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The impact of electronic cigarette liquids on human gingival cell viability – a preliminary in vitro study

Wpływ elektronicznych papierosów na żywotność ludzkich fibroblastów dziąseł – wstępne badania *in vitro*

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Abstract

Introduction. E-smoking is a new phenomenon, not only because of the opportunity for limiting or abandoning smoking, but also because it may become an introduction to traditional smoking, especially by young people, and addiction to nicotine. At the moment it is impossible to assess the advantages of e-smoking and the risk of undesirable effects, or even the toxic influence on the oral cavity tissues, due to the complexity of the phenomenon and the very few accurate clinical and laboratory studies.

Aim. The aim of the study was the impact of electronic cigarette (EC) liquids on human gingival cell (HGF-1) viability in an in vitro study.

Material and methods. Commercially available EC liquids with 11 and 24 mg/mL nicotine contents and different flavours were tested. Cytotoxicity on HGF-1 was evaluated after 24 and 48 h of exposure to EC liquids in concentrations of 0.1 and 1%.

Results. Among all the tested EC liquids only Mint-flavoured ones have a harmful effect on HGF-1 cells in a time-dependent manner. No statistically significant difference was found between EC liquids with 11 and 24 mg/mL nicotine contents.

Conclusions. Our data indicated that flavour additives in EC liquids may exert biological effects on oral cells.

Keywords: electronic cigarettes, smoking, cytotoxicity.

Streszczenie

Wprowadzenie. Palenie e-papierosów jest nowym zjawiskiem. Może być nie tylko szansą ograniczenia bądź rzucenia palenia, ale stanowi również zagrożenie jako wstęp do tradycyjnego palenia (szczególnie u ludzi młodych) i uzależnienia od nikotyny. W chwili obecnej z powodu złożoności tego zjawiska oraz niewielu badań klinicznych i laboratoryjnych nie jest możliwe porównanie korzyści wynikających z e-palenia z ryzykiem niepożądanych efektów, wśród których wymienia się niekorzystny wpływ na tkanki jamy ustnej.

Cel. Celem pracy była ocena oddziaływania liquidów do e-papierosów (EC) na żywotność ludzkich fibroblastów (linii komórkowej HGF-1) w warunkach *in vitro*.

Materiał i metody. Badaniem objęto dostępne na polskim rynku liquidy o różnych smakach z zawartością nikotyny 11 i 24 mg/ml. Cytotoksyczność wobec linii komórkowej HGF-1 była oceniana po 24 i 48 godzinach od ekspozycji na liquidy w stężeniu od 0,1 do 1%.

Wyniki. Wśród wszystkich badanych liquidów tylko miętowy wykazał szkodliwy wpływ na komórki HGF-1 w sposób zależny od czasu. Nie stwierdzono statystycznie istotnej różnicy między liquidami do e-papierosów o zawartości nikotyny 11 i 24 mg/ml.

Wnioski. Liquidy do e-papierosów mogą wykazywać biologiczny wpływ na fibroblasty w obrębie jamy ustnej.

Słowa kluczowe: elektroniczne papierosy, palenie, cytotoksyczność.

Introduction

E-smoking is a new phenomenon. Although it is an opportunity for limiting or abandoning smoking, it may also become an introduction to traditional smoking. Young people are especially in danger [1–8]. At the moment it is impossible to assess the advantages of e-smoking and the risk of undesirable effects, or even the toxic influence on the oral cavity tissues [9] due to the complexity of the phe-

nomemon and the very few accurate clinical and laboratory studies.

Habitual smokers have worst clinical results in nonsurgical and regenerative periodontal treatment. Among the negative influences of nicotine on the periodontium the following may occur: an increase in the quantity and quality of periopathogenic subgingival biofilm, and the immunomodulation of the host reaction; e.g. an intensification of the de-

structive processes in the connective tissue and the bone as well as a deficiency in the healing process [10–12]. Accordingly, nicotine is considered as the main pathological factor in certain precancerous states of the oral cavity mucosa, such as the leukoplakia and the neoplasms [13].

