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Comparative study of the effect of IQOS® aerosols and cigarette smoke on the functional activity of fibroblastic cells

Estudo comparativo do efeito dos aerossóis IQOS® e do fumo do tabaco na atividade funcional de células fibroblásticas

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Comparative study of the effect of IQOS® aerosols and cigarette smoke on the functional activity of fibroblastic cells

Dental Medical Research Article for Dissertation

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“Muito da eficiência do que fazemos, daquilo que mastigamos, depende sobretudo do que não se vê. Das raízes (…) Porque são as coisas que estão dentro de nós e em que ninguém repara quando nos olha.”

Afonso Cruz, in “O Pintor Debaixo do Lava-Loiças”

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ABSTRACT

Introduction: In addition to the association with several systemic pathological conditions, tobacco usage is one of the major causes of periodontal diseases, which results in bone loss, tooth mobility and, in more severe situations, teeth loss. To provide smokers with a potentially less dangerous alternative to traditional tobacco, IQOS® cigarettes were created. This tobacco is heated to a high temperature, which results in the production of an inhalable vapor without burning the tobacco.

In vitro experiments using human cells are very helpful for determining the potential toxicity of compounds, with a high translationality to human health. In addition, fibroblasts, the most common cell type within the periodontium, are crucial for maintaining and modifying the extracellular matrix and gingival structure.

Therefore, this study aimed to compare the effect of conventional cigarette smoke with IQOS® aerosols on the functionality of human gingival fibroblastic cell cultures.

Materials and Methods: Conventional tobacco and two different IQOS® devices – the ILUMA ONE™ and IQOS 3™ – were used to create the smoke and aerosols solutions, respectively. To produce the culture medium enriched with the aerosol/smoke, a smoking machine and the ISO 20778:2018 smoking protocol were used. The biological response to the obtained mediums was assayed in human gingival fibroblastic cell cultures. After adhesion under standard culture conditions for 24 hours, the culture medium was replaced with various dilutions of either IQOS® aerosol solutions or cigarette smoke (CS). In this work, cytotoxicity was assessed using the MTT assay at two different time points — day 1 and day 5, addressing the potential cumulative effects; and also, at day 5 with medium change at day 1, addressing the potential recovery effect — following exposure to smoke/aerosol solutions. Morphological abnormalities were also examined with fluorescence microscopy upon specific staining of the cytoskeleton.

Results: After exposure to CS, both the amount of deposited materials and the turbidity of the medium were substantially higher than that attained with IQOS® aerosol contamination. At every timepoint and for all cigarette types, the

fibroblast metabolic activity was reduced by high doses (30 and 10% extracts). Particularly on day 1, CS caused a greater drop in culture metabolic activity when assayed at high concentrations than the other groups; IQOS ILUMA ONE™ followed a similar pattern on day 5, both with and without medium change. In contrast, IQOS 3™ contamination appeared to cause the least changed metabolic profile. High levels of smoke or aerosol also had an impact on the morphology of the fibroblasts, with a pronounced loss of fibroblastic phenotypic characteristics.

Conclusion: At high concentrations, the smoke from both conventional CS and IQOS® devices aerosols had a detrimental effect on fibroblasts biological activity and morphology. The two IQOS® devices induced a dissimilar biological response of the human gingival fibroblasts.

Keywords: heated tobacco products, IQOS®, conventional tobacco, periodontal disease, fibroblastic cells

RESUMO

Introdução: Para além da sua associação com várias condições patológicas sistémicas, o consumo de tabaco é um dos maiores fatores etiológicos da doença periodontal, que resulta em perda óssea, mobilidade dentária e, em situações mais severas, na perda dentária. De forma a proporcionar aos fumadores uma alternativa ao tabaco tradicional, potencialmente menos prejudicial, surgiu no mercado a tecnologia IQOS®. Este tabaco é aquecido a temperaturas elevadas, resultando na produção de um vapor inalável, sem que haja combustão.

Os estudos *in vitro* com células humanas são bastante úteis para determinar a potencial toxicidade de compostos, com uma grande translacionalidade para a saúde humana. Adicionalmente, os fibroblastos, sendo o tipo de célula mais comum no periodonto, são cruciais para a manutenção e modificação da matriz extracelular e da estrutura gengival.

Assim, este estudo teve como objetivo comparar o efeito do fumo do cigarro convencional com os aerossóis IQOS® na funcionalidade de culturas de células fibroblásticas gengivais humanas.

Materiais e Métodos: De forma a criar as soluções de fumo e a gerar os aerossóis, foram usados uma forma de tabaco convencional e dois equipamentos IQOS® - o ILUMA ONE™ e o IQOS 3™. Para produzir o meio de cultura enriquecido com o aerossol/ fumo, foi usada uma máquina de fumo, com o protocolo de fumo ISO 20778:2018. A resposta biológica aos meios obtidos foi avaliada em culturas de células fibroblásticas gengivais humanas. Após 24h de adesão celular em condições *standard* de cultura, o meio de cultura foi substituído por diferentes diluições das soluções dos aerossóis IQOS® ou do fumo do cigarro convencional. Neste trabalho, a citotoxicidade foi avaliada, utilizando o ensaio de MTT em 2 períodos de avaliação diferentes – dia 1 e dia 5, avaliando o potencial efeito cumulativo, e o dia 5 com troca de meio ao dia 1, de forma a avaliar o potencial efeito de recuperação, após exposição às soluções de fumo/aerossol. O desenvolvimento potencial de anomalias morfológicas foi também avaliado, recorrendo a microscopia de fluorescência, após a marcação específica do citoesqueleto.

Resultados: Após a contaminação com o fumo do cigarro, quer a quantidade de matéria depositada, quer a turbidez do meio foram substancialmente elevadas na condição do tabaco convencional, em comparação com a contaminação com IQOS®. Em todos os períodos de avaliação e para todo o tipo de cigarros, a atividade metabólica dos fibroblastos foi reduzida em concentrações mais elevadas (30 e 10%). Particularmente no dia 1, o cigarro convencional causou uma maior redução na atividade metabólica da cultura, quando em concentrações mais elevadas, comparativamente aos outros grupos. O mesmo padrão foi observado para o IQOS Iluma One™, ao dia 5, com e sem troca de meio. Em contraste, a contaminação com IQOS 3™ pareceu ser a que causou menos alteração no perfil da atividade metabólica. Os níveis elevados de fumo ou aerossol – nas concentrações mais elevadas dos extratos - impactaram também a morfologia dos fibroblastos, com uma perda pronunciada de características fenotípicas.

Conclusão: Em concentrações mais elevadas, quer o fumo do cigarro convencional, quer o aerossol dos dispositivos IQOS® tiveram um efeito negativo na atividade biológica e na morfologia dos fibroblastos. Os dois equipamentos IQOS® induziram uma resposta biológica distinta nos fibroblastos humanos gengivais.

Palavras-chave: produtos de tabaco aquecido, IQOS®, tabaco convencional, doença periodontal, células fibroblásticas

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LIST OF ABBREVIATIONS

CS | Cigarette Smoke

HCI | Health Canada Intense

HGF | Human Gingival Fibroblasts

HPHC | Harmful and Potentially Harmful Compounds

HTP | Heated Tobacco Product

IQOS | I Quit Ordinary Smoking

ISO | International Organization for Standardization

MEM | Minimal Essential Medium

PAH | Polycyclic Aromatic Compounds

PBS | Phosphate-buffered Saline

PMI | Philip Morris International

INTRODUCTION

The connection between periodontitis and smoking

Periodontal diseases are the most prevalent chronic inflammatory conditions of the oral environment, being a leading cause of tooth loss in adults worldwide (1). The prevalence of severe periodontal disease in adults over the age of fifteen years ranges between 20-50% worldwide (2, 3). Due to the complex nature of periodontitis, a thorough awareness of all related risk factors is necessary for efficient disease management (4).

