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EFFECTS OF HEATED TOBACCO PRODUCTS AND CONVENTIONAL CIGARETTES ON DENTAL IMPLANT WOUND HEALING: EXPERIMENTAL RESEARCH

Running title: HTPs and dental implant wound healing

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・Dental implants are a treatment option for missing teeth.
・Heated tobacco products have been marketed worldwide.
・Evaluation of wound healing assay using a fluorescence microscope.
・Heated tobacco products may be a risk factor for dental implant treatment.

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[1]ABSTRACT

Background: Smoking affects wound healing and is associated with dental implant failure. Heated tobacco products (HTPs) appear to be less harmful than conventional cigarettes (CCs); however, there is limited analytical data to support this claim. This study aimed to compare HTPs and CCs for their impact on wound healing using L929 mouse fibroblast cells and evaluate whether HTPs also lead to failure in implant therapy.

Materials and methods: Cigarette smoke extract (CSE) was obtained from CCs (Marlboro, Philip Morris) and HTPs (Marlboro Heat Sticks Regular for IQOS, Philip Morris) and initiated a wound healing assay with a cell-free area created in the center of a titanium plate by sticking a 2-mm width line tape. The L929 mouse fibroblast cells were exposed with 2.5% and 5% CSE from HTPs and CCs and then seeded in the titanium plate. A scratch wound

[1] Abbreviations

BAT: British American Tobacco

CC: Conventional cigarette

CSE: Cigarette smoke extract

FDA: Food and Drug Administration

HPHC: Harmful and potentially harmful constituents

HTP: Heated tobacco products

JTI: Japan Tobacco International

MEM: Minimum essential medium

MMP: Matrix Metalloproteinase

PMI: Philip Morris International

TIMP: Tissue inhibitors of MMP

healing assay was initiated when all samples were at 80% confluence. The number of cells migrating to the wound site was counted after 12, 24, and 48 h.

Results: Cell migration decreased after CSE exposure from both CCs and HTPs. At each time point with 2.5% CSE, cell migration in the HTP group was less than that of the CC group. There were significant differences between the 2.5% CC and 2.5% HTP groups and the 5% CC and 5% HTP groups after 24 h. HTPs and CCs had similar effects in the wound healing assay.

Conclusion: Therefore, HTP use may be a risk factor for poor dental implant healing.

KEY WORDS: smoking, heated tobacco products, dental implants, L929 mouse fibroblast cells, scratch wound healing assay

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INTRODUCTION

Dental implants are widely used as a treatment option for individuals who have lost their teeth due to periodontal disease, injury, or other reasons [1]. Good wound healing after implant placement is critical to the success of implants because of the direct contact between the implant and soft tissues and bones. Normal wound healing involves four successive but overlapping phases: hemostasis, inflammatory, proliferative, and remodeling [2]. During the proliferative phase, fibroblasts, epithelial cells, and osteoblasts migrate and multiply at the implant interface, resulting in soft tissue attachment and osseointegration, which leads to peri-implant wound healing [3,4]. The extracellular matrix depends on fibroblasts for the production and maintenance of its structure, which is essential for cell morphogenesis, differentiation, and homeostasis [5,6]. Moreover, fibroblasts are responsible for the repair, remodeling, and regeneration of alveolar bone and cementum, and are involved in the wound healing of periodontal tissues [7].

Conventional cigarettes (CCs) affect wound healing and cause peri-implantitis and bone loss around implants, which are associated with implant loss [8-11]. Nicotine in cigarettes increases the accumulation of biofilm, a risk factor that may lead to peri-implantitis [12]. Nicotine is not only addictive but, along with cigarette smoke, contains several chemicals and compounds, including oxidants and free radicals, which induce adverse health effects [13,14].

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Heated tobacco products (HTPs) were designed to reduce exposure to harmful substances derived from combustion and reduce adverse health effects [15]. HTPs can produce aerosol by heating tobacco leaf sheets without burning them. The World Health Organization Study Group on Tobacco Product Regulation found that HTPs reduce exposure to nine specific toxicants (CO, 1,3-butadiene, benzene, benzo[a]pyrene, N-nitrosonornicotine, and 4-[methylnitrosamino]-1-[3-pyridyl]-1-butanone) compared to CCs [16-18].

