Received: 7 September 2021 | Revised: 25 September 2021 | Accepted: 12 October 2021

DOI: 10.1111/jre.12943

ORIGINAL ARTICLE

Journal of PERIODONTAL RESEARCH WILEY

# In vitro evaluation of the effect of the electronic cigarette aerosol, *Cannabis* smoke, and conventional cigarette smoke on the properties of gingival fibroblasts/gingival mesenchymal stem cells

Abir Tarek Mansour El-Mouelhy[1,2] | Sherine Adel Nasry[2] | Omnia Abou El-Dahab[1] | Dina Sabry[3,4] | Karim Fawzy El-Sayed[1,5,6]

[1]Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

[2]Department of Surgery and Oral Medicine, National Research Centre, Cairo, Egypt

[3]Department of Medical Biochemistry, Molecular Biology and Tissue Engineering Unit, Cairo University School of Medicine, Cairo, Egypt

[4]Medical Biochemistry and Molecular Biology, Faculty of Medicine, Badr University, Cairo, Egypt

[5]Stem Cell and Tissue Engineering Unit, Faculty of Dentistry, Cairo University, Cairo, Egypt

[6]Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian-Albrecht's University, Kiel, Germany

**Correspondence**
Karim M. Fawzy El-Sayed, Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, 11 Al Saraya, Old Cairo, Cairo, Egypt.
Email: karim.fawzy@gmail.com

**Funding information**
This study was partly funded by the Egyptian National Research Centre.

## Abstract

**Objective:** The current study aimed to evaluate the effect of electronic cigarette (EC) aerosol, *Cannabis*, and conventional cigarettes smoke on gingival fibroblast/gingival mesenchymal stem cells' (GF/G-MSCs) of never smokers.

**Material and Methods:** Human GF/G-MSCs ($n = 32$) were isolated and characterized using light microscopy, flow cytometry, and multilineage differentiation ability. Following the application of aerosol/smoke extracts, GF/G-MSCs were evaluated for cellular proliferation; colony-forming units (CFU-F) ability; cellular viability (using the MTT assay); mitochondrial depolarization using JC-1 dye; and genes' expression of ATM, p21, Oct4, and Nanog.

**Results:** Colony-forming units and viability (OD 450 nm) were significantly reduced upon exposure to *Cannabis* (mean ± SD; 5.5 ± 1.5; $p < .00001$, 0.47 ± 0.21; $p < .05$) and cigarettes smoke (2.3 ± 1.2 $p < .00001$, 0.59 ± 0.13, $p < .05$), while EC aerosol showed no significant reduction (10.8 ± 2.5; $p = .05$, 1.27 ± 0.47; $p > .05$) compared to the control group (14.3 ± 3, 1.33 ± 0.12). Significantly upregulated expression of ATM, Oct4, and Nanog (gene copies/GADPH) was noticed with *Cannabis* (1.5 ± 0.42, 0.82 ± 0.44, and 1.54 ± 0.52, respectively) and cigarettes smoke (1.52 ± 0.75, 0.7 ± 0.14, and 1.48 ± 0.79, respectively; $p < .05$), whereas EC aerosol caused no statistically significant upregulation of these genes compared to the control group (0.63 ± 0.1, 0.31 ± 0.12, and 0.64 ± 0.46, respectively; $p > .05$). The p21 gene was not significantly downregulated in EC aerosol (1.22 ± 0.46), *Cannabis* (0.71 ± 0.24), and cigarettes smokes (0.83 ± 0.54) compared to the control group ($p = .053$, analysis of variance).

**Conclusion:** *Cannabis* and cigarettes smoke induce DNA damage and cellular dedifferentiation and negatively affect the cellular proliferation and viability of GF/G-MSCs of never smokers, whereas EC aerosol showed a significantly lower impact on these properties.

El-Mouelhy and Nasry contributed equally to this work.

**KEYWORDS**
aerosol, *Cannabis*, cigarettes, electronic, fibroblast, gingival, mesenchymal stem cells, smoke

## 1 | INTRODUCTION

Smoking constitutes a risk factor for many serious ailments, including cancer, cardiovascular, obstructive pulmonary, and periodontal diseases.[1] Electronic cigarettes (ECs) are a drug delivery system that has been originally introduced as a safe substitute to conventional cigarette smoking and as one of the aids to help in smoking cessation.[2] It may release nicotine among other chemicals, such as propylene glycol, vegetable glycerin, and flavoring agents in the form of aerosols, while eliminating all chemicals released due to combustion by conventional cigarettes.[3] Although its most important feature is the elimination of harmful combustion products, its safety and long-term effects on human body are still not fully elucidated.[1–5] Currently, 147 million persons (2.5%) are using *Cannabis* for different reasons, including recreational and medical reasons, for pain control, or for treating neurological conditions including epilepsy.[6] In Egypt, *Cannabis* is one of the most common illegal drugs abused.[7] In a survey on drug abuse in young aged population, hashish was the most used representing almost 90% of drugs abused.[6,7] Gingival and oral tissues are the portal of entry of *Cannabis* and hence the first affected.[6] Nevertheless, the exact mechanism by which *Cannabis* causes periodontal destruction is not fully understood.[6,8]

Gingival tissues are the first to come in contact with the smoke/aerosol[6]; they are readily accessible and known for their remarkable wound healing properties without scarring.[9] Gingival fibroblasts are the most abundant cells in the gingiva.[10] Studies demonstrated that gingival connective tissue could be a reservoir for gingival fibroblast/gingival mesenchymal stem cells (GF/G-MSCs) with remarkable regenerative attributes[11–14] and resistance to infection and inflammation.[15–17] Previous studies demonstrated that smoke and aerosol extracts result in reduced proliferation and impaired function of gingival fibroblast,[18] and mesenchymal stem cells,[19] with nicotine and other by-products being primarily responsible for this hindered function.[18,20]

The aim of the present study was to evaluate and compare the effects of EC aerosol, *Cannabis*, and conventional cigarettes smoke on the proliferation, viability, and pluripotency gene expression profile (ATM, P21, Oct4, and Nanog) of human GF/G-MSCs of never smokers.

## 2 | MATERIAL AND METHODS

### 2.1 | Study design and settings

The current study was approved by the ethical committee of the Faculty of Dentistry, Cairo University (IRB:18|2|28). After signing the formal written informed consent, gingival biopsies were obtained from 32 healthy non-smoker patients ($n = 8$/group) (male/female: 3/29) during routine surgical periodontal treatment at the Department of Oral Medicine and Periodontology, Faculty of Dentistry, Cairo University.