Smoking is known to have a serious impact on periodontitis. Smokers have a more severe form of the disease, as evidenced by increased attachment loss, periodontal pocket formation, greater calculus deposits, increased gingival recession, more rapid development and progression of periodontal inflammation, and delayed periodontal healing, according to previous studies (1, 4-6). Because of this, the revised periodontal disease categorization from 2017 states that tobacco usage affects the progression of the disease (7). The severity of the disease increases with daily consumption level and/or duration of the smoking habit. This has significant therapeutic and practical implications, as periodontal tissues will improve if the patient's smoking behaviour can be changed to include reduced exposure levels (8).

Tobacco use is now believed to enhance the risk, prevalence, and severity of periodontal disease through a variety of molecular and cellular processes, including 1) reduction of the gingival perfusion, which limits the delivery of nutrients and oxygen as well as the removal of waste materials; 2) change of the host immune response by suppressing anti-inflammatory molecules and eliciting a strong pro-inflammatory response to bacterial triggers; 3) reduction of the ability of periodontal cells, such as fibroblasts, osteoblasts, and cementoblasts, to repair and regenerate the periodontium by decreasing its morphological and functional activity (9, 10); 4) change of the alveolar bone metabolism (11); 5) alteration of the oral microbiota, favouring dysbiosis and increasing infectiousness, making the oral cavity more susceptible to the spread of pathogenic bacteria like *Porphyromonas Gingivalis, Aggregatibacter Actinomycetencomitans, Bacteroides Forsythus, Prevotella Intermedia,* and

Fusobacterium Nucleatum (1, 6). Notably, many of the underlying effects of tobacco products on periodontal tissues may be caused by a direct obstruction of normal fibroblastic function (11). Prolonged exposure to cigarette smoke has been reported to impair the growth of human gingival fibroblasts and their ability to synthesize extracellular matrix molecules (12, 13).

In addition, periodontal disease has been connected to various systemic disorders and is a model of local damaging, chronic, low-grade infection, correlated with significant systemic alterations (1). As periodontitis is the leading cause of tooth loss in the adult population worldwide, those who suffer from these conditions run the risk of experiencing multiple tooth losses, edentulism, bite collapse, and masticatory dysfunction, all of which have a negative impact on their ability to eat, live a fulfilling life, and feel confident in themselves (8). Because of this, periodontitis enhanced by smoking is a serious global public health concern (14).

New marketed alternatives to tobacco smoking

Although traditional manufactured cigarettes continue to be the most popular method of tobacco use (around 75% of the total volume of sales of all tobacco products in the EU in 2020), the use of non-combustible tobacco and nicotine products, such as heated tobacco products and electronic cigarettes, has raised quickly in recent years, and their use is anticipated to continue expanding in the years to come (15). Many of these new items have been marketed as a method for adult smokers who want to keep smoking, thus minimizing the harm, or as a way to aid them in quitting (16).

In heated tobacco products (HTP), processed tobacco is heated to a point just short of combustion, producing an emission that contains nicotine and other chemicals that users inhale. Ploom (Japan Tobacco International), IQOS® (Philip Morris International), Glo (British American Tobacco), and, ultimately, lil HTP (Korea Tobacco and Ginseng Corporation) are some of the HTPs that have been commercialised thus far. Only IQOS®, which PMI introduced in 2014, was the subject of the current study since it was the first HTP to be commercially available in Portugal, it is the HTP that sells the most globally, and has undergone the most extensive research into the makeup of the emissions that

are produced (17). IQOS® is available in the majority of the countries where HTPs are now marketed (2, 18). Japan was forecast to have the highest market share for HTP in 2021, with seven of the top ten nations being in Europe i.e., Turkey, Slovakia, Portugal, Poland, Germany, Sweden and Italy (19).

IQOS® features

In the original IQOS® technology, the heat is dispersed through the tobacco plug on each puff by a blade in the heater device (often "pen-shaped") inserted into the end of the heatstick (referred to as "HEETS"). The emission then passes through a hollow acetate tube and a polymer film filter on the way to the mouth. The heating blade's temperature is electrically controlled and can only reach 350°C, whereas a regular cigarette can burn between 600°C and 800°C (20, 21). The more recent IQOS® devices (IQOS ILUMA™) heat the tobacco simply by induction and do not use a blade. The bladeless system, the lack of the requirement for cleaning in between uses, and the odourless system set these new devices apart from the older ones. The equipment model determines how long the heatsticks will stay heated (22).

The tobacco industry asserts further differences between heatsticks and conventional cigarettes, including the absence of tobacco cut-filler, much lower tobacco content, a polymer filter to cool the aerosol, and a mouthpiece made of low-density cellulose acetate to mimic the sensation of smoking. (20).

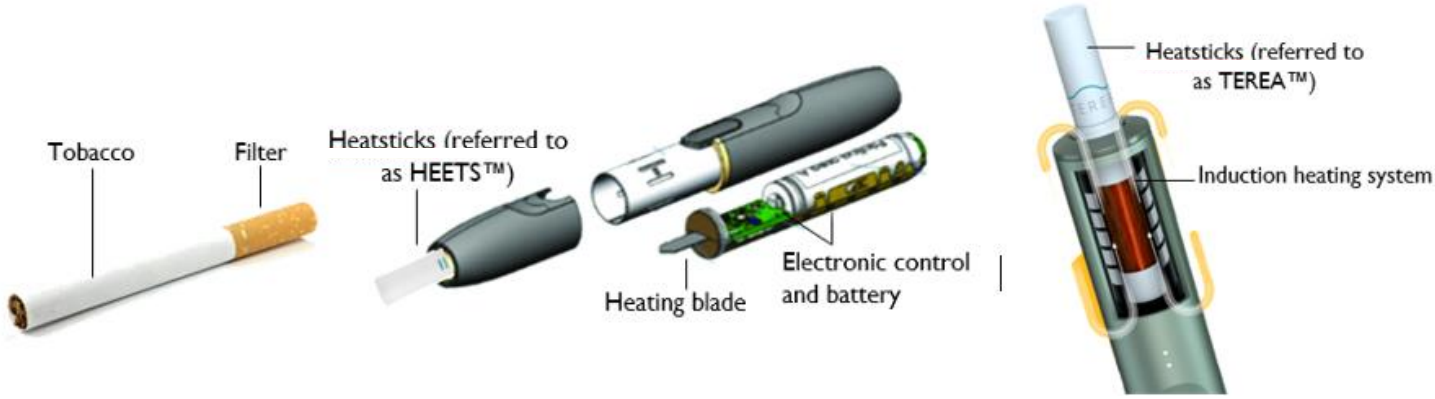

Figure 1 - From left to right: Conventional cigarette, IQOS® device, IQOS Iluma™

More independent, clinical, and long-term studies are still required to advance the state of the art regarding the potential less harmful effect of this

HTP and to provide regulatory agencies, product users, and public health professionals with information about the potential public health impact of IQOS® and other HTPs (23).

IQOS® influence on overall and oral health: controversial state of the art

According to the PMI industry, levels of toxicants and hazardous substances are significantly decreased (a mean reduction of almost 90% across a wide range of harmful and possibly harmful constituents (HPHC) compared to levels of HPHC in cigarettes, on average) (24), suggesting that the modified aerosol has lesser toxicity and less detrimental effect on cells when inhaled, compared to traditional cigarettes. (25, 26).

A systematic review by Simonavicius et al. found that HTPs reduced hazardous and potentially dangerous toxicants and particulate matter by at least 62% and 75%, respectively, while delivering up to 83% of the nicotine dose. However, they also noted that generally higher concentrations of HPHCs were released in studies on humans when compared to smoking devices.
Although evidence suggests that IQOS® only heats to 350°C, the maximum temperatures reached in heat sticks are unknown, and local hot spots may result in the formation and release of substances like phenol/cresols and polycyclic aromatic compounds (PAHs), which are typically not formed in significant quantities by the thermal breakdown of tobacco and biomass until much higher temperatures are reached (17).