However, HTPs were observed to be as toxic as cigarettes in various cells, including respiratory cells, when studies compared of the aerosols' toxicity in vitro [19,20]. Philip Morris International (PMI), Japan Tobacco International (JTI), and British American Tobacco (BAT) are the leading manufacturers in the HTP market and launched IQOS, Ploom, and Glo respectively. Overall, HTPs have lower nicotine levels than CCs, but the amount can vary by brand. The exception is IQOS, which launched a Japanese marketing pilot program in 2014 [21].

Although the relationship between CCs and dental implant failure has been widely accepted, the impact of HTPs and their relationship with dental implant failure have not yet been elucidated. Dental implants are a universally accepted treatment option, and a rapid increase in the use of HTPs has been noted worldwide [22]. Therefore, it is important that we research the risk of HTPs in implant treatment.

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In this study, we aimed to compare the impact of HTPs on wound healing after dental implant placement to CCs to assess HTPs' role in implant failure.

MATERIALS AND METHODS

Preparation of cigarette smoke extract solutions

The IQOS (PMI) was used as the test HTPs system. We prepared the CSE based on our previously reported methods [23]. To evaluate and maintain consistency of the different lots of CSE, we performed analysis by gas chromatography-mass spectrometer from the Japan Food Research Laboratories. We diluted the CSEs of CC and HTP with minimum essential medium (MEM) (Thermo Fisher Scientific, Japan) to obtain 2.5% and 5% concentrations using the following equation: (mL CSE solution/total mL) × 100. The total mL in this equation is the sum of the CSE solution (mL) and MEM (mL) volumes.

Fibroblast culture

The L929 mouse fibroblast cell line was purchased from Sumitomo Pharma (Tokyo, Japan) and cultured in MEM containing 5% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin (Thermo Fisher Scientific, Japan). All the cells were cultured in an incubator at 37°C in an atmosphere of 5% CO_2/95% air.

Implant disks

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A 500-μm-thick pure titanium plate (Ti6Al4V) was punched and polished into a 10-mm-diameter disk to match the diameter of the wells of a Falcon 48-well clear flat bottom TC-treated cell culture plate (Corning, USA). Assuming a wound healing assay, a cell-free area was created in the center of the titanium plate by sticking 2-mm-width line tape (Figure 1A, 1B). The titanium plate was sterilized with ethylene oxide gas for the experiment.

Scratch wound healing assay

Sterile implant disks were placed in 48-well plates, with one disk per well. Fibroblasts were seeded onto the titanium implant disks at 40,000 cells per disk. Thereafter, CSE solutions (2.5% and 5%) were added to the wells, and the control cells were cultured in MEM. A gap was created in a confluent monolayer of L929 cells for the scratch wound healing assay to mimic a wound by removing the line tape when each well was 80% confluent. Fibroblast growth analyses were performed at 12, 24, and 48 h after removing the line tape (n = 3 per group). Migration into the gap was imaged using a fluorescence microscope (40× magnification, BZ-9000, KEYENCE, Tokyo, Japan). Image processing and cell counting were performed using optional software (BZ-analysis application, KEYENCE). Cell migration and wound closure were calculated based on the cell numbers at 12, 24, and 48 h. We compared concentrations of CSE (e.g., 2.5% HTP vs. 2.5% CC, 5% HTP vs. 5% CC) at the three time points.

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Statistical analyses

The Wilcoxon rank sum test was used to determine the statistical differences between the cell migration numbers of 2.5% CCs and 2.5% HTPs and 5% CCs and 5% HTPs at 12, 24, and 48 h. Data are expressed as the median and interquartile range, and statistical significance was set at $p < 0.05$ using JMP Pro16 software (JMP, Cary, NC, USA).

RESULTS

Gas chromatography-mass spectrometry analysis

The nicotine concentrations in CSE were 5 ppm for both CCs and HTPs.

Whole cigarette smoke extract solutions reduced fibroblast growth

The number of migrated L929 cells decreased after exposure to CSE in both test groups. At concentrations of 2.5% CSE, cell migration in the HTP group was lower than that in the CC group at each time point. Cell migration in both comparison groups, 2.5% CC vs. 2.5% HTP and 5% CC vs. 5%, were significantly difference ($p < 0.05$ for both groups) at 24 h (Figure 2A, 2B, Table 1). The cell migration number in the 2.5% CC sample had a higher median of 334 than that noted in the 2.5% HTP sample, which had a median of 221. However, the cell migration number in the 5.0% CC sample had a lower median of 213 than that noted in the 5.0% HTP sample, which had a median of 257.