### 2.2 | Electronic cigarette aerosol, *Cannabis*, and cigarette smoke extracts preparation

Generation of EC aerosol, *Cannabis*, and cigarette smoke was done as described before.[2] Cigarettes used were widely available in the market (Cleopatra, 20 pieces with filter in soft pack with cellophane tar 15 mg/cigarette, nicotine 1.0 mg/cigarette, Eastern Company). *Cannabis* used was in the form of custom-made joints containing 0.25 g of *Cannabis* (hashish) rolled in a tobacco cigarette.[21] A third generation EC device was used to generate the aerosol using e-liquid purchased from the local market (milk and honey flavored; The Illegal Factory) that consisted of 50% vegetable glycerin, 50% propylene glycol, and 3 mg nicotine adjusted on 2.6 V and 24 W. The EC containing the liquid was attached to a 60 ml syringe carrying 10 ml of RPMI 1640 medium + 10% fetal bovine serum + Todd-Hewitt Broth. Activation of EC occurred by applying negative pressure through the syringe, and 50 ml of aerosol was bubbled through the media, followed by syringe agitation on a shaker for 12 s. The aerosol was expelled, and the procedure was repeated for 30 puffs to give 10 ml of media exposed to 1.5 L of aerosol representing the puffs' volume consumed during the vaping session,[22] over a duration of 15 min. Similar to previous study,[2] 1.5 L of aerosol extract was used to reproduce the cigarette smoke extract preparation using one cigarette. All batches of aerosol extract were made following this procedure (Supplement Figure 1).

Medias obtained from EC aerosol were sterilized and used immediately (EC group). For the *Cannabis* (Can) joints and conventional cigarettes (Cig) smoke extracts, the same method of smoke extraction was used to consume one cigarette/joint.[23] Briefly, using negative pressure, 50 ml smoke was added to a syringe with 10 ml medium, followed by 12 s of syringe agitation, then the smoke was expelled resulting in Can or Cig smoke extract (Supplement Figure 2).

One milliliter of the freshly prepared aerosol/smoke extracts was added every third day to the cells for 2 weeks.[19]

### 2.3 | Determination of nicotine, cannabidiol (CBD), and polyaromatic hydrocarbons (PAHs) concentration in the aerosol/smoke extract

#### 2.3.1 | PAHs determination using gas chromatography-mass spectrometry (GC-MS)

*Materials*

Fourteen PAHs stock solution (1000 μg/ml) containing naphthalene, fluorene, fluoranthene, benz(a)anthracene, chrysene, pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, acenaphthene, phenanthrene, anthracene, acenaphthylene, and

pyrene-d10 (surrogate standard), and reference standards were obtained from Sigma-Aldrich with purity >95%, whereas benzo(g,h,i) perylene and dibenz(a,h)anthracene were purchased as readymade preparations of 100 μg/ml in methylene chloride and indeno[1,2,3-cd]pyrene 200 μg/ml in methanol.

*Sample extraction and Chromatographic condition*

Following extraction using acetonitrile, three samples/groups were analyzed using GC-MS. The analytical parameters were performed according to a previous study[24] and reported in Table 1. The detection limit was 2 μg/L.

### 2.3.2 | Nicotine and CBD determination using high performance liquid chromatography (HPLC) method

*Materials*

(−) – Nicotine, >99%, was obtained pure from Sigma Aldrich. Certified 1.0 mg/ml CBD stock solution from Cerilliant Corporation (Sigma Aldrich) was used. Reagents and solvents used were of analytical reagent and HPLC grade, respectively.

*Sample extraction and chromatographic condition*

High performance liquid chromatography analysis was performed using an Agilent 1260 series Infinity || LC system (Agilent Corporation). A Zorbax Eclipse Plus (Sigma Aldrich) C18 column (4.6 × 250 mm i.d., 5 μm) was used. Chromatographic parameters of the HPLC method for nicotine[25] and CBD[26] are listed in Table 2.

**TABLE 1** Analytical system and parameters used for the identification of 16 PAHs in EC aerosol/Cannabis and cigarette smoke extracts preparations using GC-MS. (Analysis was performed at Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Food (QCAP), Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Giza, Egypt)

| PAHs in EC aerosol/*Cannabis* and cigarette smokes extract | GC-MS (Agilent 6890 series gas chromatography/5975 series mass selective detector) (US) |
|---|---|
| Column | Agilent GC Column (J&W HP−5 ms Ultra Inert (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness) (US) |
| Injection mode | Split-Splitless |
| Injection temperature | 260 °C |
| Injection volume | 1 μl |
| Carrier gas | Helium 99.999%, constant flow |
| Flow rate | 1.3 ml/min |
| Oven | 90°C (hold 2 min) to180°C at an increasing rate of 15°C/min (hold for 15 min), then to 250°C at an increasing rate 10°C/min (hold 2 min), and then to 290°C as a final temperature at an increasing rate of 10°C/min (hold 10 min) |
| Source temperature | 250°C |
| Interface temperature | 280°C |
| Run time | 36 min |
| Acquisition mode | SIM mode |
| Scan range | 45–450 m/z |

## 2.4 | Isolation and identification of GF/G-MSCs

Gingival fibroblast/gingival mesenchymal stem cells were isolated as described before.[27] Tissues were de-epithelialized and cut into sections (2 × 2 mm), rinsed with RPMI 1640 medium (Thermo Fischer Scientific), and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin; Biochrom) were added. The samples were placed into dry 50 ml culture flasks (Gibco, Thermo Fisher Scientific) for 30 min allowing adherence. The basic medium consisted of RPMI 1640 medium supplemented with 15% fetal calf serum (HyClone), 400 mmol/ml L-glutamine (Biochrom), 100 μg/ml penicillin, and 100 mg/ml streptomycin (basic medium). The flasks' incubation was in 5% carbon dioxide at 37°C. Phase contrast inverted microscopy was used to check the flasks, and media were changed every third day.