Some industry-sponsored studies showed that an IQOS® product did not induce vascular impairment of endothelial cell migration *in vitro* in contrast to a reference tobacco cigarette (27). However, a 2017 independent study found that IQOS® aerosol from a single heatstick can significantly and quickly damage endothelium function in rats, comparable to cigarette smoke (28). Regarding periodontal disease, an industry-sponsored study demonstrated that, in comparison to continuing to smoke, taking IQOS® instead of quitting smoking enhances both the effectiveness of periodontal therapy and overall oral health (29), which was not supported by a longitudinal study that suggested that IQOS® use was significantly associated with the prevalence of self-reported

periodontal disease, assuming a higher prevalence of periodontal diseases compared to non-users (30). Additionally, a 2020 study that assessed the acute effects of inhaling IQOS® aerosols on lung tissue damage and inflammatory changes in the lungs concluded that doing so could cause changes in the user's airways that are related to the epithelial-mesenchymal transition, as well as induced oxidative stress and inflammation, priming infections, and airway remodelling, which may also affect periodontal disease (31). The concluding observation is corroborated by additional research published in 2018, 2020, and 2023 (31-33). However, an independent study also demonstrated that IQOS® induced lesser cytokine release than combustible cigarettes, while also having lower toxicity in its emissions when compared to those of cigarettes (25).

More studies revealed that IQOS® exposure exhibited cellular toxicity with increasing concentrations, being equally harmful to human lung cells at greater doses, as CS (cigarette smoke) (34), and cell types varied in their sensitivity to IQOS® aerosol and cigarette smoke, with cells from human embryos and the respiratory system typically being more sensitive.

Why should this topic be continued to be studied?

It is clear that even though HTPs produce fewer carcinogens and toxic chemicals than traditional cigarettes, they still carry some risk because they produce new substances with unknown health effects (18), especially because they may exhibit newer damage mechanisms related to the additives, flavours, and metal nanoparticles (35). Additionally, it is crucial to keep in mind that heated tobacco product emissions may contain some of the compounds that would disintegrate either completely or partially during combustion, creating a unique chemical mixture with a unique toxicity profile that has not been sufficiently characterised before (23).

It is though critical to continue performing unbiased research on the health risks of HTPs because there is still little information available and the market is growing, particularly because consumers feel that these products are less harmful than smoking tobacco (19). Since the aerosol directly contacts the oral mucosa when it enters the oral cavity, where epithelial cells and fibroblasts

interact to preserve tissue integrity and function, this is especially important for the oral environment and associated healthcare providers.

Why use this approach?

For this study, an *in vitro* experimental protocol will be used, reaching hand of human cell cultures, considering that they are very helpful for determining the potential toxicity of compounds and/or chemicals, with a high translationality to human health, since it is an easy way to address cellular and molecular abnormalities in cellular function (36). Human gingival fibroblastic cell cultures will be used because human gingival fibroblasts are the most abundant cells in gingival connective tissue, contributing to maintaining the homeostasis of connective tissue through the secretion and degradation of components of the extracellular matrix, such as collagen (37), and inherently playing a critical role in normal turnover, repair, and regeneration of periodontal tissues. The renewal of a strong fibrillar link between the tooth root, the gingiva, and the periodontal ligament requires the modulatory activity of these cells (38).

Purpose of the investigation

The aim of the present study is to compare the effect of conventional CS with IQOS® aerosols from two distinct devices, on the functionality of human fibroblastic cell cultures. It also aims to aid in the creation of hypotheses regarding the impact of IQOS® aerosols on typical periodontium physiology and the results of switching from traditional tobacco to IQOS® products on periodontal disorders, enabling medical professionals to make recommendations based on the best available data.

MATERIALS AND METHODS

Product acquisition and storage

The following tobacco products, with various tobacco fillers and compounds, were used for the smoking experiments and creation of the aqueous extracts of cigarette smoke and IQOS® aerosols: conventional tobacco cigarettes (Marlboro red medium, Philip Morris International (PMI)) and IQOS® heatsticks (Heets blue and Terea blue, PMI). Marlboro red cigarettes were used as a comparative control for CS, as previously reported in the literature (39).

All these items were commercially available and were purchased in a local tobacco shop, unaltered, exactly as they were meant to be used by consumers.

The IQOS® 3, as well as the IQOS ILUMA ONE™, both manufactured by Philip Morris International, were the IQOS® devices utilized in this research. Before the beginning of the tests, both the cigarettes and the heatsticks were stored in a cool, dry environment, free of humidity.

Aerosol and smoke solution production

Two 250mL gas washing bottles were used in the experiment and were linked by silicone tubes. 50mL of fresh culture medium (Minimum Essential Medium Eagle - Alpha Modification, Gibco®) was placed in the first gas washing container, and 50mL of deionized water was placed in the second. The final silicone tube was attached to a high-performance peristaltic pump (YZ1515x, LbX instruments) that could be programmed for puff number, puff volume, and time between puffs. The machine was run at 434.2 rotations per minute, with an 18 # tubing size.

This set-up made it possible to use the Health Canada Intense (HCI)/ ISO 20778:2018 puffing protocol, which more accurately simulates smoker's behaviour (40). This procedure required a total puff volume of 55mL, a 2s puff, and a 30s inter-puff interval.

Starting the experiment required igniting the cigarette with a lighter or turning on the IQOS® device, while simultaneously turning on the peristatic pump to

produce smoke or aerosol (from a cigarette or heatsticks, respectively). This extraction technique mimics how a typical user would inhale.

Due to the peristaltic pump's negative pressure, the smoke or the aerosols were forced through the gas-washing bottles, contaminating the α-MEM in the first bottle and the water in the second. Each aerosol was then condensed into a liquid form that was intended to dissolve into the α-MEM and water. Up to 250 puffs of cigarettes and heatsticks were taken during this process. The cigarettes were smoked till the entire length (without reaching the filter). The heatsticks were completely consumed after four minutes of using the IQOS® device. Because the experimental system was sealed off, no air could penetrate the smoke or aerosol flow.

Each experiment was repeated three times using the different devices, to ensure three α-MEM contaminations for each product used. The aerosol was not fractionated for this research and was used unaltered since the complete trapping aerosol is what IQOS® users inhale. The culture medium that was only subjected to air served as a control.

Figure 2 - Experimental set-up: Generation of conventional cigarette smoke

Figure 3 - Experimental set-up: Generation of IQOS 3™ aerosol

Figure 4 - Experimental set-up: Generation of IQOS ILUMA ONE™ aerosol

Cell Culture

Human gingival fibroblasts (HGF) (AG09429 Fibroblast, Coriell Institute) were expanded and seeded in 96-well cell culture plates ($1.5x10^4$ cells/well) (Thermo Fisher Scientific, US) and allowed to adhere for 24h under standard culture conditions - i.e., α-minimal essential medium, containing 10% foetal bovine serum (FBS) and 100 IU/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B (all Gibco®) incubated at 37ºC, in a humidified environment with 5% CO_2.

	Cigarette smoke solution			IQOS 3™ aerosol solution			IQOS ILUMA ONE™ aerosol solution		
	Day 1 after exposure	Day 5 after exposure	Day 5 with medium change	Day 1 after exposure	Day 5 after exposure	Day 5 with medium change	Day 1 after exposure	Day 5 after exposure	Day 5 with medium change

Table 1 – Experimental timeline and timepoints of evaluation of the cell culture study.

After 24h, the culture medium was discarded and replaced with fresh culture medium with different extracts (30, 10, 3, 1 or 0.3%) of either cigarette smoke or IQOS® aerosol solutions. Cells exposed to fresh α-MEM exposed to air served as a control. Each treatment was performed in 8 replicates. The cells were incubated in the same conditions as described before. Each culture plaque was divided as described in the following scheme (fig.5).

