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Evaluation of the Effects of E-Cigarette Aerosol Extracts and Tobacco Cigarette Smoke Extracts on Human Gingival Epithelial Cells

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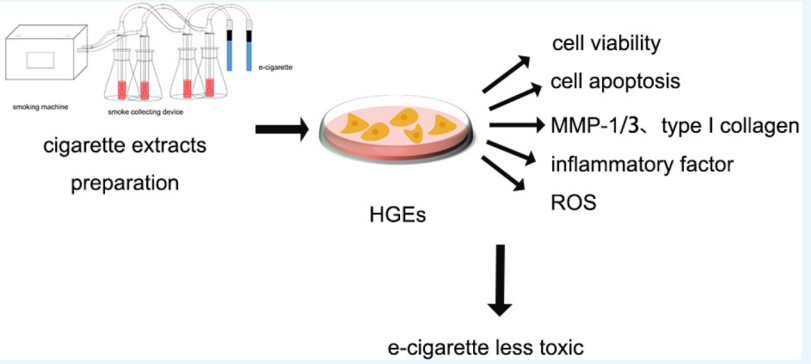

ABSTRACT: Smoking increases the risk of a number of diseases, including cardiovascular, oral, and lung diseases. E-cigarettes are gaining popularity among young people as an alternative to cigarettes, but there is debate over whether they are less harmful to the mouth than e-cigarettes. In this study, human gingival epithelial cells (HGECs) were treated with four commercially available e-cigarette aerosol condensates (ECAC) or commercially available generic cigarette smoke condensates (CSC) with different nicotine concentrations. Cell viability was determined by MTT assay. Cell apoptosis was observed by acridine orange (AO) and Hoechst33258 staining. The levels of type I collagen, matrix metalloproteinase (MMP-1, MMP-3), cyclooxygenase 2, and inflammatory factors were detected by ELISA and RT-PCR. Finally, ROS levels were analyzed by ROS staining. The different effects of CSC and ECAC on HGECs were compared. The results showed that higher nicotine concentration of CS significantly reduced the activity of HGECs. By contrast, all ECAC had no significant effect. The levels of matrix metalloproteinase, COX-2, and inflammatory factors were higher in HGECs treated with CSC than those treated with ECAC. In contrast, the level of type I collagen was higher in HGECs treated with ECAC than those treated with CSC. In conclusion, all four flavors of e-cigarettes were less toxic to HGE cells than tobacco, but further clinical studies are needed to determine whether e-cigarettes are less harmful to oral health than conventional cigarettes.

1. INTRODUCTION

Tobacco products are popular, with about 50.6 million adults in the United States using tobacco products, including e-cigarettes and cigarettes, in 2019.[1] Smoking is a worldwide problem. It is estimated by the World Health Organization that smoking causes about 6 million deaths worldwide every year.[2] In the United States alone, about 16 million people suffer from smoking-related diseases.[3] Cigarette smoking has a great impact on the occurrence and development of some systemic diseases.[4] Cigarette smoking could raise blood pressure and increase the risk of cardiovascular diseases.[5] In addition, smoking is also associated with the occurrence of chronic obstructive pulmonary disease.[6] Some additives such as menthol are used in some cigarettes to adjust the taste and reduce the pungent smell of smoke. Meanwhile, the use of menthol will increase smoking behavior and addiction to nicotine, exacerbating the deterioration of diseases.[7,8] As people pursuit a healthier life and social development, electronic cigarettes (e-cigarette) and "I quit original smoking" (IQOS) products are gradually attracting people's attention as substitutes for cigarette.[9,10] Cigarette smoke contains a variety

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of compounds, including nicotine, tar, carbon monoxide, polycyclic aromatic compounds, etc. These substances can cause oxidative damage to cells, produce toxicity, and affect the normal function of cells.[11] E-cigarette aerosols mainly include propylene glycol, glycerin, nicotine, flavoring agents, free radicals, and carbonyls. The main components of heated tobacco aerosol are nicotine, glycerin, tar, carbon monoxide, nitrosamines and so on.[11] E-cigarettes first appeared in the market for the first time in 2003 and attracted great attention in a short period of time. E-cigarettes are claimed to be safer than tobacco and to assisting smoking cessation.[10] Unlike tobacco, e-cigarettes, also known as electronic nicotine delivery systems, are built with a cartridge, nebulizer, and a chip-controlled lithium battery. E-cigarette liquid consists of glycerin, propylene glycol, nicotine, and flavoring.[10] An IQOS cigarette is a hybrid product of traditional cigarettes and e-cigarettes.[12] It reduces the harm of burning tobacco in traditional cigarettes by heating tobacco instead of burning it.[9,13] E-cigarettes have received a lot of attention as an alternative to tobacco, but there is still debate about whether they are less harmful to human health than cigarettes. Studies have shown that e-cigarettes could aid smoking cessation, and the benefits of smoking cessation may outweigh the short-term negative effects of using e-cigarettes.[14,15] However, some studies have shown that e-cigarettes may produce compounds not found in the smoke liquid during the atomization process, potentially damaging human health.[16] Therefore, more experiments are needed to evaluate the safety of e-cigarettes.

The gingiva is an important structure in the oral cavity, which protects the periodontal tissue and plays an important role in oral function.[17] Gingival epithelium is a barrier to resist the invasion of external bacteria and protect the health of periodontal tissue. T Multiple cell types exist in the gingiva, including gingival fibroblasts, gingival epithelial cells (GECs), and macrophages. GECs are an important part of gingival epithelial tissue. When bacteria invade periodontal tissue, GECs not only act as a physical barrier but also exert antibacterial effects, producing antimicrobial peptides in response to invading bacteria.[17,18] Reactive oxygen species (ROS) are a normal product of cells. When inflammation occurs, the production of ROS increases sharply. High levels of ROS cannot be balanced by the antioxidant system, thus causing cell or tissue damage.[19] Dysfunction of GECs in the oral cavity leads to the accumulation of pathogenic bacteria in periodontal tissue, which may lead to the development of periodontitis.[20]

Current studies have shown that repeated exposure to cigarette smoke condensates (CSC) and e-cigarette aerosol condensates (ECAC) is cytotoxic to oral cells and leads to increased inflammation and cellular damage.[21−23] Several studies reported that tobacco significantly inhibits the proliferation and migration of oral cells more than e-cigarette.[24] However, studies also have demonstrated that CSC could increase the proliferation of GECs.[25] E-cigarette vapor could affect the activity of epithelial cells by promoting apoptosis and damaging gingival epithelial tissue.[23] Studies on the cytotoxicity of e-cigarettes and tobacco on GECs are few and controversial. Thus, it is necessary to investigate the toxicity of e-cigarettes and tobacco on GECs.