The cells were washed with 5 ml of phosphate buffered saline (PBS; Biochrom) after reaching 80%–85% confluence. Two milliliters of 0.10% trypsin/ethylenediaminetetraacetic acid (Biochrom) were added, and the culture flasks were incubated for 10 min allowing cells detachment, followed by the addition of 5 ml of the basic medium. The cells-containing medium was transferred to sterile 50 ml polypropylene conical tubes (Becton Dickinson) and centrifuged at 643 g for 10 min. The supernatant was discarded, and the cells were resuspended in 2 ml basic medium. Cells counting and viability testing were done using MTT assay as described before.[28]

## 2.5 | Flow cytometric analysis

After reaching confluence, third passage GF/G-MSCs were characterized by flow cytometry. Primary antibodies against human CD29, CD34 (eBioscience), CD45, CD73, CD90, and CD105 (Miltenyi Biotec GmbH) were used. Cells were stained with fluorescein-isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated primary antibodies at room temperature. FITC- or PE-conjugated human IgGs were used as isotype controls at a concentration similar to the specific primary antibodies.[27] The analyses were done using Cytomics FC 500 Flow Cytometer (Beckman Coulter) and analyzed using CXP Software version 2.2.

## 2.6 | Multilineage differentiation assays

Third passage GF/G-MSCs were cultured in osteogenic medium for 14 days, in adipogenic medium for 21 days, and in chondrogenic

**TABLE 2** Chromatographic parameters of the HPLC method for nicotine and CBD (Analysis was performed at the Chromatography lab, at the National Research Center, Giza, Egypt)

| Parameters | Conditions for nicotine | Conditions for CBD |
|---|---|---|
| Solvent used for sample extraction | Dichloromethane | Methanol 90% |
| Mobile phase | acetonitrile (ACN): sodium hydrogen carbonate (25:75, v/v) (pH = 10.0.03 M) | Methanol 90% |
| Flow rate condition | Isocratic method | Isocratic method |
| Flow rate | 1 ml/min | 1 ml/min |
| Column temperature | 40°C | 25°C |
| Injection volume | 10 μl | 5 μl |
| Ultraviolet detector | 250 nm | 220 nm |
| Run time | 13 min | 8 min |

medium for 35 days or in basic medium for the same durations as respective controls. Osteogenic differentiation was assessed, using Alizarin Red staining. Areas of osteogenic mineralization were observed as dark orange-red patches, using the inverted light microscopy.[19] Intracellular presence of lipid drops indicating adipogenic differentiation was evaluated, using Oil Red O stain. Chondrogenic pellets formed were fixed with 4% formaldehyde solution, rinsed with PBS, and stained with 1% Alcian Blue solution prepared in 0.1 N HCL for 30 min.[29]

## 2.7 | Experimental groups

The study groups comprised GF/G-MSCs obtained from healthy non-smokers. Cells were grouped according to the aerosol/smoke extract to which they were exposed into four groups:

1. EC group: GF/G-MSCs were exposed to EC aerosol extract,
2. Cig group: GF/G-MSCs were exposed to cigarette smoke extract,
3. Can group: *Cannabis* smoke extract was applied to the GF/G-MSCs, and
4. Control group: only basic medium was added to the GF/G-MSCs.

## 2.8 | Colony-forming unit assay

Cells from all groups were cultured. Clusters of 50 or more cells were recorded as colonies (CFU-F). On the twelfth day, cultures were fixed using 4% formalin, and colonies' numbers were counted and statistically evaluated.[29]

## 2.9 | MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the metabolic activity of GF/G-MSCs. Seeding was in duplicates onto flat bottom 96-well plates for all groups. Cells in 90 μl media were incubated with 10 μl of 5 mg/ml MTT substrate solution, thiazolyl blue tetrazolium bromide salt (Sigma- Aldrich) for 4 h. Stopping the reaction occurred by adding 100 μl of solubilizing solution (12 mmol/L HCl, 346 mmol/L SDS, and 5% isobutanol) and incubation for 15 min at room temperature. Absorbance was measured at OD450 nm using a microplate reader [19].

## 2.10 | Mitochondrial membrane potential

In all groups, mitochondrial membrane potential (ΔΨm) was assessed using Mitochondrial Permeability JC-1 dye (JC-1 Mitochondrial Membrane Potential Assay Kit, Cayman Chemical Company). After reaching 75%–80% confluence, cells were cultured in 96 well culture plate at a density of $5 \times 10^4$–$5 \times 10^5$ cells/well in 100 μl of culture medium. Ten microliter of JC-1 staining solution was added to each well and gently mixed. Cells were incubated in carbon dioxide incubator at 37°C for 20 min. The culture plate was centrifuged for 5 min at 400 *g* at room temperature, and the resultant supernatant was carefully aspirated. Two hundred microliters of assay buffer was added to each well followed by plate centrifuge for 5 min at 400 *g* at room temperature; the resultant supernatant was also carefully aspirated. The last step was repeated twice, followed by adding 100 μl of assay buffer to each well. Fluorescent plate reader was used to directly analyze the cells. Healthy cells with mainly JC-1 J-aggregates (red) were detected with filter sets designed to detect rhodamine (excitation/emission =540/570 nm), while apoptotic or unhealthy cells with mainly JC-1 monomer (green) were detected using filter sets designed to detect FITC (excitation/emission =485/535 nm). Changes in the ΔΨm were determined as aggregates:monomer ratio, using a fluorescent plate reader.[30]

## 2.11 | Molecular analysis

Total mRNA were extracted from GF/G-MSCs, using GeneJET RNA Purification Kit (Thermo Scientific), according to the manufacturer's instructions 14 days following the exposure in the respective groups. The extracted mRNA was reversely transcribed with

the RevertAid first strand cDNA synthesis kit (Thermo Scientific), using random primers according to the manufacturer's instructions. Using Step One Real-Time PCR system (Applied Biosystems), cDNA was amplified. The cycling conditions were 95°C for 3 min then 40 amplification cycles (95°C denaturation for 3 s, annealing for 30 s at the primers' melting temperature, and extension at 72°C for 30 s), followed by a final cycle at 72°C for 5 min. Relative expression of target genes was conducted according to the comparative ΔΔCt method, using human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene for normalization. Gene expression levels were evaluated for ATM, P21, Oct4, and Nanog (Table 3).

### 2.12 | Statistical evaluation

Data were coded and entered using IBM SPSS Statistics for Windows, Version 22.0. IBM Corp. Released 2015. Data were checked for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally, distributed data were expressed using means and standard deviation. Comparisons between the four studied groups were done using analysis of variance with multiple comparisons post hoc test. Nonparametric data were expressed using medians and range, and comparisons were done using Kruskal-Wallis test. Statistical significance was considered when $p < .05$.

## 3 | RESULTS

### 3.1 | Gingival tissues donors

Gingival tissues were obtained from 32 patients. Male to female ratio was 3:29 with mean age of 21 ± 2.6 years. Each group consisted of eight patients.