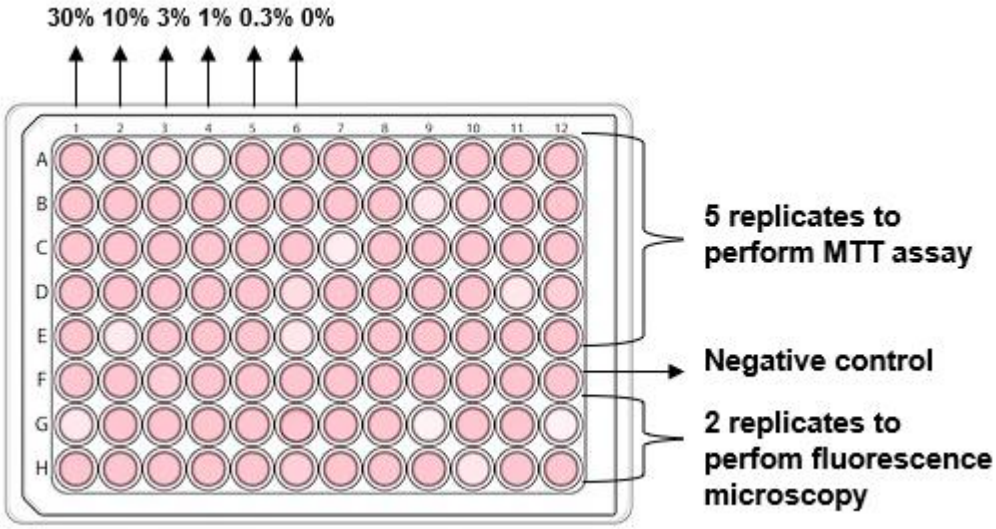

Figure 5 - Cell culture on 96-well cell culture plates (Thermo Fisher Scientific, US) shown schematically.

In this experiment, two distinct time points – day 1 and day 5 – following exposure to smoke/aerosol solutions were employed to evaluate cytotoxicity and cell morphology. In one experimental setting, the culture medium was not changed throughout the 5-day period, allowing the assessment of potential cumulative effects. In another experimental setting, the culture medium was

changed on day 1, with fresh supplemented α-MEM, to evaluate the potential capability of the cell culture to recover from the early exposure.

Cytotoxicity assay

The MTT assay, a colorimetric assay that detects mitochondrial dehydrogenase activity, was used to evaluate the metabolic activity of the cultures and inherently, cell viability/proliferation.

A violet, water-insoluble compound known as formazan is created when metabolically active cells reduce the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide). Fewer viable cells result in decreased mitochondrial enzyme activity, which can be directly correlated with the amount of formazan produced (41). On day 1 or day 5 following exposure, 10μL of MTT substrate was added to the growing cells. After 2h of incubation under the same conditions as before, the MTT-containing culture medium was removed. Dimethyl sulphoxide (DMSO) was used to dissolve MTT-derived formazan crystals, and spectrophotometric quantification was conducted at 550 nm with a microplate reader (Synergy HT; BioTek). Results were reported as the mean ± standard deviation, n=5.

Statistical analysis was conducted to evaluate the difference between groups. Data normality was assessed by the Shapiro-Wilk test. Regarding normal data sets, one-way ANOVA was performed, followed by multiple comparisons using Turkey's test. For non-parametric data sets, the Kruskal-Wallis test was performed, followed by multiple comparisons using Dunn's tests. p values<0.05 were considered significant.

Morphological evaluation

The wells used for fluorescence microscopy were fixed with formaldehyde for 15 minutes on the first- and fifth-day following exposure. Following this, the formaldehyde was removed, and cultures were washed with PBS, and the cells were stored in the refrigerator, at 4ºC, with PBS. For marking the cell structures, 0.1% triton X-100 diluted in PBS was initially applied to permeabilize the cells. Following, a 1% bovine serum albumin (BSA) solution, likewise dissolved in PBS, was added to block non-specific interactions. The

nucleus was stained with 8 g/mL of a nuclear dye (Hoechst 33342, MedChemExpress LLC, US), and the actin filaments were stained with a green marker (Flash Phalloidin™ Green 488, BioLegend, US), diluted at 1:150. Stained cells were observed with a fluorescence microscope (CELENA S digital imaging system - Logos Biosystems).

RESULTS

Macro-analysis of the deposit left on the gas washing container following exposure

With the same number of puffs, the colour of the medium changes. A significantly increased turbidity, and the amount of deposited matter on the gas washing container, seems to be higher after exposure to CS, than after IQOS 3™ and IQOS ILUMA ONE™ aerosols.

The differences in the deposit matter between the two IQOS® devices are hardly noticeable. The alteration in colour is likewise extremely minor (fig.6).

Figure 6 - Deposit matter following different exposures (from left to right: control, CS, IQOS 3™, IQOS ILUMA ONE™)

Cytotoxicity assay

As shown in the fig. 7, the cell viability/proliferation decreased with higher concentrations (30 and 10%) for all cigarette varieties on the first day after exposure, whether a trend for reduced MTT activity may be verified already at the 3% concentration. The conventional tobacco cigarette induced a poorer overall performance, with significantly lower values being attained with 3 and 10% concentrations, as compared to the other groups. The differences are less significant at lower concentrations, and the contamination with IQOS 3™ appears to be less cytotoxic. Additionally, at lower concentrations, the mitochondrial activity for experimented groups was more similar to the one of control. Statistically, the mean was significantly different from the control group at high concentrations. 3 and 10% from IQOS 3™ aerosol were significantly different from the same concentration of CS. For the concentration of 3% IQOS 3™ and IQOS Iluma One™ were significantly different.

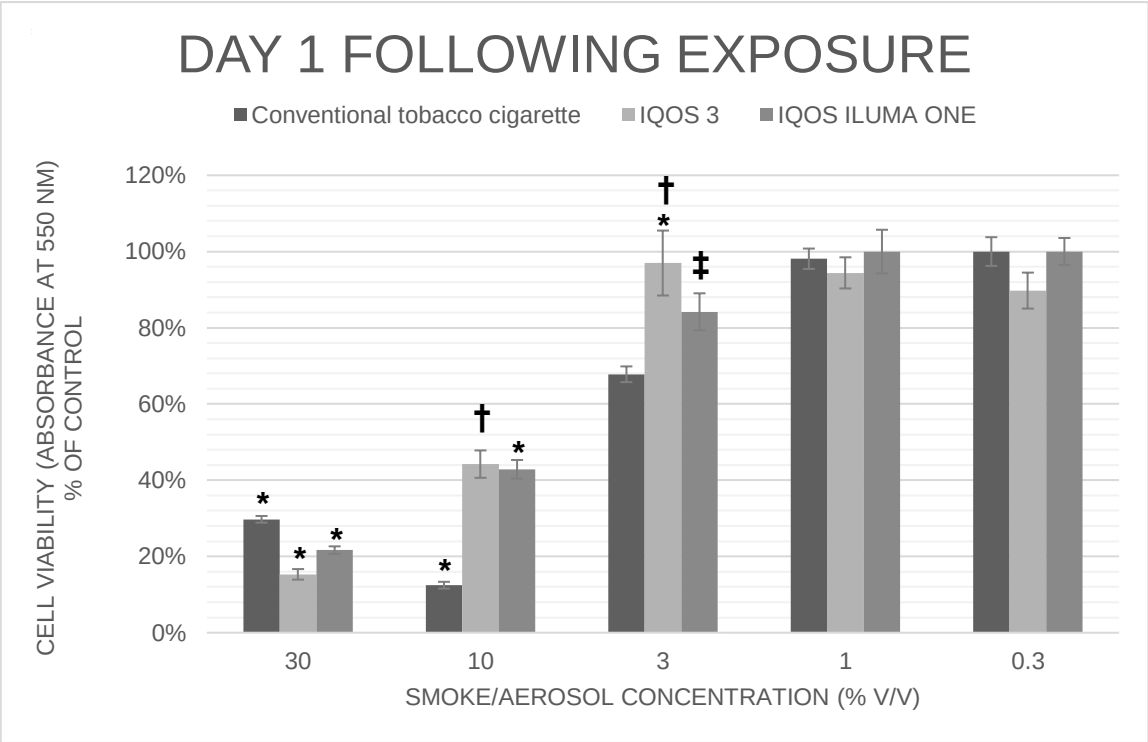

*Figure 7 - Effects of using different dilutions of conventional cigarette smoke and IQOS® aerosol on human gingival fibroblasts viability at day 1, using MTT assay. The results are expressed as % of control, and presented as mean ± SD. *Significant differences between each individual group and the control group; † significant differences between IQOS® aerosols and CS; ‡ significant differences between IQOS 3™ and IQOS Iluma One™.*