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DISCUSSION AND CONCLUSION

Fibroblasts, epithelial cells, and osteoblasts migrate to the surface of the dental implant material to achieve soft tissue attachment and osseointegration in peri-implant wound healing [3,4]. These are natural processes of wound healing and fibroblasts are important contributors to this process [5,6], and thus poor healing can lead to implant failure.

HTPs are marketed as being less harmful because they do not involve the combustion present in CCs [15]. However, HTPs were observed to be as toxic as cigarettes at the cellular level [19,20].

In the present study, at both 2.5% and 5% concentrations, CCs and HTPs prevented cell migration on titanium plates. However, HTPs prevented migration more than CCs at a 2.5% concentration. This may suggest that HTPs can be toxic, even at a lower concentration of 2.5%. Results from gas chromatography-mass spectrometry analysis indicate that nicotine is present in HTPs at the same concentrations. Cotinine, the major metabolite of nicotine, has been found in the serum and saliva of smokers [24]. Cotinine decreases the ability of fibroblasts to migrate and adhere to the radicular surfaces of the teeth [25].

In their marketing materials, PMI cites the Food and Drug Administration (FDA) list of 93 harmful and potentially harmful constituents (HPHCs) to report that the levels of 40 HPHCs were lower in HTPs compared to CCs. However, the levels of 56 other constituents

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were higher in HTPs, including 22 constituents that were 200% higher than CCs and 7 were 1000% higher. The substances include α, β-unsaturated carbonyl compounds (e.g., 2-cyclopentene-1,4-dione), 1,2-dicarbonyl compounds (e.g., cyclohexane, 1,2-dioxo-), furans (e.g., 2 (5H)-furanone), and epoxides (e.g., anhydrolinalool oxide), all of which have high levels of cytotoxicity. [15,26] Additionally, HTP devices produce genotoxic compounds (e.g., formaldehyde; acetaldehyde; acrolein), propylene glycol, and glycerin via the dehydration and oxidation of humectants [27,28]. Furthermore, compared to HTPs, IQOS contained higher levels of nicotine and harmful substances [21], and it was considered that their effects are similar to those of CCs.

Our study suggests that HTPs are risk factors for wound healing of dental implants and may lead to implant failure.

Limitations

In the present study, the in vitro cells received CSE through direct exposure. However, in vivo, cells are subject to exposure through cigarette smoke. Therefore, the results of this study cannot be generalized to real-world settings. To address this, cigarette smoke effects can be clearly observed in vitro by considering the "air-liquid interface culture" method, which uses Vitrocell Exposure Systems (Vitrocell systems, Germany) to incubate cells with external air of cigarette smoke.

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At all-time points (12, 24, and 48 h), CCs simply showed a trend toward increased cytotoxicity in a concentration-dependent manner due to the accumulation of toxic substances generated by combustion. Notably, HTPs tended to be more toxic than CCs when compared at a concentration of 2.5%. The toxic substances in HTPs are generated by device heating and incomplete combustion, the mechanism of which is complex and is assumed to have led to these results. However, to elucidate this difference, future research is needed to identify the causative agents.

Future directions

This study revealed that, similar to CCs, HTPs affect wound healing of implants by preventing fibroblast migration. We are currently conducting experiments in two directions to elucidate the mechanism.

Matrix metalloproteinases (MMPs) act to degrade components of the extracellular matrix, including fibroblasts, and, thus, have a major impact on wound healing. The balance of expression of MMPs and tissue inhibitors of MMPs (TIMPs) maintain extracellular matrix homeostasis and remodeling [29,30]. Therefore, analyzing their gene expression in CSE-exposed cells is valuable.

In the present study, HTPs and CCs had similar effects in the wound healing assay. The substances assumed to be responsible for the inhibition of wound healing include nicotine,

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formaldehyde, o-toluidine, 2-naphthylamine 1,3-butadiene, benzene, benzo[a]pyrene, N-nitrosonornicotine, and 4-[methylnitrosamino]-1-[3-pyridyl]-1-butanone which are contained in aerosols. Therefore, we are working to elucidate the causative agents.