Minimizing nicotine intervention becomes an important part of any prophylactic and therapeutic action. Nicotine replacement therapy may include adhesive plasters, tablets or chewing gum with nicotine. In recent years attention has been focused on so-called electronic cigarettes containing nicotine [14–17]. E-smoking is still not a well-known phenomenon, and it may carry the risk of unexpected side effects or even toxic influences [17].

According to the World Health Organization, tobacco smoking is one of the most serious health threats in the world. It concerns not only general health problems, such as cardiovascular disorders or breathing system disorders, but also oral cavity health including periodontal and oral mucosa conditions.

Many studies indicate that smokers are choosing ECs for the same reasons as other nicotine substitutes – to limit their smoking and to reduce the symptoms from stopping [9, 15, 18, 19]. The unanswered question is whether the regular use of e-cigarette liquids is free of risks to the health?

Aim

The aim of the study was to investigate the impact of electronic cigarette liquids on human gingival cell viability in an in vitro study.

Material and methods

E-cigarette refill fluids

All the fluids are popular and easily available to e-cigarette users in local shops in Poland. Seven bottles of refill fluids containing various flavourings (Pepper Mint, Fresh Ice Mint, Juicy Cherry, Very Strawberry, Black Tea, Black Currant, Natural Tobacco) in two nicotine concentrations (11 and 24 mg/mL) were evaluated (**Table 1**).

Cell culture

An HGF-1 cell line was obtained from the American Type Culture Collection (ATCC-HBT-55) and maintained as a monolayer culture in T-75 cm² tissue culture flasks. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich), a high glucose medium (4.5 g/L) containing sodium pyruvate (110 mg/L), and supplemented with 10% fetal bovine serum, 6 µg/mL penicillin-G, and 10 µg/mL streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 95% O₂, 5% CO₂ [20]. When confluent, the cells were detached enzymatically with trypsin-EDTA and sub-cultured into a new cell culture flask. The medium was replaced every 2 days.

Cell exposure

The concentrations of refill fluids used in the experiments were carefully chosen according to the results obtained from preliminary experiments and literature data. The e-cigarette refill fluids were diluted to appropriate concentrations in a serum free (SF) cell culture medium and filtered through a 0.22 µm membrane filter at the time they were added to the cells. The HGF-1 cells were then incubated with the refill solutions (0.1% and 1.0%, final concentration of nicotine: 11, 110; 24, 240 µg/mL, respectively) for 48 hours. The concentration of refill fluids was based on preliminary data obtained for concentrations of 0.001%, 0.01%, 0.1%, 0.5% and 1.0%. Control cells were incubated with the same volume of SF cell culture medium (DMEM).

Cytotoxicity of e-cigarette refill fluids evaluation – MTT assay and cellular morphology study

The HGF-1 cells were seeded in triplicate at a density of 2x10⁴ cells/100 µL of cell culture medium into a 96-well. The next day, CRL-2014 cells were exposed to different concentrations of e-cigarette refill fluids as above for 24 and 48 hours. This assay evaluates mitochondrial activity (assesses cell growth and cell death) and is performed by adding a pre-mixed optimized dye solution to the culture cells. Absorbance was recorded at 570 nm (FLUOstar OPTIMA). Results from the treatment groups were calculated as a percentage of control values (unexposed cells) according to the following equation: % of viability = (experimental absorbance [abs] 570 nm of exposed cells – background of experimental abs 570 nm/abs 570 nm of unexposed cells) × 100. Absorbance values were corrected for background (refill fluid blank used for each concentration).

The morphology of HGF-1 cells in the presence of EC fluids was visualized by a light microscope at 100× magnification (ALTRA20 microscopy and CELIA Acquisition software, Olympus, Japan). Control cells were unexposed to EC fluids.

The experimental results were expressed as mean ± SD for the triplicate determination of 3–4 separate experiments. The results were analysed using one-way ANOVA and Tukey's post hoc test with a p value < 0.05 being considered statistically significant.

Results

After an incubation time of 24 h none of the tested EC liquids exerted a statistically significant cytotoxic effect on HGF-1 cells (**Table 1**).