In this study, the null hypotheses of the study are e-cigarettes have less effect on GECs than traditional cigarettes. We measured the effects of exposure to CSC and ECAC on the survival rate and apoptosis of GECs and levels of ROS, inflammatory factors, collagen, and matrix metalloproteinase (MMP) and demonstrated that CSC is more toxic to HGECs than ECAC.

2. MATERIALS AND METHODS

2.1. Cell Culture. Human GECs (HGECs) were from Otwo Biotech Inc., Shenzhen, and mung flavors and cigarettes and cultured in culture dishes with Dulbecco's modified Eagle's medium (DMEM) (Genview) supplemented with 10% calf serum (Sigma-Aldrich) at 37 °C in an incubator with 5% CO_2. The cells were passaged after digestion with 0.25% trypsin (EDTA) (Genview), observed under a Nikon inverted microscope (Nikon CKX41, Japan),[23] and used in experiments at 4−6 generations.

2.2. Preparation of Electronic Cigarette Aerosol Condensate and Cigarette Smoke Condensate. ECAC and CSC were prepared as previously described from selected commercially available RELX e-cigarettes and tobacco.[26] In brief, e-cigarettes with grape, watermelon, cola, and mung flavors and cigarettes were smoked through a smoking machine at frequencies of 10 times and 160 times per hour, respectively, to simulate the average smoking times of people. The ECAC and CSC produced by smoking were dissolved in 20 mL of cell culture medium without any additives and analyzed using high-performance liquid chromatography. Nicotine concentration was calculated by comparing to the standards as previously reported.[26] CSC and different types of ECAC with nicotine concentrations of 0.03125, 0.125, 0.5, 2, 8, and 32 μg/mL were used to treat HGECs, respectively. Moreover, air, after smoked through a smoking machine, was used as the control.

2.3. Cell Viability Assay. Cell viability was determined using the MTT method.[26] In brief, digested HGECs were seeded in 96-well plates and treated with CSC and ECAC at different concentrations for 24 and 48 h, respectively. Followed by incubation with 5 mg/mL MTT (M5655 Sigma-Aldrich, China) for 4 h, 100 μL of dimethyl sulfoxide (DMSO) (Solarbio Science & Technology Co. Ltd., Beijing, China) was added. The cells were sealed with tin foil and incubated under shaking for 10 min. The absorbance at 570 nm was measured using a SpectraMAX ABS Microplate Reader (Molecular Devices, LLC, CA). Cell viability was calculated as cell viability (%) = ($OD_{570\ nm}$ of the sample group − $OD_{570\ nm}$ of the blank group)/($OD_{570\ nm}$ of the control group − $OD_{570\ nm}$ of the blank group) × 100%.

2.4. Cell Apoptosis Assay. Cells were seeded in a confocal culture dish. After treatment with CSC and ECAC at a nicotine concentration of 0.125 μg/mL for 48 h, cells were stained with 0.01% acridine orange (AO, Fluka) for 5 min at room temperature, washed with PBS, and observed under a fluorescence microscope (Nikon Ci-L, Japan).

Cells in the logarithmic growth phase were seeded in a confocal culture dish. After being cultured for 12 h, cells were treated with CSC and ECAC with a nicotine concentration of 0.125 μg/mL for 48 h, stained with a 10 μg/mL Hoechst 33258 probe (Solarbio Science & Technology Co. Ltd., Beijing, China) at 37 °C dark for 15 min, washed three times with PBS, and observed under a fluorescence microscope (Nikon Ci-L, Japan).

2.5. Enzyme-Linked Immunosorbent Assay (ELISA). Cells were seeded in 24-well plates and treated with CSC and ECAC for 24 and 48 h, respectively. Cell supernatant was collected and used to measure interleukin (IL)-6, IL-8, IL-1β, tumor necrosis factor-α (TNF-α), type I collagen, MMP-1, and

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Table 1. Primers Used in RT-PCR

genes	sense	antisense
COL1A1	5′-GTGCGATGACGTGATCTGTGA-3′	5′-CGGTGGTTTCTTGGTCGGT-3′
TNF-α	5′-AGCTGGTGGTGCCATCAGAGG-3′	5′-TGGTAGGAGACGGCGATGCG-3′
IL-8	5′-AACTGAGAGTGATTGAGAGTGG-3′	5′-ATGAATTCTCAGCCCTCTTCAA-3′
IL-6	5′-CACTGGTCTTTTGGAGTTTGAG-3′	5′-GGACTTTTGTACTCATCTGCAC-3′
IL-1β	5′-GCAGAAGTACCTGAGCTCGCC-3′	5′-CCTTGCTGTAGTGGTGGTCGG-3′
MMP-1	5′-CACAGCTTTCCTCCACTGCTGCTGC-3′	5′-GGCATGGTCCACATCTGCTCTTGGC-3′
MMP-3	5′-GAAAGTCTGGGAAGAGGTGACTCCAC-3′	5′-CAGTGTTGGCTGAGTGAAAGAGACCC-3′
COX-2	5′-CACAGGCTTCCATTGACCAGA-3′	5′-GTGCTCCAACTTCTACCATGG-3′
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′-GAAGATGGTGATGGGATTTC-3′