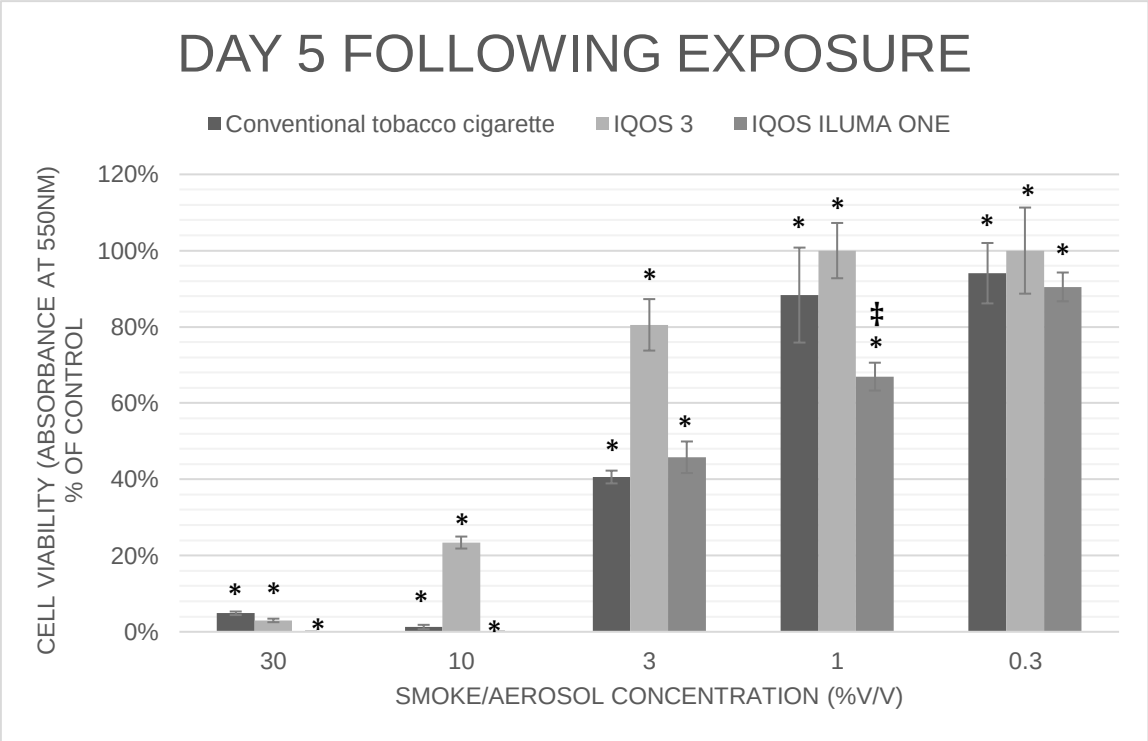

*Figure 8 - Effects of using different dilutions of conventional cigarette smoke and IQOS® aerosol on human gingival fibroblasts viability at day 5, using MTT assay. The results are expressed as % of control, and presented as mean ± SD. * Significant differences between each individual group and the control group; † significant differences between IQOS® aerosols and CS; ‡ significant differences between IQOS 3™ and IQOS Iluma One™.*

Similar to the findings on day 1 following exposure, the mitochondrial activity significantly declines at high smoke/aerosol concentrations (30 and 10% extracts), at day 5. As illustrated in fig.8, IQOS ILUMA ONE™ appears to be the most cytotoxic, because it regularly results in the lowest MTT reduction values within the assayed concentrations, as compared to control. Conventional CS follows closely with a similar trend, inducing a significant reduction in fibroblasts' functionality. IQOS 3™ seems to allow, comparatively, for the highest MTT reduction values, within the assayed concentrations. The differences between the groups are less significant and more comparable to the control group at lower concentrations. Statistically, all the means group were significantly different from the control group. At the 1% extract IQOS 3™ was significantly different from IQOS Iluma One™.

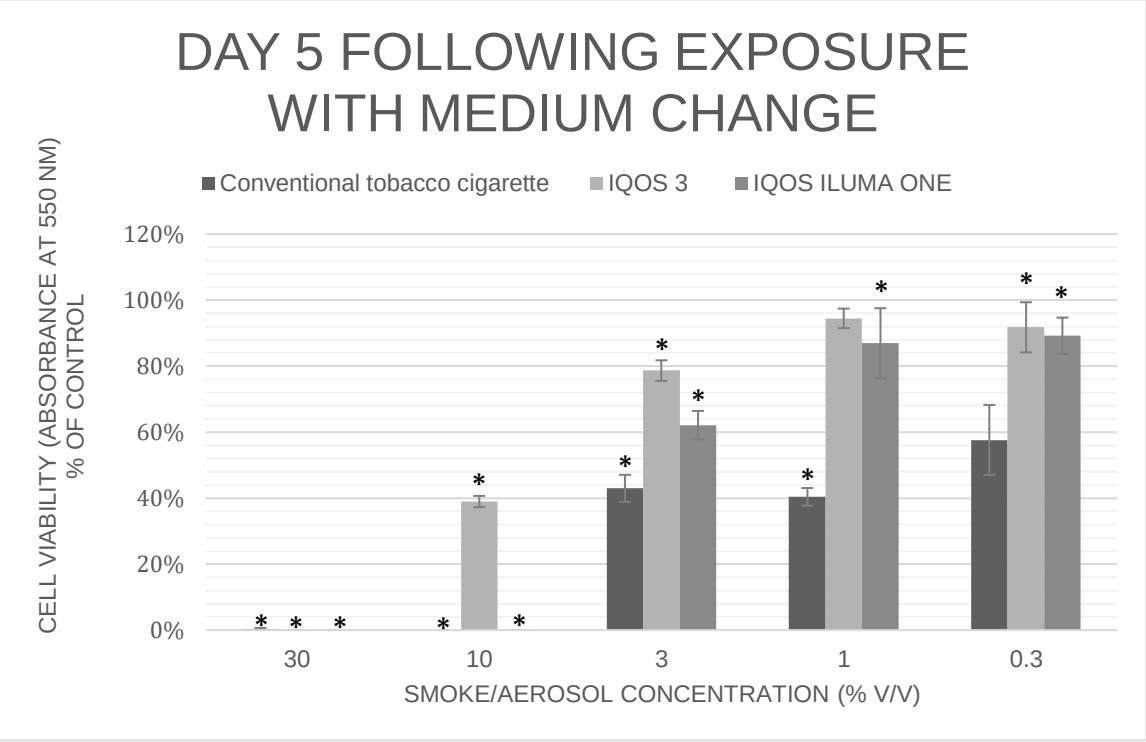

*Figure 9 - Effects of using different dilutions of conventional cigarette smoke and IQOS® aerosol on human gingival fibroblasts viability at day 5 after medium change at day 1, using MTT assay. The results are expressed as % of control and presented as mean ± SD. * Significant differences between each individual group and the control group; † significant differences between IQOS® aerosols and CS; ‡ significant differences between IQOS 3™ and IQOS Iluma One™.*

After changing the medium on day 1, with supplemented α-MEM, to assess the potential recovery of the cultures' functionality, a trend similar to the one attained on day 5 without medium change was verified. This decline

appears to be connected to high aerosol/smoke concentrations. Fig. 9 shows the same pattern as the previous figures, with the differences between the groups becoming less pronounced and more identical to the control group at lower concentrations. Statistically, following the same tendency as the previous graphs, almost all the means were significantly different from the control group.