### 3.2 | GF/G-MSCs characterization

Third passage GF/G-MSCs showed plastic adherent fusiform morphology (Figure 1A). Cells showed successful osteogenic, adipogenic, and chondrogenic differentiation. Osteogenic differentiation was evident by the calcium deposits stained with Alizarin Red stain. Adipogenic differentiation was demonstrated by the presence of intracellular lipid droplets stained with Oil Red O stain. Chondrogenic differentiation was shown by the formation of glycosaminoglycans stained with Alcian Blue stain (Figure 1B). GF/G-MSCs were positive for CD29, CD73, CD90, and CD105 and negative for CD34 and CD45 (Figure 1C).

### 3.3 | Nicotine, CBD, and PAHs concentration in the aerosol/smokes extract

The standard curve of nicotine showed linearity in the range of 12.5–50 μg/ml with $r^2 = .999$, while that of CBD was in the range of 2.5–10 μg/ml, with $r^2 = .999$ as obtained from the linear regression analysis. The analysis revealed a statistically significant difference between the median concentration of nicotine in the EC aerosol extract (1.7 (1.35–2.25) μg/ml) (median (minimum-maximum)), cigarette smoke extract (8.2 (6.53–9) μg/ml), and *Cannabis* smoke extract (4.17 (3.65–4.31) μg/ml; $p = .02$). Median CBD concentration in the *Cannabis* smoke extract was 0.59 (0.45–1.4) μg/ml.

The standard curve of PAHs showed linearity in the range of 2–500 μg/L with $r^2 = .998$. The total of 16 PAHs (Σ16 PAHs) concentration as Benzo(a)pyrene was calculated according to the total equivalent toxic factor for conversion.[31] The median Σ16 PAHs concentration as Benzo(a)pyrene in *Cannabis* smoke extract was 0.883 (0.501–1.414) μg/L. All analytes were below the detection limit in the EC aerosol and the cigarettes smoke extracts.

### 3.4 | Cellular proliferation, mitochondrial membrane potential, and viability following smoke and aerosol exposure

Compared to the control group (14.3 ± 3), CFU-Fs were significantly reduced in the Cig (5.5 ± 1.5, $p < .00001$) and Can groups (2.3 ± 1.2, $p < .00001$), with no statistically significant difference to the EC group (10.8 ± 2.5, $p = .05$). CFUs in Cig and Can groups were significantly different to the EC group ($p = .001$ and $p < .00001$, respectively; Figure 2A).

**TABLE 3** Primers used for RT-PCR and their IDs

| Gene | | Sequence | Accession ID |
|---|---|---|---|
| ATM | Forward | 5′-TCCTGCAGTATGCTGTTTGACTTTGGC–3′ | NM_000051.4 |
| | Reverse | 5′-CTGTGAAGAATTGGAGGCACTTCTGT GC–3′ | |
| P21 | Forward | 5′-TGTCCGCGAGGATGCGTGTTC–3′ | NR_164656.1 |
| | Reverse | 5 ′-GCAGCCCGCCATTAGCGCAT–3′ | |
| Oct4 | Forward | 5′-TTC TCA GGG GGA CCA GTG TC–3′ | LC006948.1 |
| | Reverse | 5′-CCC ATT CCT AGA AGG GCA GG–3′ | |
| Nanog | Forward | 5′-CCA GTG ACT TGG AGG CTG C–3′ | NG_054758.1 |
| | Reverse | 5′-AAG GAT TCA GCC AGT GTCC–3′ | |