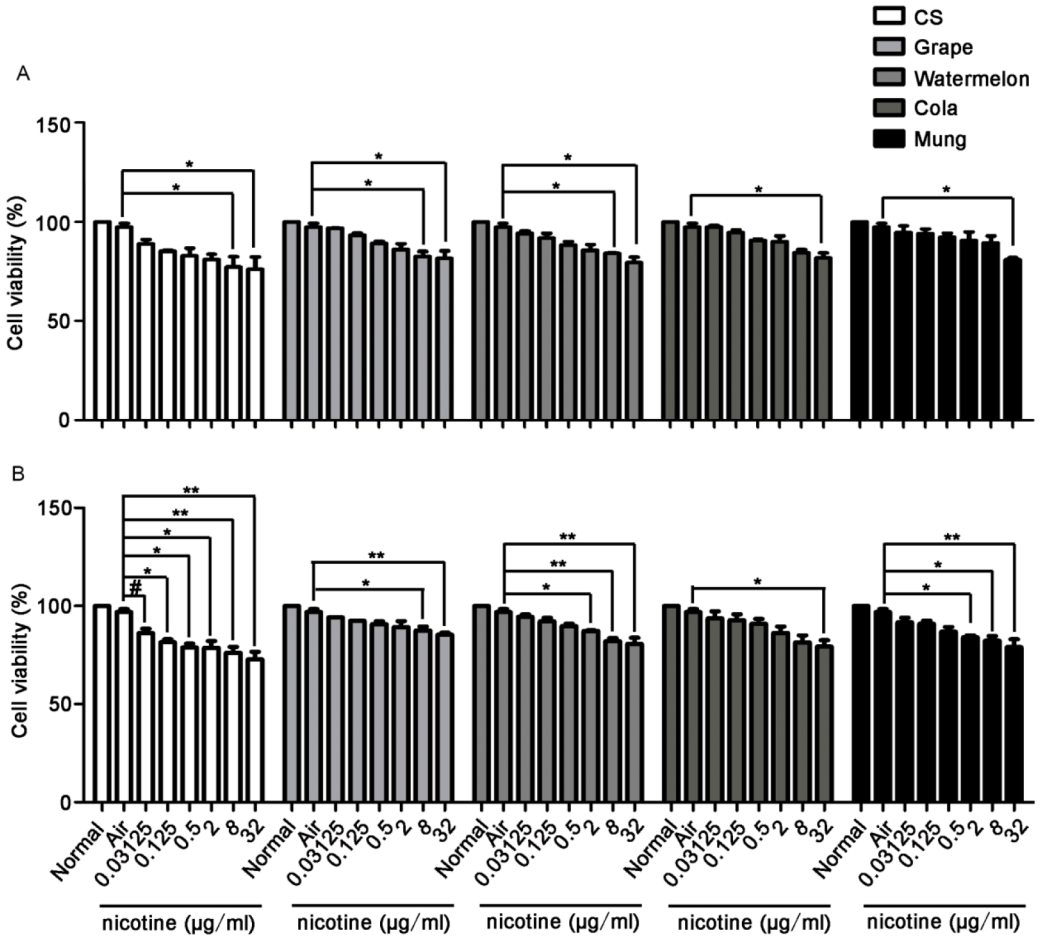

Figure 1. Viability of HGECs treated with different concentrations of cigarette smoke condensates or e-cigarette aerosol condensates. HGECs were treated with different kinds of ECAC or CSC with different nicotinic concentrations (0.03125, 0.125, 0.5, 2, 8 μg/mL or 32 μg/mL) for 24 h (A) or 48 h (B). The cell viability was detected by MTT assay. Data are expressed as means ± S.E., #$p > 0.05$, *$p < 0.05$ and **$p < 0.01$, $n = 3$. CSC: cigarette smoke condensates. ECAC: e-cigarette aerosol condensates.

MMP-3 using ELISA kits according to the manufacturer's instructions (1110602, 1110802, 579409, and 1117203, Dakewei Biotechnology Co., Ltd., Beijing, China; SEKH-0401, Solarbio Science & Technology Co. Ltd., Beijing, China; KE00223, KE00160, Proteintech).

2.6. Real-Time Fluorescence Quantitative Polymerase Chain Reaction (RT-PCR). RNAs were extracted from cells at 4 °C using Trizol (Thermo Fisher Scientific Co., Ltd., USA) and chloroform and converted to cDNA using a reverse transcription kit (ABclonal Co, Ltd., Wuhan, China). Real-time quantitative PCR was performed in triplicate using RT-PCR kits (ABclonal Co., Ltd., Wuhan, China) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The level of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The sequence of primers used for RT-PCR is shown in Table 1.

2.7. ROS Level Detection. Cells in the logarithmic growth phase were seeded in a confocal culture dish. After being cultured for 12 h, cells were treated with CSC and ECAC with a nicotine concentration of 0.125 μg/mL for 48 h. After that, cells were incubated with 1 μg/mL DCFH-DA (2.7-dichlorofluorescein dihydrodiacetate) fluorescent probe (EMD Millipore Corp., USA) in DMEM medium (Genview, USA) for 30 min in the dark, observed under a fluorescence microscope (Nikon Ci-L, Japan), and photographed. The images were analyzed using the Image-J software, and the

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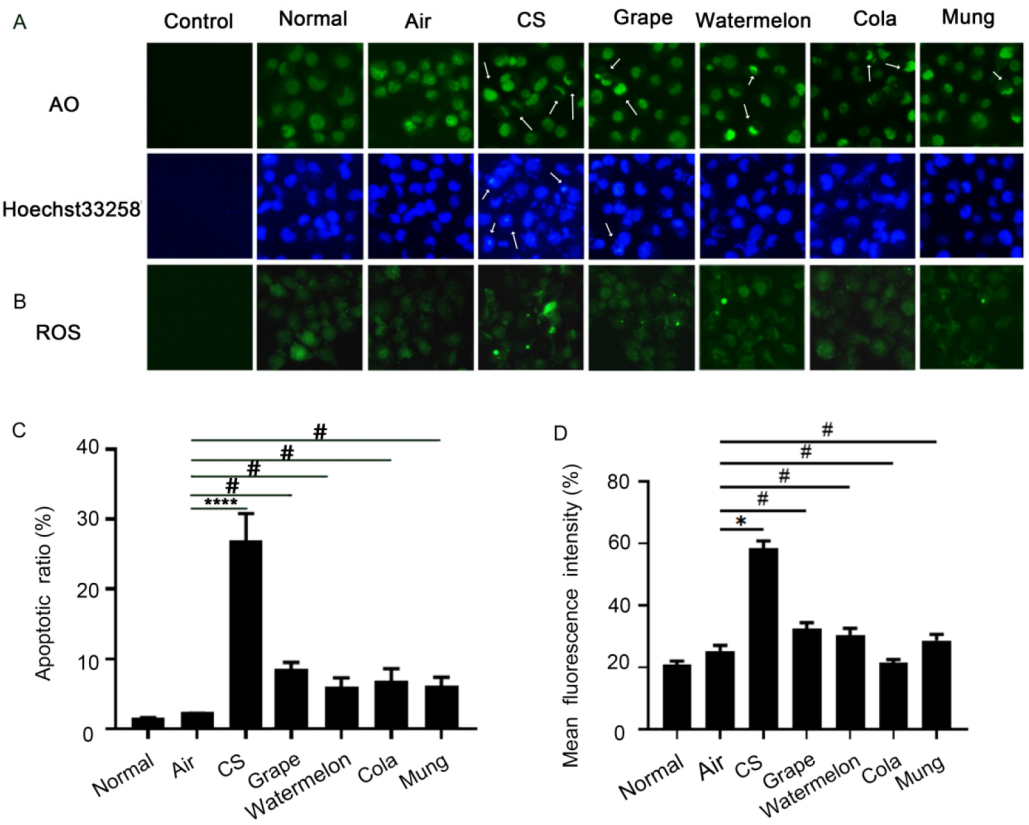

