=0.5271	22.34, P=<.0001	0.33, P=0.5657	7.02, P=0.0081	2, P=0.1573	2, P=0.1573				

B-basal cells, DF--differentiated cells, CC-cells with condensed chromatin, KARRC-karriothetic cells, PC-pycnotic cells, KYC-karyotic cells, BN-binucleated cells; counted in 1000 cells; MN_micronucleus, BE-broken egg, NB-nuclear bud; counted in 2000 DF, frequency (freq.) at 1000 DF; hi-square and Pearson with p

subjects reported higher alcohol consumption, this small group could not be examined further. If the cumulative effect and the synergistic effect of all components of Listerine are considered together, these results would mean devastating consequences for tissues exposed to Listerine (Bassole & Juliani, 2012). The same components of Listerine have shown toxic effects *in vitro* and *in vivo*, and the greatest contributor may be due to the large amount of ethanol in Listerine Cool Mint, which is considered to be the most responsible for the possible toxic effects of prolonged exposure to Listerine. Although consumed ethanol should only be metabolised in the liver, there is evidence that the microorganisms in the oral cavity can also metabolize ethanol and the first metabolite derived from the alcohol dehydrogenase enzyme activity is acetaldehyde that is as toxic as ethanol itself in *in vitro* conditions and cellular models (Homann et al., 1997; Obe & Ristow, 1977). Acetaldehyde remains longer in the the oral cavity saliva and can affect the decrease in basal cell count, and thus cause epithelial atrophy (Mascrec et al., 1984). In the oral cavity there are microorganisms that can metabolize ethanol and convert it into acetaldehyde, which also has toxic effects on cells *in vitro* and *in vivo* and according to carcinogen classification is placed in group 2B. Ethanol metabolism starts already in the mouth by bacteria (Homann, 1997) and its first metabolite acetaldehyde exhibits even more intense toxicity, as demonstrated in some cellular and animal models (Homann et al., 1997; Obe & Ristow, 1977). A one-time use of mouthwash containing ethanol resulted in increase in the acetaldehyde level in the saliva to the level normally present after consuming alcoholic beverages (Lachenmeier et al., 2009). After adding 0.5 grams of alcohol per kilogram of body weight corresponding to the consumption of half a liter of wine, the acetaldehyde level was between 50-100 μ M, which is the range of concentrations that can cause mutagenic effects, such as inherited changes in the cell genome. However, Seitz and Stickel (2007) have shown that after using alcohol-containing mouthwash for two weeks, the acetaldehyde concentration is reduced by approximately 30-50%, suggesting that the reduction of the presence of oral bacteria decreases the

concentration of toxic substances in the mouth. Mechanisms of acetaldehyde genotoxic activity are adduct formation in DNA molecules, cross-linking of DNA chains, DNA-protein crosslinking, and increased frequency of sister chromatids exchange (Seitz & Stickel, 2007). The IARC (International Agency for Cancer Research) proclaimed the aldehyde as a possible human carcinogen and placed it in group 2B (IARC Monographs, 1999). Vlachojannis et al. (2016) reviewed 19 studies on Listerine mouthwash. Of these 19 studies, 16 focused on the efficacy of Listerine, and only 3 investigated the potential harm of the solution. Although the FDA (Health and Human Services) rated Listerine as safe and effective in 8 of the 16 clinical studies (efficacy, non-harm) conducted until then, doubts still exist because, according to the findings of this study, the guideline hardly exceeds the presumed margin of harm, and only 8 of the above confirmatory studies were performed at clinically significant conditions for 6 months (Vlachojannis et al., 2016).

Listerine, unlike other mouthwash formulations such as 0.2% chlorhexidine, the gold standard among the mouthwashes that is always used as comparator, shows no toxicity in short term (a period of a few days) but reaches its maximum after two weeks, when the effect 0.2% of chlorhexidine and Listerine is equal, as shown by Haerian-Ardakani et al. (2015) and this reduction of bacterial count goes down to a factor of 2. The authors have shown that Listerine eliminates harmful effects of bacteria during this period to a sufficient extent, thus preventing the formation of acetaldehyde. Therefore a treatment of at least two weeks allows the evaluation of the effects of ethanol itself on the buccal cells.

There are studies that show that oral exposure to ethanol increases the risk of developing oral cavity cancer (Wight & Ogden, 1998; Fioretti et al., 1999; Schlecht et al., 1999) and that additional exposure to smoking increases the risk of developing malignant neoplasms (Schlecht et al., 1999).

The results obtained in our study showed no significant genotoxic effect of Listerin exposure, although the values for micronuclei were higher after the treatment, but also showed that individuals who consume larger amounts of alcohol are more sensitive and this group also includes smokers. Reis

et al. (2002, 2006) demonstrated that chronic exposure to alcohol causes an increased incidence of micronuclei even in non-smokers, but this change was not statistically significant (Reis et al., 2002, 2006).