Conclusions

In a wound healing model using a scratch assay on a pure titanium plate, HTPs and CCs prevented fibroblast migration. This study suggests that HTP may be a risk factor equivalent to CCs for dental implant treatment by inhibiting fibroblast migration to wounds.

Further studies are required to identify the responsible substances and obtain molecular biological evidence that elucidate how they inhibit wound healing.

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PROVENANCE AND PEER REVIEW
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Figure 1A: Pure titanium plate

Figure 1B: Control image immediately after line tape removal (40× magnification). A 2-mm-width line tape is applied to the center of the titanium plate.

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Figure 1A, 1B

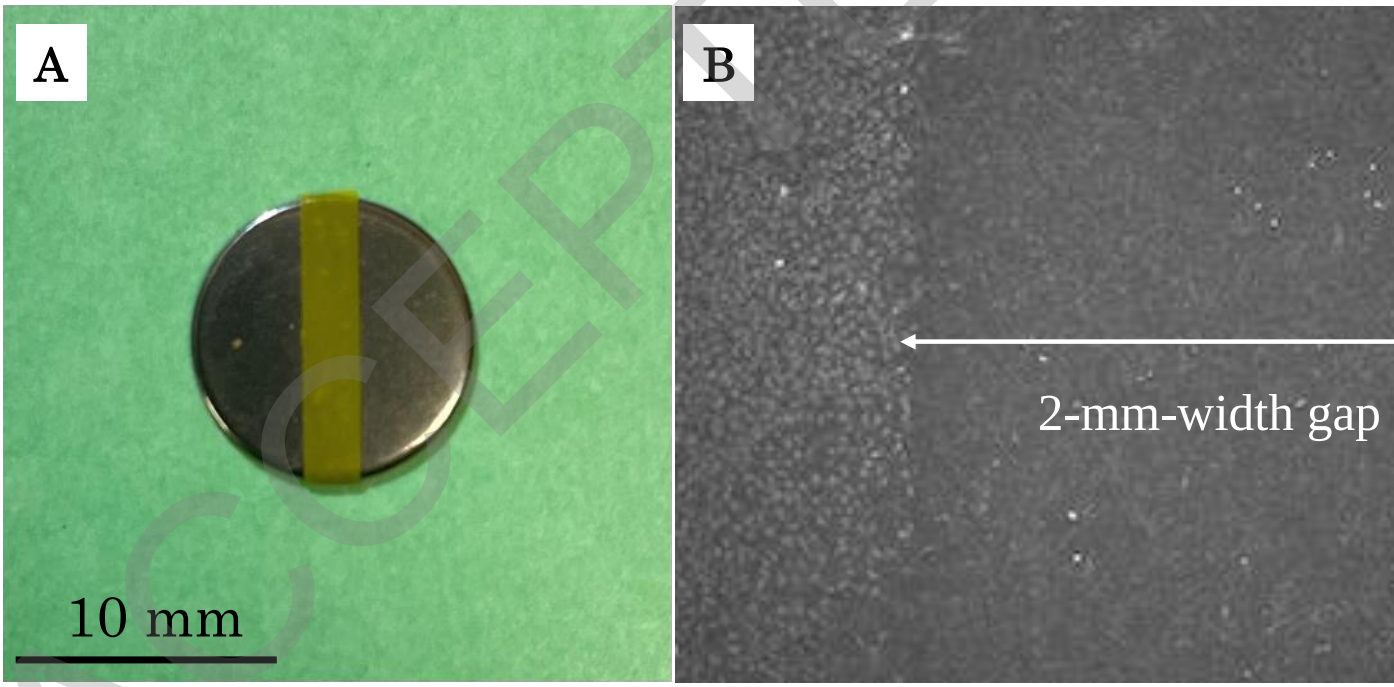

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Figure 2A: Scratch wound healing assay

Images from scratch assay experiments at different time points (40× magnification). Migrated cells are shown in red.