Figure 2. Apoptosis and ROS levels of HGECs treated with cigarette smoke condensates or e-cigarette aerosol condensates. (A) Cells were treated with ECAC and CSC with nicotine concentration of 0.125 μg/mL were cultured for 24 h and then stained with AO and Hoechst33258. Arrows indicate apoptotic cells. The control group is the background image without cells. (B) Effects of ECAC and CSC on ROS levels in cells treated with 0.125 μg/mL nicotine for 24 h. The fluorescence intensity of ROS in the cells was detected under a fluorescence microscope. The brighter spots are ROS. The control group is the background image without cells. (C) Apoptotic ratio after 24 h of treatment was quantified after Hoechst 33258 staining. The cells showed condensed chromatin and nuclear fragmentation were much brighter in the Hoechst 33258 staining and recognized as apoptotic cells. (D) Average fluorescence intensity was calculated by Image-J software and expressed as means ± SEM # $p > 0.05$, and *$p < 0.05$. $n = 3$.

amount of ROS was quantified as the relative fluorescence intensity of DCFH per cell and presented as the average fluorescence intensity of X cells.

2.8. Statistical Analysis. All experiments were repeated at least three times. Statistical analysis was performed using SPSS v22 (Chicago, IL). One-way ANOVA was used to compare all data and the results were expressed as mean ± standard deviation. $p < 0.05$ was considered a significant difference. GraphPad Prism (GraphPad Software, La Jolla, CA) and Adobe Photoshop (Adobe, San Jose, CA) were used to process images.

3. RESULTS

3.1. Cell Viability Assessment. In our study, ECAC was extracted from RELX e-cigarettes with grape, watermelon, cola, and mung flavors. HGECs were treated with different ECAC and CSC of different nicotine concentrations for 24 and 48 h, and cell viability after treatment were determined using the MTT assay. As shown in Figure 1A, 24 h of treatment with CSC and ECAC dose-dependently decreased HGEC viability, showing significant differences between the air control group and CSC treatment groups with nicotine concentration at 8 and 32 μg/mL, ECAC with grape and watermelon flavors with nicotine concentration at 8 and 32 μg/mL, and ECAC with cola and mung flavors with nicotine concentration at 32 μg/mL ($p < 0.05$ for all), respectively. As shown in Figure 1B, 48 h of treatment with CSC and ECAC also dose-dependently decreased the viability of HGECs, showing significant differences between the air control group and CSC treatment groups with nicotine concentration at 0.125, 0.5, 2, and 8 μg/mL ($p < 0.05$) and 32 μg/mL ($p < 0.01$), ECAC with grape flavor with nicotine concentration at 8 μg/mL ($p < 0.05$) and 32 μg/mL ($p < 0.01$), ECAC with watermelon flavor and mung flavor with nicotine concentration at 2 μg/mL ($p < 0.05$) and 8 and 32 μg/mL ($p < 0.01$), and ECAC with cola flavor with nicotine concentration at 32 μg/mL ($p < 0.05$), respectively. Overall, these results showed that the threshold of cell survival rate was different after different treatments. The threshold of CSC treatment was the lowest. Thus, CSC and ECAC with a nicotine concentration of 0.125 μg/mL were used in subsequent experiments.

3.2. Cell Apoptosis Determination. We then examined whether the decreased cell viability was due to cell apoptosis. To observe the changes in cell nucleus and morphology, cells were stained with acridine orange (AO) and Hoechst before and after being treated with CSC and ECAC with a nicotine concentration of 0.125 μg/mL. The results showed that cells presented nuclear condensation and fragmentation, which are characteristics of apoptosis (Figure 2A). In addition, condensed chromatin was found in cell nuclei with much brighter Hoechst 33258 staining, which is considered an indicator of cell apoptosis (Figure 2A). The proportion of

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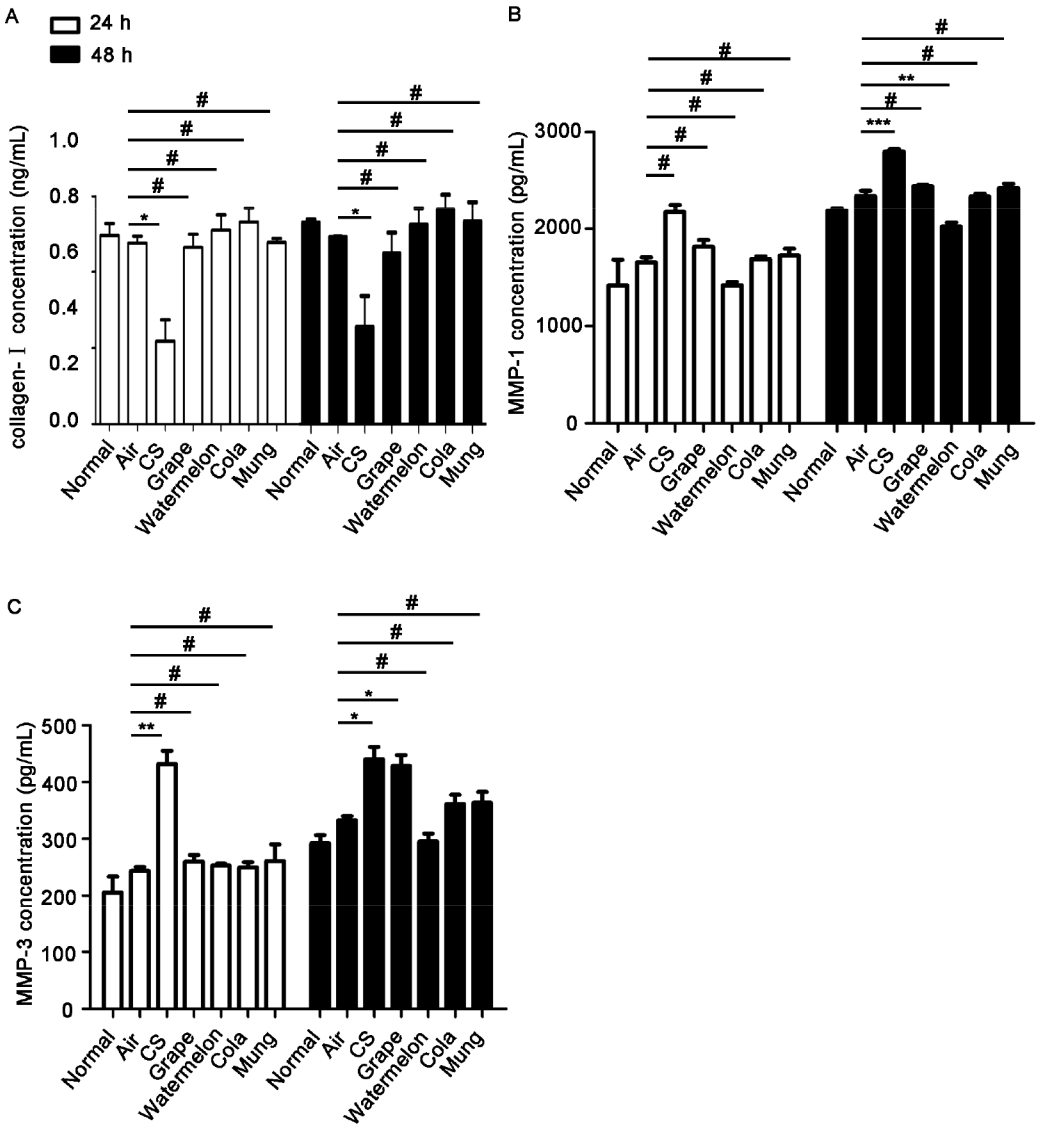