Morphological evaluation

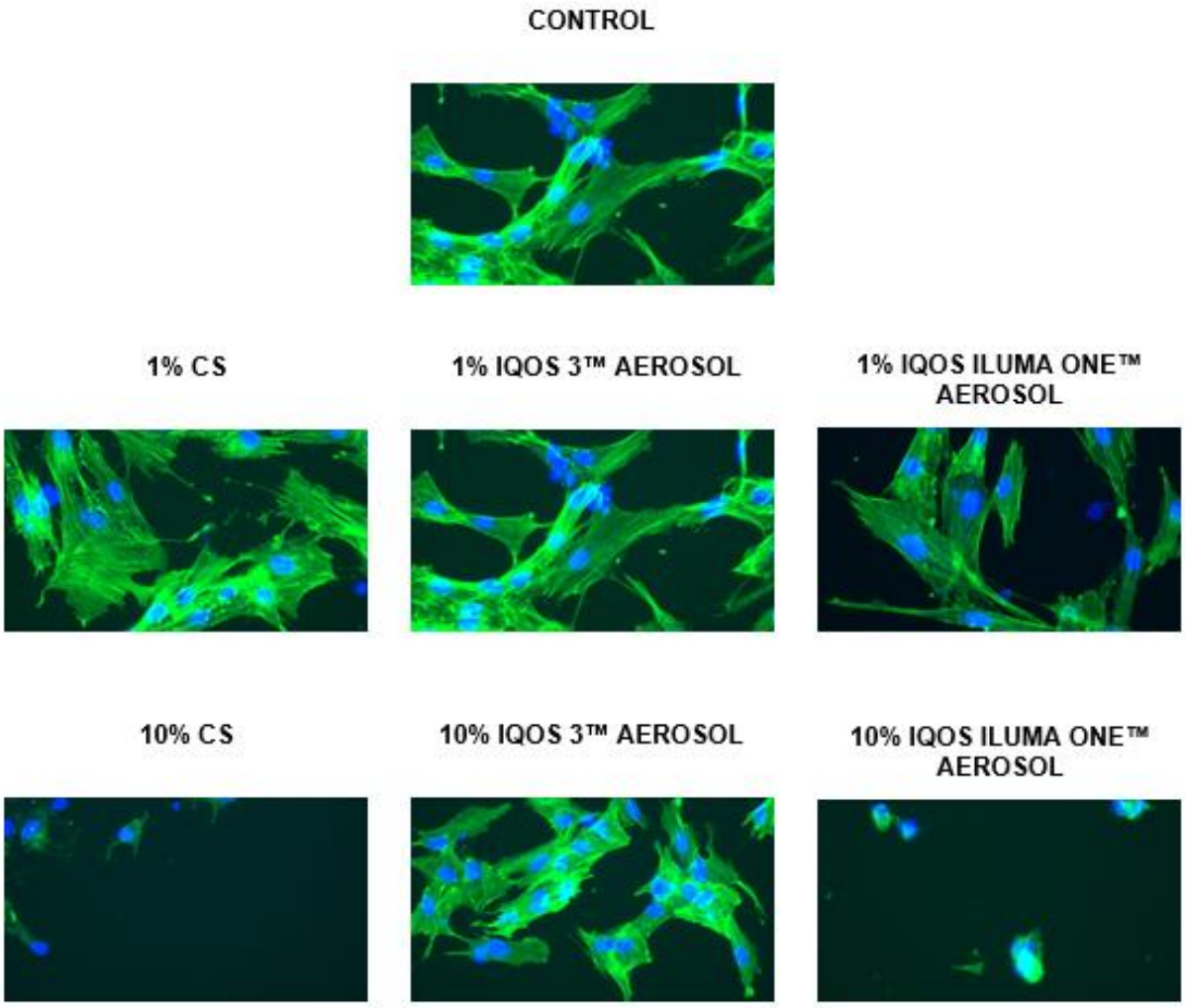

Figure 10 - Representative micrographs of human gengival fibroblasts exposed to different dilutions of conventional cigarette smoke and IQOS® aerosol at day 1, by using fluorescence microscopy. Actin cytoskeleton is marked in green, and the nucleus in blue. Objective magnification: 20x

Human gingival fibroblasts (HGF) with a characteristic fibroblastic phenotype may be seen in the control group's image. The cells exhibited a flattened, striated, and elongated shape, with extended cytoplasmatic processes, and a well-defined cell body. The nucleus had an oval form. The actin filaments were well arranged and created a web of fibers throughout the

cytoplasm. The cells revealed initial intercellular contact. The treatment with 1% of CS and IQOS® aerosols, after 1 day of exposure, barely influenced the normal morphological aspect of the cells. However, the normal morphological characteristics of the cells appear to be significantly affected by the treatment with 10% CS and IQOS® aerosols. The CS-treated cells exhibited an abnormal morphology with loss of fibroblastic cells features, such as their elongated form. They also had a sparse quantity of cytoplasm and a disordered cytoskeleton. The cells treated with 10% IQOS Iluma One™ aerosol displayed the same pattern. The HGF exposed to 10% of IQOS 3™ aerosol were smaller and less elongated than those in the control group. The actin filaments were partially disorganized but maintained the typical fibroblastic organization, despite the trend for a smaller cytoplasmic arrangement (Fig.10).

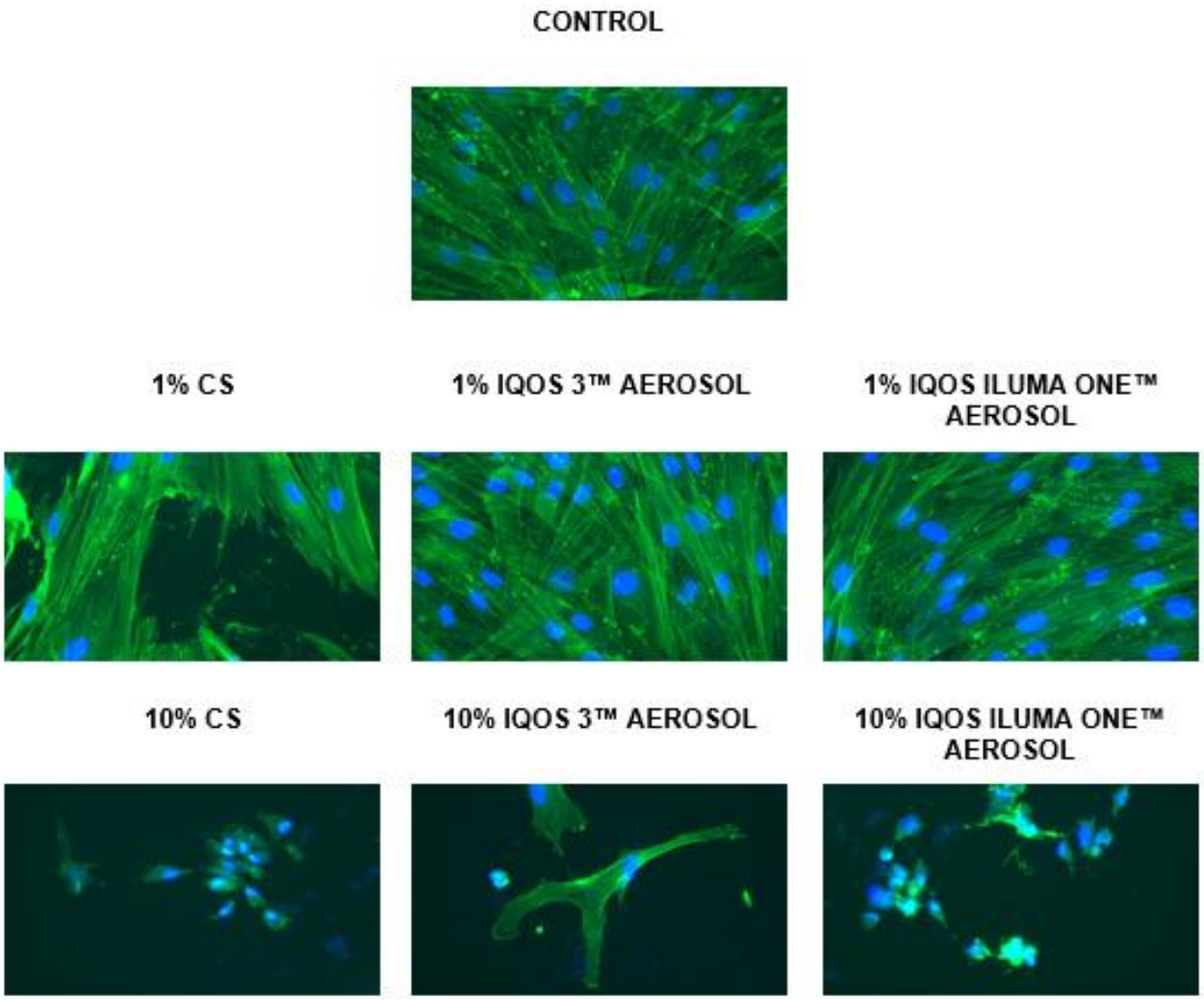

Figure 11 - Representative micrographs of human gengival fibroblasts exposed to different dilutions of conventional cigarette smoke and IQOS® aerosol at day 5, by using fluorescence microscopy. Actin cytoskeleton is marked in green, and the nucleus in blue. Objective magnification: 20x