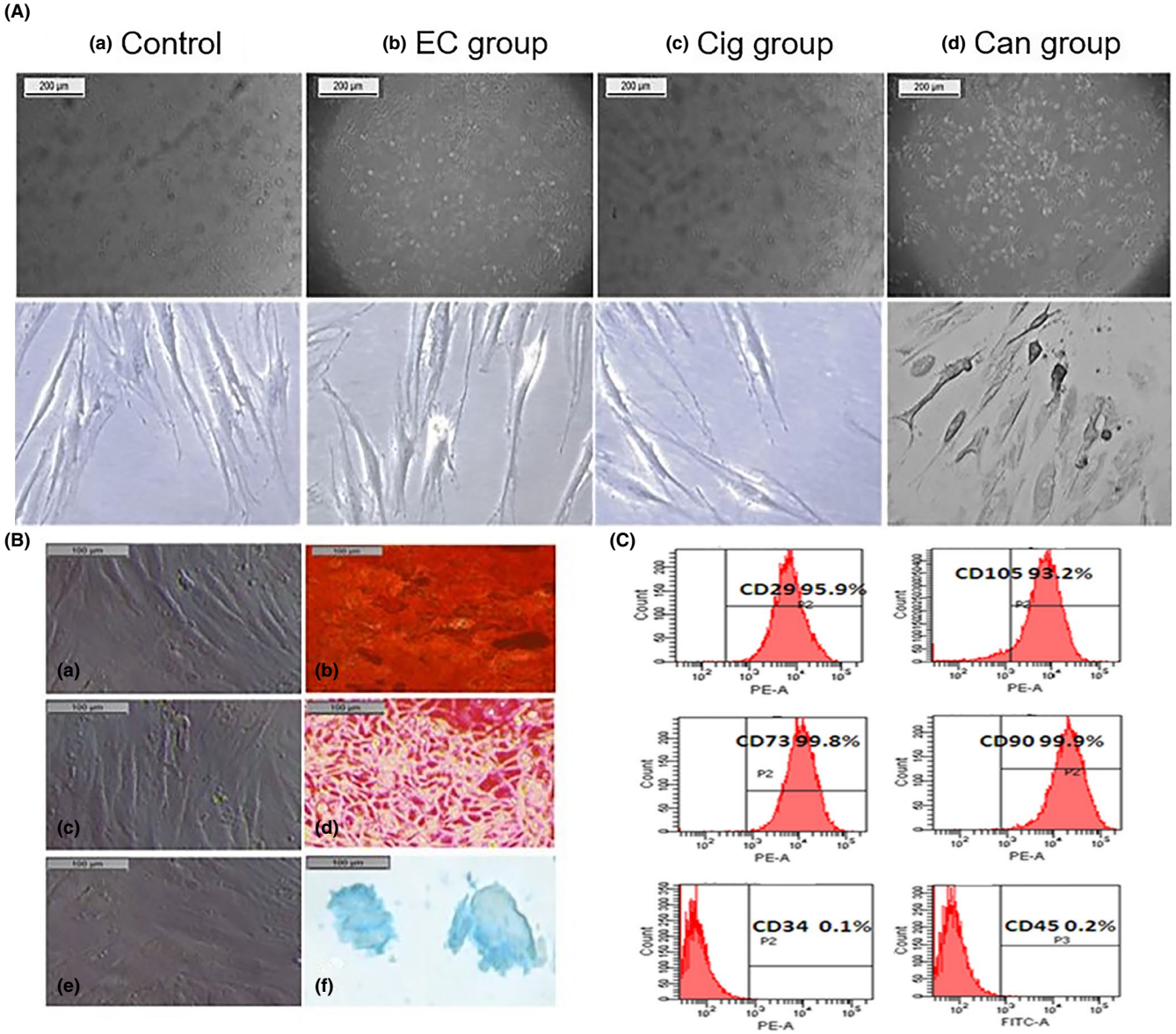


**FIGURE 1** Phase contrast inverted light microscopy images (magnification 200x) showing fusiform morphology of GF/G-MSCs. EC, Cig, and Can smoke extracts alter cells morphology. High magnification DIC images are shown in the lower panel (1A). Phase contrast inverted light microscopy images (magnification 100x) showing multilineage differentiation potential of GF/G-MSCs (1B) GF/G-MSCs control (a) and their Alizarin Red staining of the osteogenic stimulated GF/G-MSCs (b). GF/G-MSCs control (c) and their Oil Red O staining of the adipogenic stimulated GF/G-MSCs (d). GF/G-MSCs control (e) and their Alcian Blue staining of the chondrogenic stimulated GMSCs (f). Flow cytometric analysis (1C)

Mitochondrial membrane potential assay showed significant decrease of JC-1 aggregates fluorescence ratio, indicating reduced mitochondrial potential in Can (0.47 ± 0.05, $p$ = .001) and Cig (0.36 ± 0.04, $p$ = .001) compared to control group (1.74 ± 0.26), with no difference to the EC group (1.6 ± 0.4, $p$ = .09). Moreover, Can and Cig groups exhibited significant decrease compared to EC group ($p$ = .008 and $p$ = .006, respectively; Figure 2B).

MTT assay showed significant reduction in cell viability (OD450 nm) at 24 and 48 h in Can (0.47 ± 0.21, 0.77 ± 0.28) and Cig (0.59 ± 0.13, 0.85 ± 0.46) compared to control group (1.33 ± 0.12, 2.06 ± 0.5, $p$ < .05), with no difference to the EC group (1.27 ± 0.47, 2.17 ± 0.69, $p$ = .9). Can and Cig groups showed a significantly lower cellular viability compared to EC ($p$ < .001; Figure 2C).

## 3.5 | Gene expression

Significant upregulation of ATM gene expression (gene copies/GADPH) was notable in Can (1.52 ± 0.75) and Cig (1.5 ± 0.42) compared to the control group (0.46 ± 0.26; $p$ = .001), with no elevation in the EC group (0.63 ± 0.1, $p$ > .05). Compared to EC group, Can and Cig groups exhibited significant upregulation in

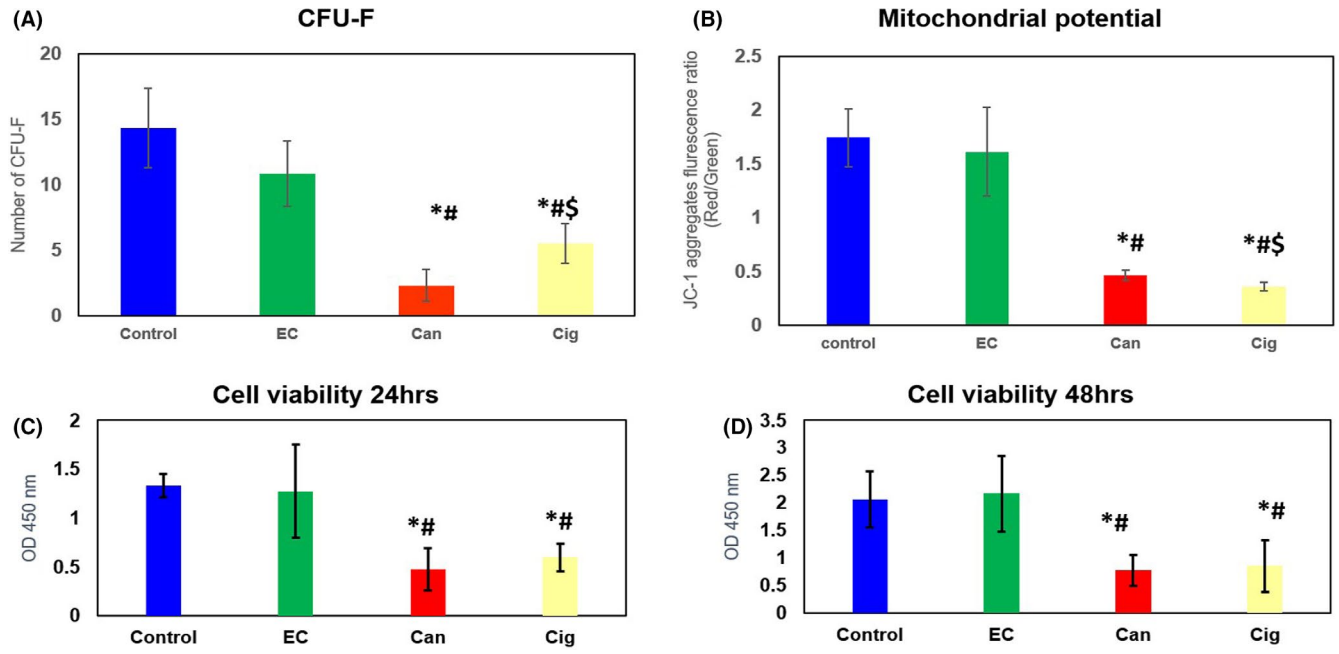


FIGURE 2 Colony-forming unit assay (A). Comparison of mitochondrial membrane potential in all studied groups (B). Comparison between cellular viability of all studied groups by MTT assay at 24 and 48 h (C). Comparison between cellular viability of all studied groups by MTT assay at 48 h (D). Data were expressed as Mean ± SD; $p$ value < .05 was significant ($n$ = 8). * denotes significant difference versus control group, # denotes significant difference versus EC group, and $ denotes significant difference versus Can group