Figure 3. Effects of CSC and ECAC on matrix metalloproteinase and collagen levels in HGECs. Levels of type I collagen (A), MMP-1 (B), and MMP-3 (C) were determined by ELISA after the cells were treated with ECAC and CSC at a nicotine concentration of 0.125 μg/mL for 24 and 48 h, respectively. Data were expressed as means ± SEM; #$p > 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ ($n = 3$).

apoptotic cells was 26.97% after CSC treatment, 12% after treatment with ECAC with grape flavor, 11.4% after treatment with ECAC with watermelon flavor, 14.3% treatment with ECAC with cola flavor, and 12.4% treatment with ECAC with mung flavor (Figure 2C).

3.3. Comparison of ECA and CS on Reactive Oxygen Species Levels in HGECs. To examine the effect of ECAC and CSC on ROS levels, HGECs were stained with DCFH-DA. As shown in Figure 2B, the fluorescence intensity of cells treated with CSC was much higher than that of the air control group. The average fluorescence intensity calculated by Image-J software was 20.8%, 25.1%, 58.5%, 32.4%, 30.4%, 21.5%, and 28.6% for HGECs in the normal group, air control group, CSC group, ECAC with grape, watermelon, cola, and mung flavor groups, respectively (Figure 2D), indicating that only CSC significantly increased ROS levels.

3.4. Detection of Matrix Metalloproteinase and Collagen Levels. The levels of MMP-1, MMP-3, and type I collagen in cell supernatants were determined by ELISA. The level of type I collagen was significantly lower in the CSC group than in the air control group ($p < 0.05$) but showed no significant differences between the air control group and all ECAC treatment groups (Figure 3A). The level of MMP-1 did not change significantly after 24 h of CSC treatment but significantly increased after 48 h of CSC treatment compared with that in the air control group ($p < 0.001$). In contrast, the level of MMP-1 decreased significantly after treatment with ECAC with watermelon flavor ($p < 0.01$) (Figure 3B). Moreover, 24 and 48 h of CSC treatment significantly increased MMP-3 levels. In contrast, only 48 h of treatment with ECSC with grape flavor, but not other flavors, significantly increased MMP-3 level compared with the air control ($p < 0.05$) (Figure 3C).

The mRNA levels of collagen type I, MMP-1, MMP-3, and COX-2 were determined by RT-PCR. Although the results of RT-PCR and ELISA were generally similar, there were differences between RT-PCR and ELISA in MMP-1 results in HGECs treated with ECSC with watermelon flavor, MMP-3 results in HGECs treated with ECSC with grape, cola, and mung flavors, and in type I collagen in HGECs treated with

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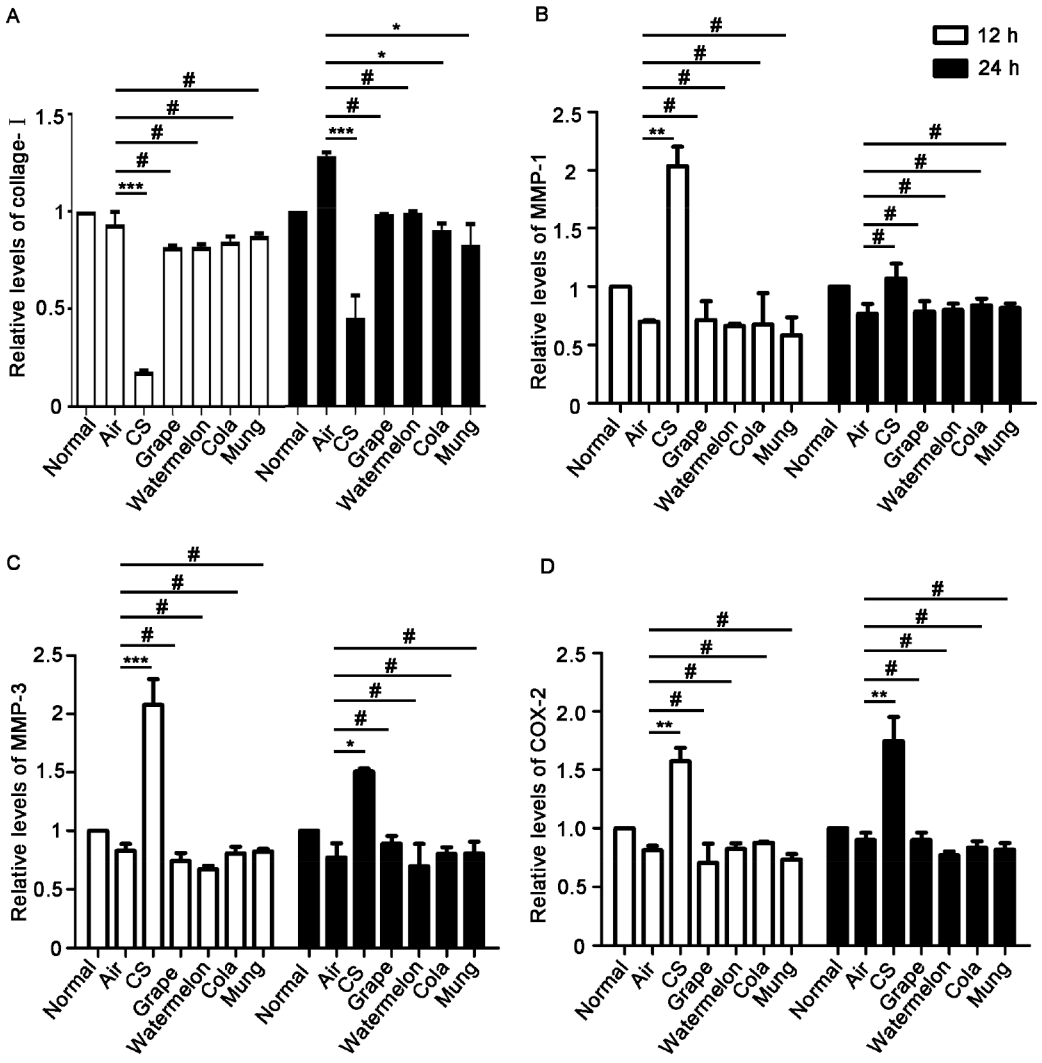