Figure 2A

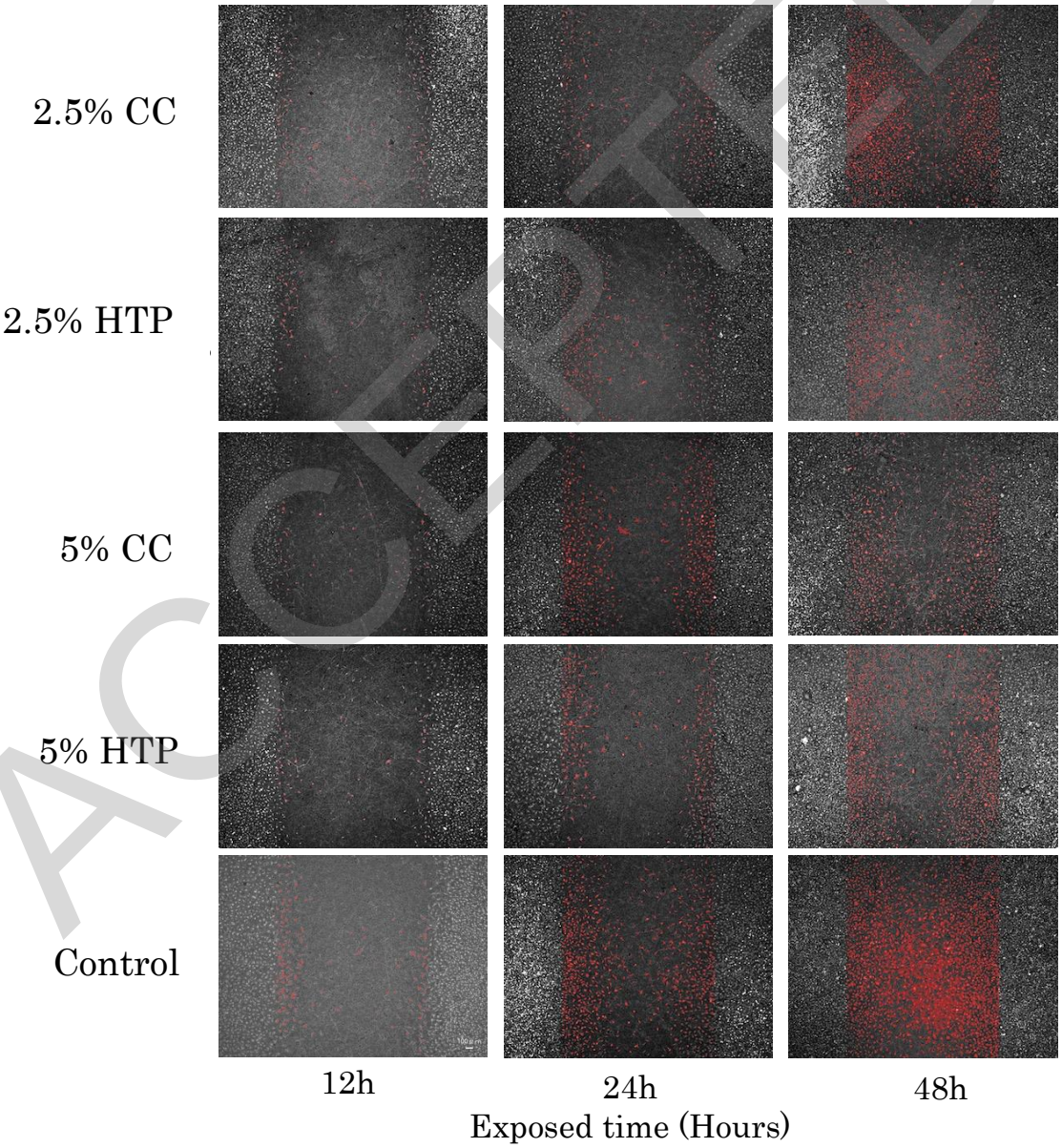

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Figure 2B: Number of L929 cell migration into a cell-free area

The number of L929 cells decreased on exposure to cigarette smoke extract from HTPs and CCs (n = 3)