The cell density was found to be higher on day five following exposure, than it was on day one. The HGF still exhibited the typical fibroblastic pattern following treatment with 1% CS, but the cytoplasmatic processes were smaller than those in the control group, and there was a fewer cell number overall. When compared to the control group, the fibroblasts treated with 1% of IQOS 3™ and 1% of IQOS Iluma One™ exhibited no significant alterations. After being treated with 10% CS and IQOS Iluma One™ aerosol, the fibroblastic morphology was aberrant, with little or no cytoplasm and actin strands. When 10% of IQOS 3™ was applied, the cell shape become abnormal with minor cytoplasmatic processes, but a large cell area with extended cytoplasm, and actin filaments showing a partial organization, despite the abnormally large cytoplasmic spreading and reduced cell number (Fig. 11).

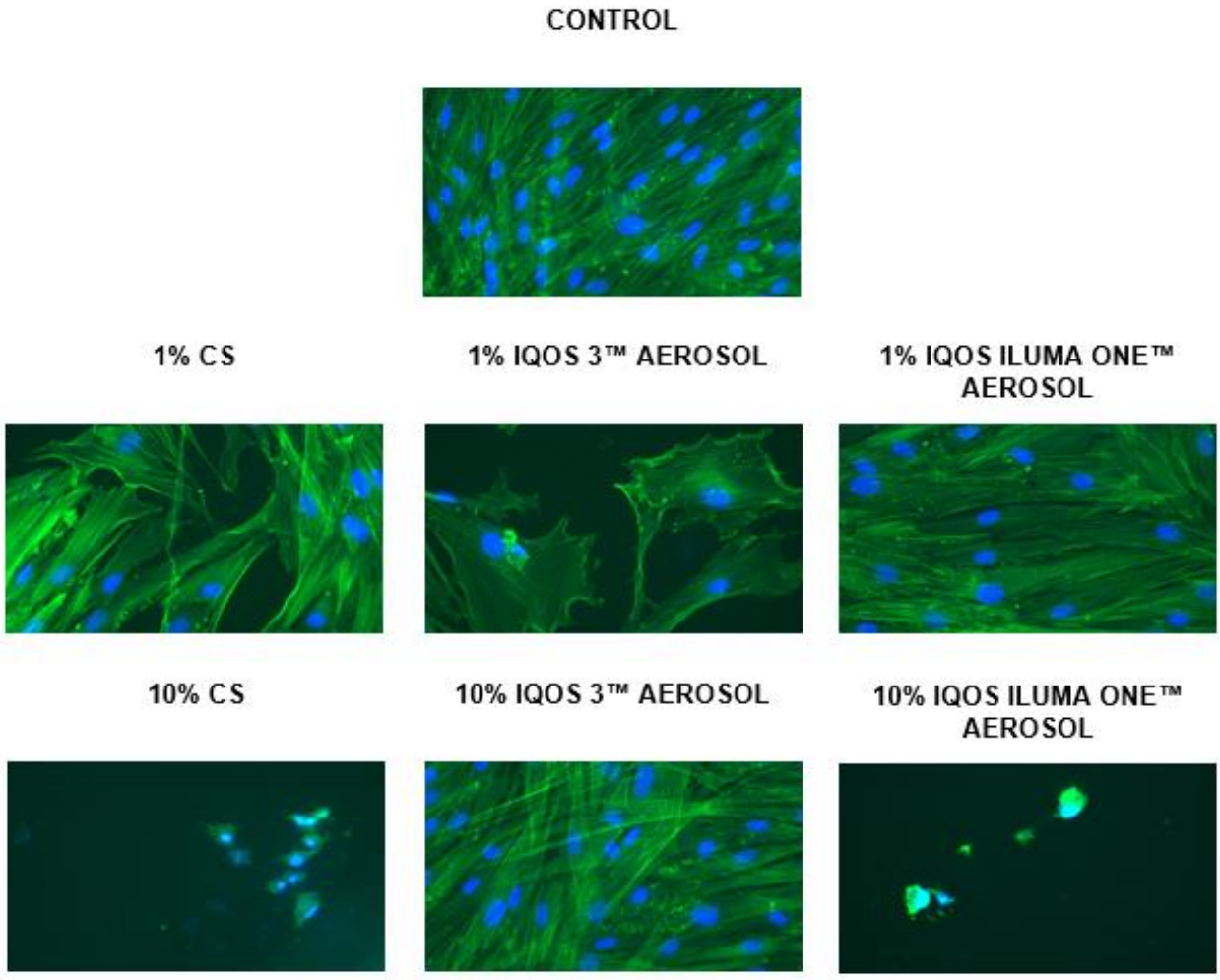

Figure 12 - Representative micrographs of human gengival fibroblasts exposed to different dilutions of conventional cigarette smoke and IQOS® aerosol at day 5 with medium change at day 1, by using fluorescence microscopy. Actin cytoskeleton is marked in green, and the nucleus in blue. Objectifive magnification: 20x

In general, the cells exhibited the same morphological aspect on day 5 after treatment with a medium change on day 1, as they did on day 5 after exposure with no medium change. The fibroblasts CS-treated at 1% exhibited a defective cell contour, and a reduced number of cells. On the other hand, the ones treated with 1% IQOS® aerosols presented a fibroblastic morphology similar to the control, with the number of cells being the only difference in comparison with the control. After the treatment with 10% of CS and 10% of IQOS Iluma One™ aerosols, the fibroblasts also presented an anomalous aspect, with aggregated spheric cells, and minor cytoplasmic spreading or cytoplasmatic processes. The fibroblasts exposed to 10% of IQOS 3™ aerosol presented a dysmorphic structure, with a faulty contour, and a reduced number of cells (Fig. 12).

DISCUSSION

In this study, the effects of CS smoke and IQOS® aerosols, on the functionality of human gingival fibroblastic cells were compared. First, CS and IQOS® aerosols' contaminated cell culture mediums were prepared with an establish and standardized protocol. The HGFs were then seeded in the culture medium that had been supplemented with CS and IQOS® aerosols and allowed to adhere, grow and multiplicate. On the first and fifth day after exposure, the cultures' metabolic activity and morphology were assessed.

The colour of the medium changed noticeably and there was a lot of deposited matter in the gas washing container right away after the fresh medium was contaminated with the CS produced by the vacuum pump, which did not happen after the contamination with IQOS® aerosols. However, there was little difference in the effect on fibroblasts' functionality. At all timepoints, IQOS® aerosols and CS had a negative impact on the metabolic activity of fibroblasts at high concentrations. There is a definite pattern of viability reduction with an increase in smoke/aerosol concentration, even while the viability of the cells exposed to 30% of CS is higher than that of the cells exposed to 10% of CS, which may be connected to the stress that cells were subjected to.

The same outcomes—a significative decrease in cell viability at high concentrations (30% and 10% of CS and IQOS® aerosols, respectively)—can be seen on day 5 after exposure. The largest decrease in the viability of fibroblasts was caused by the IQOS Iluma One™ extract at day 5.

The timepoint at day 5 with the medium change was made to check if the cells could recover from the exposure. The findings revealed no differences between the groups with and without medium change. Overall, there was no difference between the groups in terms of the influence on the HGF for lower concentrations, and IQOS Iluma One™ aerosol and CS smoke had the greatest effect on the viability reduction.