Figure 4. Effects of CS and ECA on levels of matrix metalloproteinase and collagen genes in HGECs cells. The mRNA levels of type I collagen (A), MMP-1 (B), MMP-3 (C), and COX-2 (D) were detected by RT-PCR after HGECs were treated with different kinds of ECAC and CSC at nicotine concentration of 0.125 μg/mL for 12 and 24 h, respectively. Data are expressed as means ± SEM; #$p > 0.05$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ ($n = 3$).

ECSC with cola and mung flavors. It was speculated that the inflammatory cytokine activity of cells was affected by internal or external stimuli, thus affecting the release of MMP.[27] The level of collagen type I was significantly lower in cells treated with CSC with a nicotine concentration of 0.125 μg/mL than in the air control group ($p < 0.001$). Moreover, the level of type I collagen was significantly decreased by treatment with ECAC with cola and mung flavors for 24 h ($p < 0.05$) (Figure 4A). Furthermore, MMP-1, MMP-3, and COX-2 levels were decreased by CSC treatment but not by ECAC with all flavors (Figure 4B,D).

3.5. Determination of Inflammatory Factors. The levels of IL-6, IL-8, IL-1β, and TNF-α were determined by ELISA. Treatment with ECAC with all flavors did not change the levels of all these inflammatory factors compared with the air control. Moreover, TNF-α level was increased after treatment with CSC for 24 and 48 h ($p < 0.05$), IL-6 level was increased by treatment with CSC for 48 h ($p < 0.01$), and IL-1β and IL-8 levels were increased by treatment with CSC for 24 h (Figure 5).

The mRNA levels of IL-6, IL-8, IL-1β, and TNF-α were examined by using RT-PCR assay. The results were similar to the ELISA results. IL-1β, IL-8, and TNF-α mRNA levels were consistent with their protein levels, while IL-6 mRNA level was different from its protein level. Studies have shown that IL-6 production is regulated by IL-1β, and it is precisely because the regulation of IL-1β that IL-6 mRNA level is different from its protein level.[28] Treatment with ECAC with all flavors did not change mRNA levels of all these inflammatory factors compared with the air control. In contrast, CSC treatment for 24 and 48 h increased IL-6 and TNF-α mRNA levels, and CSC treatment for 24 h increased IL-1β and IL-8 mRNA levels ($p < 0.001$) (Figure 6).

4. DISCUSSION

E-cigarettes are currently gaining popularity among young people.[15] The dangers of smoking tobacco are gradually recognized. Traditional smoking increases the risk of systemic diseases such as cardiovascular diseases.[29]

For teenagers, the use of nicotine-containing devices during adolescence can impair brain development by affecting the way synapses are formed between brain neurons. The skeletal effect is most pronounced during adolescence, when smoking

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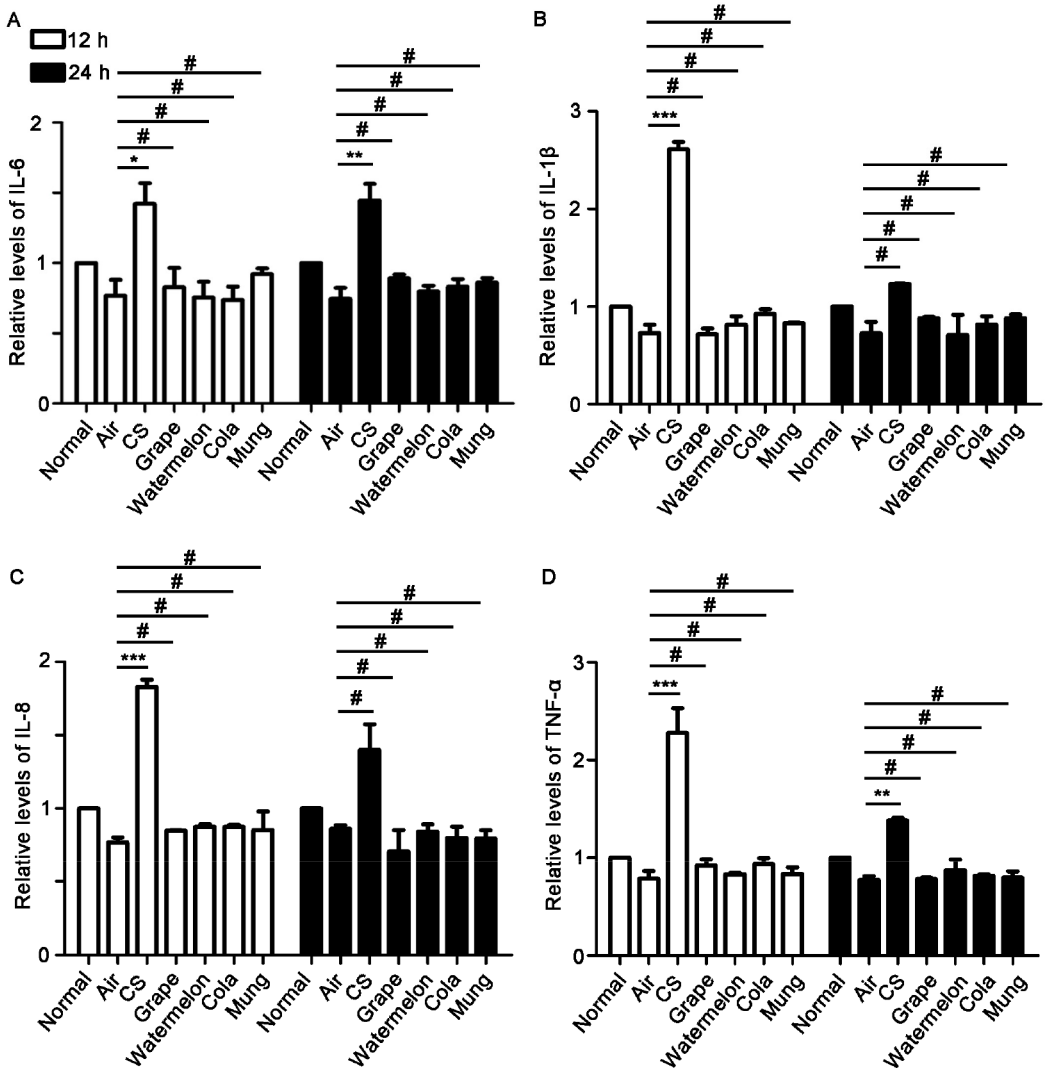