After 48 h of exposure, among all the tested liquids, only Mint EC fluids caused a marked reduction in HGF-1 cells viability. Compared to Fresh Ice Mint-flavoured liquids, the Pepper Mint-flavoured liquids were observed to exert a stronger cytotoxic effect on HGF-1 cells. The content of nicotine did not significantly influence the cytotoxicity of EC liquids in HGF-1 cells (**Table 1**). Significant morphological changes in HGF-1 cells were observed after 48 h HGF-1 exposure to Mint-flavoured EC

(Pepper Mint, Fresh Ice Mint). These were characterized by cell shrinkage and irregular shapes when compared with control cells (**Figure 1**).

The phase-contrast micrographs were indicative of cell death induced by Mint-flavoured EC because HGF-1 cells detached from the cell culture dish after exposure to EC liquids.

Discussion

ECs are relatively new products and it is still not clear what influence EC refill fluids have human

oral cells. This study shows that EC refill fluids demonstrated harmful effect on human gingival fibroblast cells in a time- and flavour-dependent manner. Behar et al. [21] proved that flavourings in refill fluids are linked to cytotoxicity in human embryonic stem cells, human pulmonary fibroblasts and mice neural stem cells. It has been found that among all the tested flavours only mint (Fresh Ice Mint and Pepper Mint) reduced HGF-1 cell viability. Additionally, the Pepper Mint flavoured liquid is observed to be much more cytotoxic than Ice Mint. Also, Wil-

Table 1. The impact of e-cigarette liquids on HGF-1 cell viability

Tabela 1. Wpływ liquidów do e-papierosów na żywotność linii komórkowej HGF-1

EC liquid	Viability of HGF-1 cells (%)			
	Concentration of e-cigarette liquids (%) and time of incubation (h)			
	0.1 (%)		1 (%)	
	24h	48h	24h	48h
Control	98.6 ± 6.1	97.3 ± 4.8	98.6 ± 6.1	97.3 ± 4.8
Very Strawberry*	96.5 ± 6.4	98.9 ± 7.6	92.6 ± 7.7	81.4 ± 7.9
Very Strawberry**	91.3 ± 8.1	90.8 ± 8.5	90.1 ± 8.6	83.6 ± 5.0
Fresh Ice Mint *	98.2 ± 4.2	76.1 ± 6.5 [#]	87.4 ± 7.1	69.1 ± 7.1 [#]
Fresh Ice Mint **	97.8 ± 5.9	70.9 ± 4.1 [#]	82.6 ± 5.3	64.3 ± 6.4 [#]
Natural Tobacco *	98.3 ± 6.3	90.3 ± 6.4	94.2 ± 9.2	87.2 ± 9.1
Natural Tobacco **	93.1 ± 7.9	87.4 ± 5.9	87.3 ± 9.6	80.9 ± 7.3
English Black Tea *	91.6 ± 3.8	86.3 ± 6.0	89.4 ± 7.4	83.5 ± 4.8
English Black Tea **	96.2 ± 8.2	87.7 ± 4.8	88.2 ± 4.2	81.2 ± 6.6
Black Currant *	97.2 ± 5.8	94.1 ± 9.1	95.1 ± 5.9	83.1 ± 7.1
Black Currant **	94.3 ± 7.3	93.4 ± 8.3	90.4 ± 6.8	81.7 ± 8.9
Pepper Mint*	97.9 ± 4.8	44.6 ± 3.2 ^{#,a}	89.7 ± 9.0	37.2 ± 3.4 ^{#,a}
Pepper Mint**	90.6 ± 4.1	38.1 ± 2.1 ^{#,a}	86.2 ± 8.2	30.9 ± 4.1 ^{#,a}
Juicy Cherry*	97.8 ± 9.1	93.8 ± 8.6	98.0 ± 4.1	90.7 ± 8.3
Juicy Cherry**	93.2 ± 6.3	97.9 ± 5.3	99.4 ± 6.2	88.6 ± 6.4