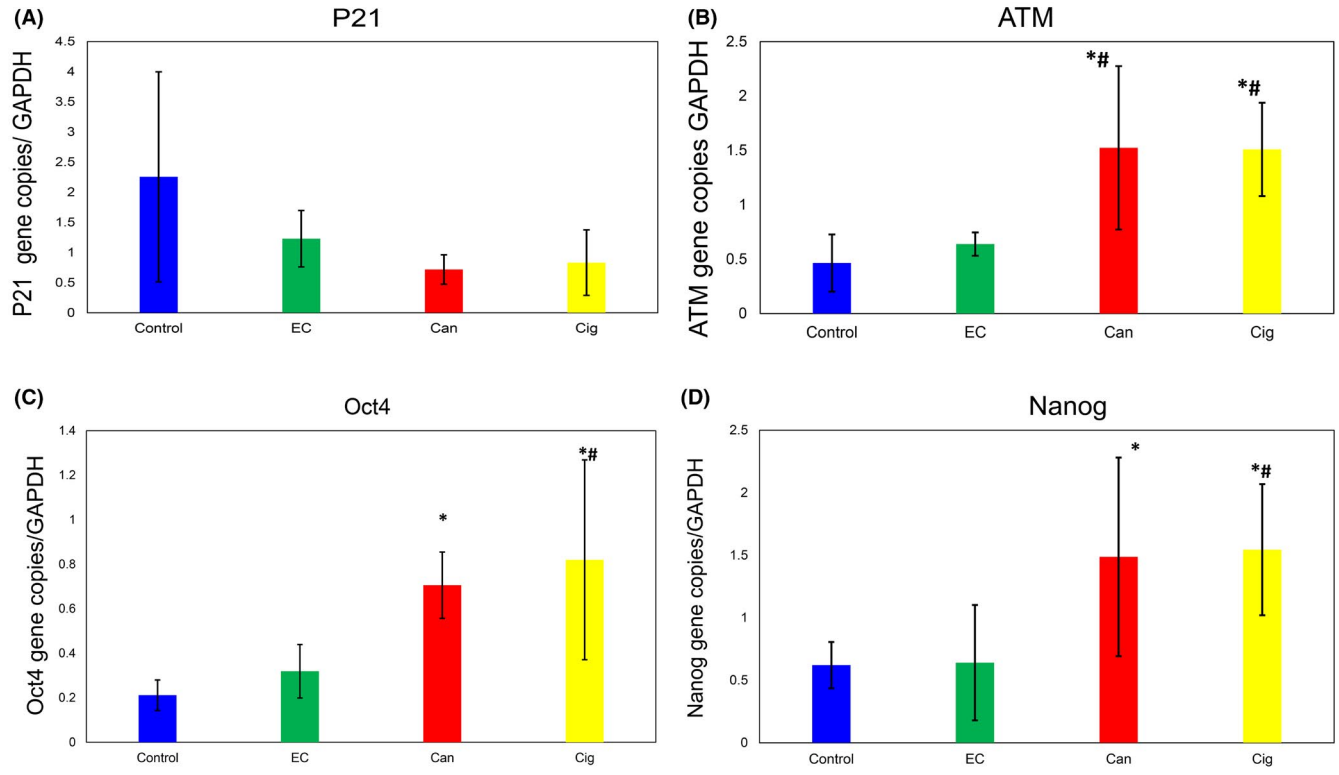


FIGURE 3 Comparison of p21 gene (A), ATM gene (B), Oct4 gene (C), and Nanog gene (D) expression in all studied groups. Data were expressed as Mean ± SD, $p$ value < .05 was significant. *Denotes significant difference versus control group; # denotes significant difference versus EC group

ATM expression ($p$ = .001), with no difference between Can and Cig groups ($p$ > .05). EC (1.22 ± 0.46), Can (0.71 ± 0.24), and Cig groups (0.83 ± 0.54) downregulated p21 gene compared to the control group (2.25 ± 1.74, $p$ = .053). Can (0.7 ± 0.14) and Cig (0.82 ± 0.44) significantly upregulated Oct4 gene expression compared to the control group (0.21 ± 0.06, $p$ = .001). Only Cig

significantly upregulated Oct4 gene expression compared to EC group (0.31 ± 0.12, $p = .01$).

Can (1.48 ± 0.79) and Cig (1.54 ± 0.52) significantly upregulated Nanog gene expression compared to the control group (0.62 ± 0.18, $p = .007$), while EC (0.64 ± 0.46) group failed to show significant difference (Figure 3).

## 4 | DISCUSSION

Gingival fibroblast/gingival mesenchymal stem cells' are key players in periodontal repair and regeneration.[13,32] Their unique properties make them ideal candidate for tissue engineering, regenerative procedures, and cellular and gene therapies.[13,33] Previous investigation suggested that cigarette smoke increases the permeability of the oral epithelial cells by upregulating the desquamation and affecting the tight junctions between cells, leaving the underlying GF/G-MSCs prone to the negative effects of smoke.[34] The present study aimed to evaluate and compare the effects of EC aerosol, *Cannabis*, and cigarettes smoke on the proliferation, viability, and gene expression profile (ATM, P21, Oct4, and Nanog) of GF/G-MSCs of never smokers.

Similar to earlier studies, GF/G-MSCs were positive for CD29, CD73, CD90, CD105, while being negative for CD34 and CD45.[13,14] Moreover, they showed the capacity of colony formation, along with remarkable multilineage differentiation ability into osteogenic, adipogenic, and chondrogenic directions.[14,29]