Figure 5. Effects of CSC and ECAC on the contents of inflammatory factors in HGECs. The levels of IL-6 (A), IL-1β (B), IL-8 (C), and TNF-α (D) were determined by ELISA after cells were treated with ECAC and CSC at nicotine concentration of 0.125 μg/mL for 24 and 48 h. Data are expressed as means ± SEM; #$p > 0.05$, *$p < 0.05$, and **$p < 0.01$ ($n = 3$).

increases the risk of cavities in permanent teeth.[30,31] Smoking affects oral health and increases the risk of periodontal diseases. A study of smoking and periodontal disease in South Korea found that traditional smokers have a higher incidence of periodontal disease than nonsmokers.[14] *In vitro* studies of oral cells have shown that cigarette smoke is toxic to oral cells, causing oxidative damage, promoting apoptosis, affecting cell migration, and inhibiting repair.[22,32,33] A study of mouse embryonic cell models shows that cigarette smoke is harmful to embryonic cells.[34] Many people use e-cigarettes as a means of quitting smoking. However, there is still a lot of debate about the safety of e-cigarettes. Nicotine is a common ingredient in tobacco and e-cigarettes, and it is present in tobacco and most e-cigarettes. Studies have shown that nicotine may inhibit the production of inflammatory factors in oral epithelial cells,[35] while other studies have shown that nicotine can also be produce cytotoxicity to human periodontal cells and induce human periodontal cells to produce IL-1β and IL-8.[36] E-cigarettes are cytotoxic to a variety of cells, including gingival fibroblasts, gingival mesenchymal stem cells, and gingival epithelial cells, and affect cell proliferation, but e-cigarettes have less effect on cell proliferation and viability than tobacco.[37] Therefore, more in-depth research is needed to explore the safety of e-cigarettes and how they compare to tobacco. Our results showed that compared with e-cigarettes, the cell viability was decreased by lower concentration of traditional cigarettes, the levels of ROS, matrix metalloproteinase, COX-2, and inflammatory factors were higher in HGECs treated with CSC and the level of type I collagen was higher in HGECs treated with ECAC than those treated with CSC.

In this study, CSC and ECAC with diluted nicotine concentrations were used to treat cells for the same time to compare their effects on HGECs.[26] The results are basically consistent with the null hypothesis. HGEs play an important role in oral gingival tissue, being the first cells to be exposed to the environment,[33] acting as a barrier against invading bacteria and acting as an antibacterial agent.[18] The decreased survival rate of HGEs will destroy the homeostasis of oral microorganisms, leading to the accumulation of oral pathogenic microorganisms, the formation of dental plaque and the development of periodontitis.[38] From our results, a high concentration of CS and e-cigarettes both reduced cell survival. Thus, CS had a greater impact on cell survival which suggested

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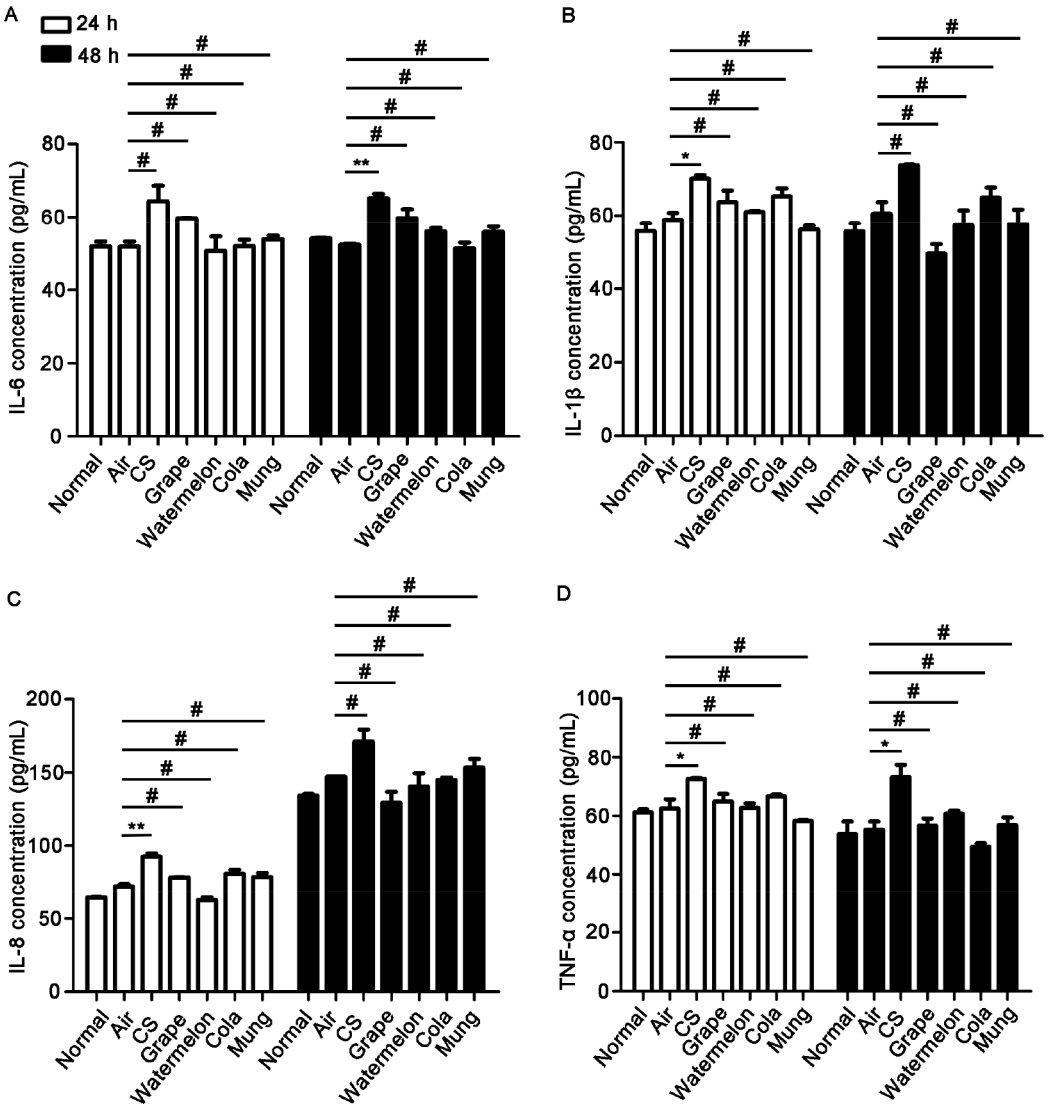