These results were supported by morphological examination, which revealed that at a concentration of 10% of smoke/ IQOS® aerosol, there was a substantial change with the loss of fibroblastic phenotype, although, at a concentration of 1% of Smoke/ IQOS® aerosols, the differences with the control

group were not that significant. The impact of IQOS 3™ seems to be less harmful to the morphological structure of the HGF.

The findings of this experiment are supported by a further study that evaluated the cytotoxicity of IQOS® aerosols and smoke from two different conventional cigarettes on eight distinct cell types. The study's findings that the cell response to exposures was concentration-dependent and that all treatments were cytotoxic at high concentrations (42) concur with those of a previous study that examined the cytotoxic effects of IQOS® aerosols on human bronchial epithelial cells. That study found that exposure to IQOS® aerosols damaged the cells more than exposure to air controls, even though the cytotoxicity was less than for conventional CS (43). Additionally, another investigation found that IQOS® exposure was as cytotoxic as CS smoke at some concentrations, by using two different cytotoxicity assays (MTT and LDH) (34) which is also in agreement with a study that suggests that short-term inhalation of IQOS® aerosol causes lung damage and proinflammatory changes (44).

However, the outcomes of this investigation do not support an *in vitro* study that showed that various dilutions of IQOS® extracts did not cause cytotoxicity in either oral fibroblasts or keratinocytes, and that cell morphology was unaffected. The smoking protocol (4s of puff duration, 55mL each puff, and only 10 puffs altogether) or the fact that the cells were only cultured in the enriched medium for one day may be responsible for these contradictory results (45). A different study conducted by the same research team found that IQOS® aerosols were not as harmful to cells as CS, which only caused some functional alterations without inducing cell death or severely affecting their morphology (46). Another independent study that looked at the effects of IQOS® on the osteogenic differentiation of mesenchymal stem cells and primary osteoblasts in comparison to conventional cigarettes came to the opposite conclusion of the present investigation, finding that IQOS® aerosol had less of an impact on cell viability than conventional CS (40). Studies conducted by the industry claim that IQOS® aerosols are less cytotoxic than CS aerosols (47, 48).

Periodontal disease is a chronic inflammatory condition that affects the tissues surrounding the teeth, involving complex interactions among the host immune response, bacterial pathogens, and tissue remodelling processes.

Cellular metabolism plays a significant role in periodontal infections and inflammation. Oxidative stress, resulting from an imbalance between oxidants and antioxidants, can cause tissue damage and trigger chronic inflammation. Therefore, it is crucial to understand the impact of reduced mitochondrial reductase activity, as measured by the MTT assay. Reduced activity of mitochondrial reductase can lead to mitochondrial dysfunction and an altered redox balance, thereby affecting cellular metabolism. Additionally, it can increase oxidative stress levels. These mechanisms have the potential to promote tissue damage, inflammation, and impair the remodelling and repair processes of periodontal tissues (49, 50). In addition, since actin filaments are essential for cell migration (affecting the structural basis of protrusions called lamellipodia and filopodia), extracellular matrix remodelling (restraining the secretion and organization of its constituents, such as collagen and fibronectin), wound healing & tissue repair, and cell adhesion, a disorganized cytoskeleton without cytoplasmatic processes may compromise the motile events of fibroblastic cells (51). Each of the above processes is essential for periodontal tissue remodelling, wound healing, and maintaining periodontal tissue integrity.

This study compared two different IQOS® devices and includes the new machine on the Portuguese market, providing new insight into the influence of IQOS® aerosols on fibroblastic cell cultures. It is curious that IQOS 3™, one of the earliest versions of the IQOS® technology, produces different reactions than IQOS Iluma One™ while being slightly less damaging to cell viability and morphology. The key distinction between these two models is that the IQOS Iluma One™ uses induction heating instead of a blade like the IQOS 3™ and does not require cleaning after every 20 smokes.

This study was conducted as short-term research, involving only two different time points, day 1 and day 5 following exposure, to a limited quantity of 20 cigarettes. To comprehensively understand the effects of chronic conditions, long-term research is necessary to assess the cumulative impact of continuous exposure to CS/IQOS® aerosol. Additionally, the absence of a standardized approach for comparing *in vitro* toxicity between emerging tobacco products and traditional cigarettes poses a challenge. Some authors have utilized the same exposure time or puff number across different aerosols, while others have focused on comparable nicotine exposure.

Moreover, this study solely employed human gingival fibroblasts. However, expanding the cellular models to include other cell types important for periodontal tissues could enhance the reliability and reproducibility of the experimental findings.

Furthermore, it is crucial to consider the type of heatsticks used, as different additives and compounds present in various products may result in distinct toxic products. Consequently, a thorough physical and chemical characterization of the enriched medium after exposure with CS or IQOS® aerosol (by using gas chromatography-mass spectrometry, high-performance liquid chromatography, or Fourier transform infrared spectroscopy, for example) would be highly relevant and informative.

Based on these results, it is not safe to assert that switching to IQOS® devices is a viable strategy for reducing traditional cigarette usage. Consequently, clinicians should refrain from endorsing the use of these products and continue to emphasize the importance of not smoking at all as the most optimal approach.

CONCLUSION

Based on the obtained results, it is evident that both regular tobacco cigarettes and IQOS® devices impacted the biological activity of fibroblastic cells, particularly at high concentrations. However, at low concentrations, there was a minimal disparity observed between the different groups. Notably, when present in significant quantities, conventional cigarette smoke demonstrated a further reduction in fibroblast viability. Similarly, the IQOS Iluma One® aerosol exhibited a comparable pattern of decline. Moreover, it is noteworthy that the two IQOS® devices exhibited distinct effects on the functionality of fibroblasts.

To understand the clinical relevance of these findings in the health of periodontal tissues and their potential impact on periodontal disorders, long-term investigations, and clinical approaches are required.

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ANNEXES

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DECLARAÇÃO
Mestrado Integrado em Medicina Dentária

Monografia/Relatório de Estágio

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Identificação da publicação

Dissertação de Mestrado Integrado (Monografia) [X] **Relatório de Estágio** [ ]

Título completo

"Comparative study of the effect of IQOS® aerosols and cigarette smoke on the functional activity of fibroblastic cells"

Orientador Professora Doutora Marta dos Santos Resende

Coorientador Professor Doutor Pedro Sousa Gomes

Palavras-chave heated tobacco products; IQOS; conventional tobacco; periodontal disease; fibroblastic cells

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Justificação para a não autorização imediata Publicação de artigo

Data 22 / 05 / 2023

Assinatura Rafaela Maria Fernandes da Silva

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Anexo II: Declaração de autoria do trabalho apresentado

Monografia/Relatório de Estágio

Declaro que o presente trabalho, no âmbito da Unidade Curricular Monografia/Relatório de Estágio integrado no Mestrado Integrado em Medicina Dentária, da Faculdade de Medicina Dentária da Universidade do Porto, é da minha autoria, e todas as fontes foram devidamente referenciadas.

Porto, 22 de maio de 2023

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(A Estudante)

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Anexo III: Parecer do Orientador para entrega definitiva do trabalho apresentado

Informo que o trabalho de Monografia de Investigação desenvolvido pela estudante Rafaela Maria Fernandes da Silva, com o título: "Comparative study of the effect of IQOS® aerosols and cigarette smoke on the functional activity of fibroblastic cells" está de acordo com as regras estipuladas pela Faculdade de Medicina Dentária da Universidade do Porto, foi por mim conferido e encontra-se em condições de ser apresentado em provas públicas.

Porto, 22 de maio de 2023

(O/A Orientador/a)

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UNIVERSIDADE DO PORTO

Anexo IV: Parecer do Coorientador para entrega definitiva do trabalho apresentado

Informo que o trabalho de Monografia de Investigação desenvolvido pela estudante Rafaela Maria Fernandes da Silva, com o título: "Comparative study of the effect of IQOS® aerosols and cigarette smoke on the functional activity of fibroblastic cells" está de acordo com as regras estipuladas pela Faculdade de Medicina Dentária da Universidade do Porto, foi por mim conferido e encontra-se em condições de ser apresentado em provas públicas.

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