CCs, conventional cigarettes, HTPs, heated tobacco products

Figure 2B

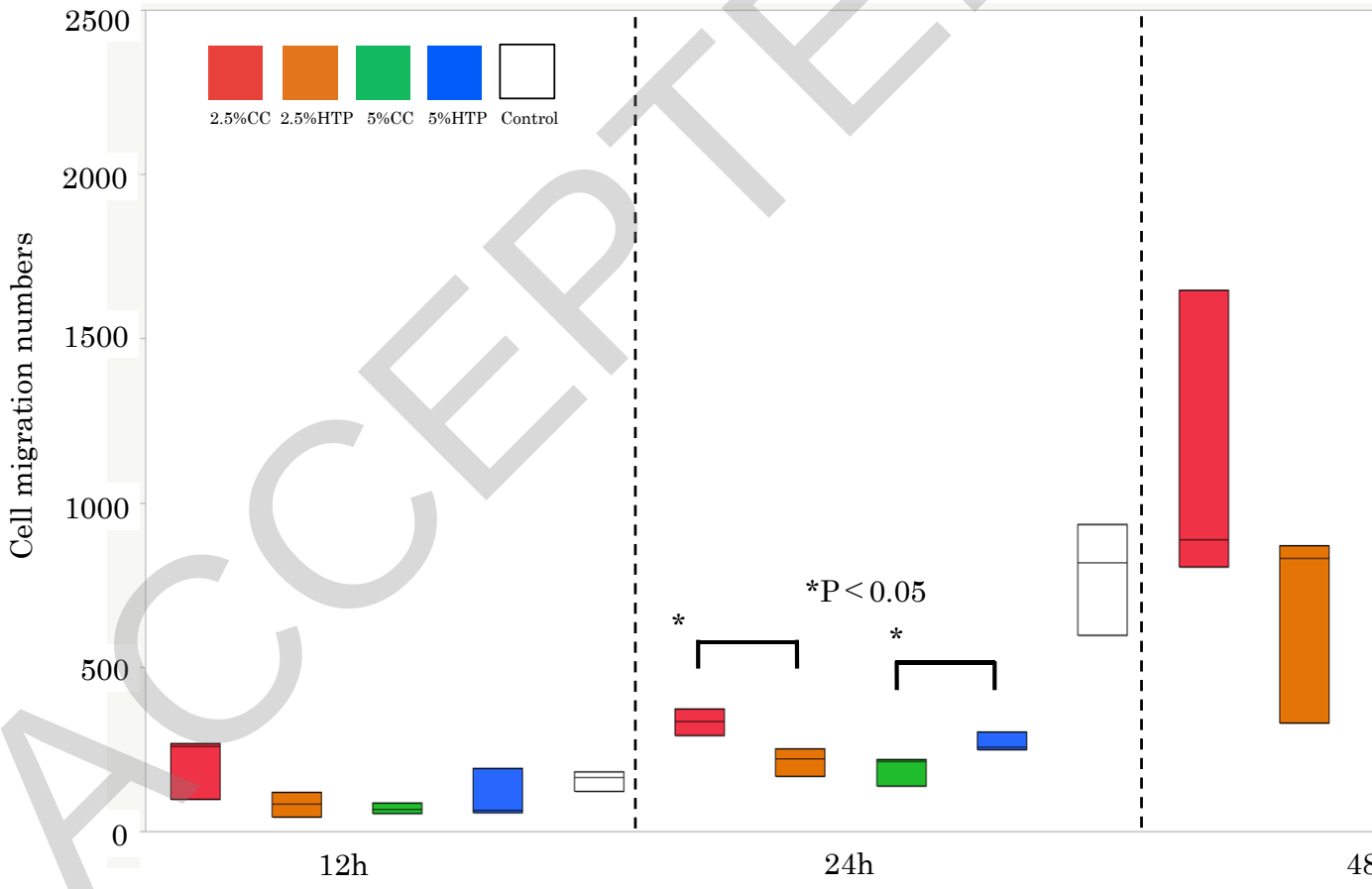

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Table 1: Median (interquartile range) and p values

Median (IQR)						
	12 h	P-value *	24 h	P-value**	48 h	P-value***
2.5% CC	260 (98–268)		334 (292–372)		888 (805–1647)	
2.5% HTP	83 (42–118)	0.127	221(168–252)	0.049	832 (330–870)	0.275
5% CC	67 (54–86)		213 (138–218)		831 (752–846)	
5% HTP	66(57–192)	0.827	257 (248–302)	0.049	692 (549–864)	0.513
Control	165(122–180)		817 (598–933)		2377 (1421–2415)	

* P-value for 2.5% CC vs. 2.5% HTP and 5% CC vs. 5% HTP at 12 h.

** P-value for 2.5% CC vs. 2.5% HTP and 5% CC vs. 5% HTP at 24 h.

*** P-value for 2.5% CC vs. 2.5% HTP and 5% CC vs. 5% HTP at 48h.

IQR, interquartile range, CC, conventional cigarette, HTP, heated tobacco product

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