Concerning smokers, in our research, no particular conclusions or important correlations could be reached-because heavy smokers (more than 40 cigarettes per day) were at the same time the consumers of strong alcoholic beverages so that the genotoxic effects could not be attributed solely to the influence of tar and nicotine. According to this logic, alcohol would also be unacceptable as genotoxin, but other authors' research provided enough evidence that ethanol is taken as a major factor, and is also present in the Listerine content in sufficient proportion to be taken into account. Since ethanol is not carcinogenic, the mechanisms are yet to be clarified how ethanol influences genomic instability, and thus the development of tumor lesions. Although our research has shown that after the treatment the whole group had slightly elevated frequencies of micronuclei, these results were within the limits of normal values.

However, there is a large individual variation in the resulting lesions and cell differentiation, indicating that there are probably some other mechanisms that cause such great differences. People who consume regular quantities of strong alcoholic beverages showed higher values for micronuclei after the treatment (Pastorino et al., 2018), indicating that an additional source of ethanol other than that of Listerine two times daily may increase the effect on genomic stability.

Vlahojannis et al. (2015) showed that 27% ethanol has higher antimicrobial activity than Listerine. Alcohol in Listerine was also responsible for the cytotoxic effect of Listerine on gingival fibroblast (Eick et al., 2011) and stem cells (Park et al., 2014) and reduction in the number of primary human gingival fibroblasts and primary human nasal epithelial cells (Schmidt et al., 2016).

Vlachojannis et al. (2016) reviewed the results of the research on all types of Listerine mouthwashes and found that in 16 studies Listerine improved health and maintainance of oral hygiene, but that this still does not mean that Listerine is safe from the toxicological view in short and especially long term

use. Assays for long term exposure should include factors affecting metabolism and prolonged exposure to harmful ethanol metabolites, such as the genetic polymorphisms. Namely, there are 5 types of alcohol dehydrogenase enzyme in humans, of which two enzymes (ADH2 and ADH3) may have polymorphic (non-mutated) forms that can affect faster or slower metabolism of ethanol and thus shorter or longer exposure to this harmful agent. Polymorphic ADH3 strongly affects the metabolism of ethanol in acetaldehyde, and ADH3 1 allele carriers can metabolise ethanol faster than ADH3 2 allele carriers. Also, people with this enzyme deficiency have an increased risk of developing oral cancer associated with heavy alcohol ingestion (Carretero et al., 2004).

Due to the lack of understanding of cancer mechanisms, the scientific community has not yet ruled out the use of alcoholic antiseptics as an actor in the development of oral cancer (American Dental, 2009; Boyle et al., 2014), although there have been studies that provide evidence for this link (Currie and Farah, 2014). In our study, interindividual differences could mask actual relationship. In the future studies, the volunteers should be chosen with similar life styles in order to reduce the effect of variables that contribute to cytometry variations before exposure to the selected substance. This logic has even greater weight when it comes to the buccal mucosa, which is highly adaptive tissue when it comes to environmental pressures, and exhibits the most diverse profiles in healthy persons. But even if subjects are classified as healthy, the styles and the place of life dictate the whole line transition from "healthy" to "prone", for example by using a water-based Listerine which components themselves have proven to have an adverse effect.

However, considering that the correlation between the use of alcoholic antiseptics and oral cancer development has not been unambiguously and fully demonstrated, but not completely rejected as yet, dentists should not recommend long-term use of alcohol-based antiseptics.

Some vulnerable groups of people such as the smokers, people with alcohol intolerance and alcohol dehydrogenase deficiency and other people with higher risk of developing oral cancer should limit such use if needed.

Conclusions

The buccal micronucleus cytome test proved to be a sensitive method for analysing changes in cell differentiation, the frequency of apoptotic/necrotic cells, and changes in genomic stability in a 2 week exposure to Listerine Cool Mint mouthwash according to the manufacturer's instructions. The results of this small study conducted on ten individuals did not demonstrate statistically significant effect of this mouthwash on the differentiation and genomic stability of the buccal cells, although the entire group had higher micronuclei frequency and showed a significantly higher incidence of apoptosis. Inter-individual differences have shown some indications and guidelines for similar research on a large number of people. Individuals who regularly enjoy hard liquor had higher number of microanalysis and nuclear buds but did not express distinct differences in differentiated cells, indicating that alcohol did not affect rapid apoptosis, but Listerine along with additional concentrations of alcohol from alcoholic beverages demonstrated a synergistic effect. Also, greater values of genomic instability were observed in smokers. This knowledge should be verified on a large number of people with similar habits (regular consumers of hard liquor with and without smoking habits) to assess the extent to which lifestyle affects genomic stability. Genome-specific SNP (single nucleotide polymorphisms) variants associated with metabolism of ethanol to acetaldehyde should also be included in the following studies.

Conflict of interest

Authors declare no conflict of interest.

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