The present investigation demonstrated a significantly reduced CFU-F ability in GF/G-MSCs exposed to cigarette smoke compared to controls, in accordance with a previous study on CFU-F from mice stem cells[35] and mice embryonic stem cells.[36] It could be attributed to several mechanisms, including the oxidative stress resulting in reactive oxygen species (ROS) production, which affects the equilibrium between proliferation and apoptosis, genetic damage, as well as the effect of the aldehydes content, resulting in reduced defense mechanisms, cell death, and direct cell membrane damage.[37] To our knowledge, this is the first study on the effect of *Cannabis* (hashish) smoke on GF/G-MSCs. The detrimental effect of the *Cannabis* smoke on the colony formation ability of GF/G-MSCs could be partly attributed to the nicotine content in the joint and the negative effect of CBD on cell proliferation,[38] in addition to 9-tetrahydrocannabinol (THC) as a mitochondrial inhibitor, negatively affecting their viability and proliferation,[39,40] with the least viability notable in the *Cannabis* group. Additionally, cellular exposure to *Cannabis* smoke results in reduced levels of IL-8 and ΔΨm, which are involved in wound healing.[40] Yet, the current findings are in contrast to previous results by Sazmand et al.,[41] where the addition of different concentration of *Cannabis* to rat bone marrow MSCs resulted in an increased cell viability compared to the unexposed controls at 24 h period. The results could be attributed to the beneficial effect of *Cannabis* on growth factors, as well as its antioxidant and anti-inflammatory effects. The contradictory results seen in the present study during the 24 h period might be due to the different method of *Cannabis* application: plant in powder form in the previous study versus smoke in the present study allowing the delivery of harmful combustion products in addition to the THC.[42] In the present study, the EC group demonstrated the highest cellular viability and a nonsignificant reduction in the number of CFU-F compared to the control group. These findings are in agreement with Shaito et al.,[19] demonstrating that the effects of EC aerosol on MSCs proliferation were significantly lower than those of cigarette smoke. It was suggested that EC aerosol could have a cytotoxic effect on human cells, resulting in necrotic cell death.[2] Necrotic cells lose the integrity of their membrane, releasing their intracellular content, stimulating pro-inflammatory cytokines production such as IL-1, IL-6, IL-8, and PGE2,[43,44] which could amplify the reparative actions of stem cells, and upregulating their proliferation and differentiation capacities.[16] This could explain the similarity in the cells proliferation rate between the EC and control groups. This could be further attributed to the low levels of nicotine in the EC aerosol extract, thus reducing its negative effects on the cells' proliferation.

ΔΨm is a key factor that evaluates mitochondrial function, and reduced ΔΨm with increased depolarization is connected to apoptosis.[30,45,46] In that context, control and EC groups demonstrated similar results in agreement with a previous investigation.[30] On the contrary, a recent investigation showed reduced ΔΨm and increased mitochondrial depolarization, when epithelial cells were exposed to cinnamon flavored EC aerosol.[47] The difference between the results could be attributed to the cinnamon flavor, being an aldehyde capable of reacting with propylene glycol to form acetals resulting in oxidative stress.

The ATM gene has a crucial role in repairing double strands breaks of DNA, while the p21 gene inhibits DNA synthesis and plays a role in apoptosis and DNA repair.[48] The least ATM gene expression was observed in EC group suggesting the absence of DNA damage and reduced amount of ROS production, whereas the *Cannabis* and cigarette groups showed significantly higher expression, indicating the role of ATM gene in repairing damaged DNA,[49] as well as its role in controlling ROS levels,[50] thus protecting other biomolecules in the cells from the oxidative damage caused by cigarette and *Cannabis* smoke.[45,51] The current findings contradict a previous investigation, where ATM gene expression was significantly downregulated in the epithelial cells of the oral mucosa of chronic smokers, which could be attributed to the advanced maturation stage of epithelial cells and their lower metabolic activity compared to the GF/G-MSCs used in this study.[52]

Normally, p21 gene is downregulated in response to severe DNA damage, to allow the induction of the apoptotic process.[53] In accordance with an earlier investigation,[54] present results revealed that the lowest p21 gene expressions were in the *Cannabis* and cigarette groups, which can be attributed to the high levels of oxidative stress released by the smoke. These findings contradicted a previous study,[55] where an upregulation of p21 gene expression was observed in mouse lung cells exposed to cigarette smoke. This difference might be explained by the variation in cell types (GF/G-MSCs versus lung cells), the culture condition (in vivo vs. in vitro), and the method of exposure to smoke.

Oct4 and Nanog are pluripotency genes regulating and maintaining the cellular pluripotency and self-renewal. Their expression is limited to pluripotent cells and is reduced upon differentiation.[56] Oct4 and Nanog genes should be maintained in a balanced state.[57,58] The EC group showed almost similar expression to the control group, suggesting no effect of ECs on the pluripotency of GF/G-MSCs. In accordance with a recent investigation,[59] the highest value of Oct4 and Nanog genes were found in the cigarettes and the *Cannabis* groups. This upregulation of the pluripotency markers may direct the cells' dedifferentiation into a more primitive form, which eventually could result in malignant transformation.[60]

The results presented here should be cautiously interpreted in light of the current study's limitations. First, using positive control groups like gingival tissues collected from smokers/vapers could have made the present results more credible and could be recommended for future studies. Second, examining multiple concentrations of the used extracts would have allowed further investigation of the effects of the different chemical compounds on the used cells. Third, the exploration on protein level, not only their mRNA, could have been conducted to give a better understanding of the cells' response to these chemicals. Finally, the in vitro trials never full reproduce the complex biodynamic systems of the human body.

## 5 | CONCLUSION

The present investigation shows that *Cannabis* and cigarette smoke extracts negatively affect the cellular proliferation, viability, and mitochondrial membrane potential of GF/G-MSCs, in contrast to the specifically used concentration of EC aerosol extract in the current investigation, which had less effect on these attributes. The difference observed in gene expression further underlines the damaging effects of the *Cannabis* and cigarette smoke extracts on the GF/G-MSCs, driving their dedifferentiation. Within the limits of the current investigation, an adverse change in GF/G-MSCs mediated healing process could be anticipated in *Cannabis* and cigarette users.

### CONFLICT OF INTERESTS

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

All data will be available from the authors upon reasonable request.

### ORCID

*Abir Tarek Mansour El-Mouelhy* https://orcid.org/0000-0002-0109-8391
*Sherine Adel Nasry* https://orcid.org/0000-0002-3764-9562
*Omnia Abou El-Dahab* https://orcid.org/0000-0003-4882-7616
*Dina Sabry* https://orcid.org/0000-0002-6720-3385
*Karim Fawzy El-Sayed* https://orcid.org/0000-0002-6261-3609