* – 11 mg/mL nicotine; 24 mg/mL nicotine

** – $p < 0.001$ compared with control

a – $p < 0.001$ Pepper Mint-flavored EC compared with Fresh Ice Mint-flavored EC

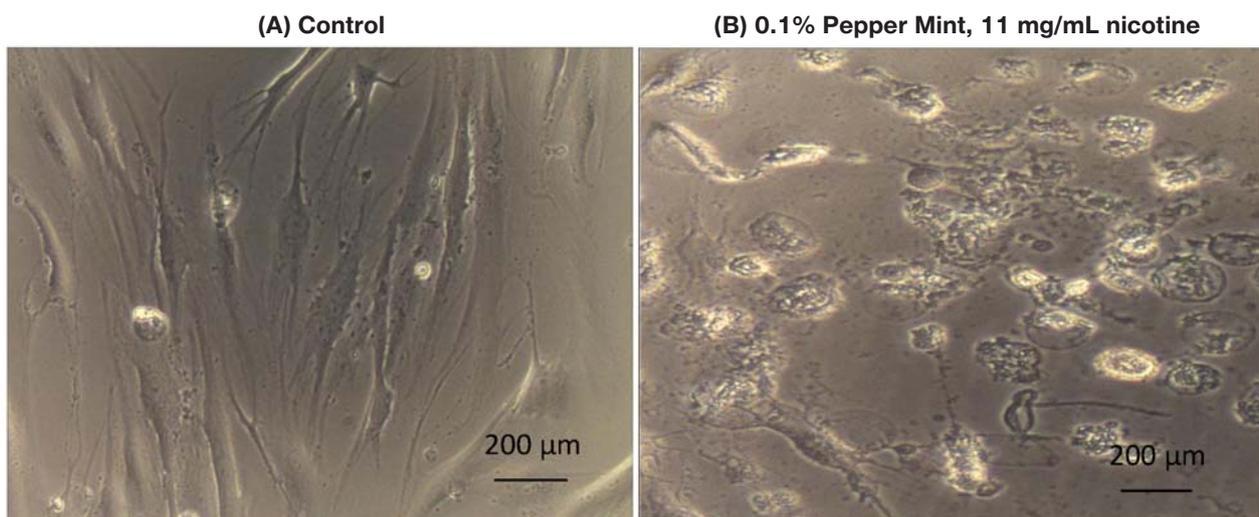


Figure 1. Morphological changes in HGF-1 cells exposed to 0.1% Mint-flavoured EC liquid with 11 mg/mL nicotine for a period of 48 h followed by phase-contrast microscopy; (B) shows cell death as compared with untreated control cells (A) (bar, 200 µm)

Rycina 1. Morfologiczne zmiany obserwowane w mikroskopie kontrastowym w linii komórkowej HGF-1 eksponowanej na 0,1% miętowy liquid do elektronicznych papierosów z zawartością 11 mg/ml nikotyiny w okresie 48 godzin. (B) komórki uszkodzone w porównaniu z komórkami kontrolnymi, nie eksponowanymi na liquid miętowy (A) (bar, 200 µm)

Willershausen et al. [22] observed that the addition of menthol may lead to a reduction in the proliferation rate of human periodontal ligament fibroblasts. On the other hand, a 13-week smoke inhalation study indicated that the addition of menthol to tobacco had no effect on the biological responses normally associated with the inhalation of cigarette smoke in rats [23]. According to studies performed by the tobacco industry, both menthol and non-menthol cigarette smoke have similar levels of cytotoxicity [24–26]. Menthol is a very popular tobacco flavouring because it masks the bitter taste of cigarette smoke [27]. What is important, though, is that L-menthol as a cigarette additive may promote smoking initiation and nicotine addiction [27]. However, currently it has been found that flavour additives do attract people, especially the young, to electronic cigarettes [28, 29].

In conclusion, the authors have found that flavour additives to EC liquids exerted a biological inhibiting effect on oral HGF-1 cells in a flavour- and time-dependent manner rather than the nicotine content.

Further epidemiological, clinical and molecular research linked to the present study is recommended.

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Conflict of interest statement

The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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