Figure 6. Effects of CSC and ECAC on the expression of inflammatory factor genes in HGECs. mRNA levels of IL-6 (A), IL-1β (B), IL-8 (C), and TNF-α (D) in HGECs were detected by RT-PCR after cells were treated with ECAC and CSC at nicotine concentration of 0.125 μg/mL for 12 and 24 h. Data are expressed as means ± SEM; #p > 0.05, *p < 0.05, **p < 0.01, and ***p < 0.001 (n = 3).

that tobacco might be more likely to cause periodontitis than e-cigarettes.

ROS generally refers to free radicals and nonfree radicals derived from oxygen[39] and are natural byproducts of normal oxygen metabolism. When inflammation occurs, the production of ROS increases which breaks the balance between ROS production and elimination and leads to oxidative stress.[40] Excessive ROS can regulate the intracellular JAK2 pathway in HGECs, promote IL-6 and TNF-α production, damage epithelial cells, and aggravate gingival inflammation.[41] Our staining results showed that ROS level was much higher in cells treated with CSC than in cells treated with ECAC. Thus, CSC is more likely to trigger oxidative stress than e-cigarettes, leading to the development of oral inflammation.

COX-2 is a proinflammatory mediator that mediates inflammation progression. The level of COX-2 is very low in normal epithelial tissues, but it will be expressed at a high level when stimulated by external factors such as bacteria and their products, cytokines, and growth factors.[42] Studies have shown that COX-2 can promote cell proliferation and play a role in cell apoptosis and tumorigenesis.[43] Overexpression of COX-2 further stimulates the inflammatory response of HGECs, leading to the destruction of connective tissues. Excessive activation of the cytokine network, including IL-1β, IL-6, IL-8, and TNF-α, will lead to gingival epithelial tissue damage and alveolar bone loss and promote the development of periodontitis.[44] IL-1β is a proinflammatory cytokine and an important regulator of microbial infection along with TNF-α.[45] IL-6 is a key factor in periodontal destruction and plays a certain role in collagen degradation. IL-8 is a chemokine secreted by HGECs to regulate osteoclast formation, leading to the development of periodontitis.[46,47] Increased levels of these cytokines will damage the local tissues of the oral cavity and further development of periodontitis. Our results showed that CSC promoted the gene expression of COX-2 and inflammatory factors more than e-cigarettes. Tobacco users may have higher levels of cycloxygenase 2 (COX-2) and inflammatory factors in tobacco users might be higher than that in e-cigarette users. Thus, tobacco smokers are more likely to suffer from oral inflammation and oral tissue damage than e-cigarette users.

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Type I collagen, the most abundant protein in the human body,[48,49] is one of the main structural components of the periodontal extracellular matrix and plays an important role in maintaining the homeostasis of periodontal connective tissue.[50] Decreased collagen levels increase the fragility of the periodontal blood vessels, leading to bleeding gums. MMPs degrade the protein components of the extracellular matrix. Overexpression of MMPs degrades collagen and disrupts the junctions between gingival epithelial cells,[51] leading to gingival attachment loss and periodontal pocket formation. MMP-1 is a key enzyme in collagen degradation, which can promote collagen degradation.[51] Our results showed that smoking tobacco and watermelon-flavored e-cigarettes significantly increased MMP1/3 levels and reduced type I collagen levels, suggesting that increased MMP-1/3 levels by smoking tobacco and watermelon-flavored e-cigarettes might degrade collagen levels and contribute to the bleeding gum and other diseases. Thus, some flavored e-cigarettes may also have an adverse effect on the mouth.

E-cigarettes consist of glycerin, propylene glycol, nicotine, and flavoring agents. Propylene glycol and glycerin have been shown to be toxic to cells cultured *in vitro*.[27] In addition, the high concentration of flavors in e-cigarettes are also cytotoxic, with one *in vitro* study suggesting that chocolate flavor agents can affect cellular functions.[52,53] Burning cigarettes produces a variety of compounds, including tar, nicotine, carbon monoxide, carbonyl compounds, and more. Studies have shown that the higher the tar content, the greater the risk of lung cancer.[54] Carbon monoxide produced by cigarette smoke binds to hemoglobin and reduces the ability to transport oxygen, thereby affecting the function of cells or tissues.[55] Cigarettes and e-cigarettes not only contain nicotine but also produce carbonyl compounds such as formaldehyde and acrolein when burned.[56] Studies have shown that formaldehyde and acrolein can damage cellular DNA and induce cytotoxicity and genotoxicity.[57] Thus, in order to evaluate the safety of cigarettes and e-cigarettes, toxicological analysis of e-cigarettes and other components of cigarettes should also be conducted.

There are some limitations in this study. The treatment time of cigarette smoke and e-cigarette sol for HGECs is short, and the observation time is short. Further long-term studies are needed to clarify the safety comparisons between e-cigarettes and tobacco. In addition, we only demonstrated the effect of e-cigarettes and conventional cigarettes on apoptosis, but a specific study on the mechanism of apoptosis did not, which is what we will continue to do in the future.

5. CONCLUSION

We compared the effects of tobacco and e-cigarette on HGECs. The results showed that compared with cells treated with ECAC, CSC-treated HGECs showed lower viability, lower collagen levels, higher apoptosis rates, and higher levels of inflammatory factors. Therefore, we concluded that e-cigarette is less toxic to HGECs than tobacco. Some studies have shown the effects of tobacco and e-cigarettes on oral cell viability and apoptosis, proving that e-cigarettes are less cytotoxic than tobacco, which is consistent with our result.[22,24,37]

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Notes

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