### REFERENCES

1. Rouabhia M, Park HJ, Semlali A, Zakrzewski A, Chmielewski W, Chakir J. E-cigarette vapor induces an apoptotic response in human gingival epithelial cells through the caspase-3 pathway. *J Cell Physiol*. 2017;232(6):1539-1547. https://doi.org/10.1002/jcp.25677
2. Hwang JH, Lyes M, Sladewski K, et al. Electronic cigarette inhalation alters innate immunity and airway cytokines while increasing the virulence of colonizing bacteria. *J Mol Med (Berl)*. 2016;94(6):667-679. https://doi.org/10.1007/s00109-016-1378-3
3. Franco T, Trapasso S, Puzzo L, Allegra E. Electronic cigarette: role in the primary prevention of oral cavity cancer. *Clin Med Insights Ear Nose Throat*. 2016;9:7-12. https://doi.org/10.4137/CMENT.S40364
4. Ramôa CP, Eissenberg T, Sahingur SE. Increasing popularity of waterpipe tobacco smoking and electronic cigarette use: implications for oral healthcare. *J Periodontal Res*. 2017;52(5):813-823. https://doi.org/10.1111/jre.12458
5. Yu V, Rahimy M, Korrapati A, et al. Electronic cigarettes induce DNA strand breaks and cell death independently of nicotine in cell lines. *Oral Oncol*. 2016;52:58-65. https://doi.org/10.1016/j.oraloncology.2015.10.018
6. Singh H, Katz J, Saleh W, Cha S. Impact of Cannabis on the port of entry-oral tissues: an overview. *Int J Oral Dent Health*. 2019;5:98. https://doi.org/10.23937/2469-5734/1510098
7. Abdel-Salam OME, Galal AF, Elshebiney SA, Gaafar AEDM. Chapter 12 - International aspects of cannabis use and misuse: Egypt. In: Preedy VR, ed. *Handbook of Cannabis and Related Pathologies*. Cambridge, MA: Academic Press; 2017:110-121.
8. Le A, Palamar JJ. Oral health implications of increased cannabis use among older adults: another public health concern? *J Subst Use*. 2019;24(1):61-65.
9. Fawzy El-Sayed KM, Dörfer CE. Gingival mesenchymal stem/progenitor cells: a unique tissue engineering gem. *Stem cells Int*. 2016;2016:7154327.
10. Lee I-K, Lee M-J, Jang H-S. The interrelationship between human gingival fibroblast differentiation and cultivating time. *Tissue Eng Regen Med*. 2013;10(2):60-64.
11. Fawzy El-Sayed KM, Mekhemar MK, Beck-Broichsitter BE, et al. Periodontal regeneration employing gingival margin-derived stem/progenitor cells in conjunction with IL-1ra-hydrogel synthetic extracellular matrix. *J Clin Periodontol*. 2015;42(5):448-457. https://doi.org/10.1111/jcpe.12401
12. Fawzy El-Sayed KM, Paris S, Becker ST, et al. Periodontal regeneration employing gingival margin-derived stem/progenitor cells: an animal study. *J Clin Periodontol*. 2012;39(9):861-870. https://doi.org/10.1111/j.1600-051X.2012.01904.x
13. Gamal A, Al-Berry N, Hassan A, Rashed L, Iacono V. In vitro evaluation of the human gingival fibroblast/gingival mesenchymal stem cell dynamics through perforated guided tissue membranes: cell migration, proliferation and membrane stiffness assay. *J Periodontal Res*. 2017;52(3):628-635.
14. Mitrano TI, Grob MS, Carrion F, et al. Culture and characterization of mesenchymal stem cells from human gingival tissue. *J Periodontol*. 2010;81(6):917-925.
15. Fawzy El-Sayed KM, Dorfer CE. Gingival mesenchymal stem/progenitor cells: a unique tissue engineering gem. *Stem Cells Int*. 2016;2016:7154327. https://doi.org/10.1155/2016/7154327
16. Fawzy KM, Elahmady M, Adawi Z, et al. The periodontal stem/progenitor cell inflammatory-regenerative cross talk: a new perspective. *J Periodontal Res*. 2019;54(2):81-94. https://doi.org/10.1111/jre.12616
17. Zhang F, Si M, Wang H, Mekhemar MK, Dorfer CE, Fawzy El-Sayed KM. IL-1/TNF-alpha inflammatory and anti-inflammatory synchronization affects gingival stem/progenitor cells' regenerative attributes. *Stem Cells Int*. 2017;2017:1349481. https://doi.org/10.1155/2017/1349481

18. Alanazi H, Park HJ, Chakir J, Semlali A, Rouabhia M. Comparative study of the effects of cigarette smoke and electronic cigarettes on human gingival fibroblast proliferation, migration and apoptosis. *Food Chem Toxicol*. 2018;118:390-398.
19. Shaito A, Saliba J, Husari A, et al. Electronic cigarette smoke impairs normal mesenchymal stem cell differentiation. *Sci Rep*. 2017;7(1):14281. https://doi.org/10.1038/s41598-017-14634-z
20. Faddy MJ, Cullinan MP, Palmer JE, Westerman B, Seymour GJ. Ante-dependence modeling in a longitudinal study of periodontal disease: the effect of age, gender, and smoking status. *J Periodontol*. 2000;71(3):454-459.
21. Kögel CC, Balcells-Olivero MM, López-Pelayo H, et al. The standard joint unit. *Drug Alcohol Depend*. 2017;176:109-116.
22. Lee YO, Nonnemaker JM, Bradfield B, Hensel EC, Robinson RJ. Examining daily electronic cigarette puff topography among established and nonestablished cigarette smokers in their natural environment. *Nicotine Tob Res*. 2018;20(10):1283-1288.
23. McEachern EK, Hwang JH, Sladewski KM, et al. Analysis of the effects of cigarette smoke on staphylococcal virulence phenotypes. *Infect Immun*. 2015;83(6):2443-2452.
24. Khorshid M, Souaya ER, Hamzawy AH, Mohammed MN. QuEChERS method followed by solid phase extraction method for gas chromatographic-mass spectrometric determination of polycyclic aromatic hydrocarbons in fish. *Int J Anal Chem*. 2015;2015:1–7.
25. Bansal M, Sharma M, Bullen C, Svirskis D. A stability indicating HPLC method to determine actual content and stability of nicotine within electronic cigarette liquids. *Int J Environ Res Public Health*. 2018;15(8):1737.
26. Saingam W, Sakunpak A. Development and validation of reverse phase high performance liquid chromatography method for the determination of delta-9-tetrahydrocannabinol and cannabidiol in oromucosal spray from cannabis extract. *Rev Bras De Farmacogn*. 2018;28(6):669-672.
27. Van Pham P, Tran NY, Phan NL-C, Vu NB, Phan NK. Vitamin C stimulates human gingival stem cell proliferation and expression of pluripotent markers. *In Vitro Cell Dev Biol Anim*. 2016;52(2):218-227.
28. Assem M, Kamal S, Sabry D, Soliman N, Aly RM. Preclinical assessment of the proliferation capacity of gingival and periodontal ligament stem cells from diabetic patients. *Open Access Maced J Med Sci*. 2018;6(2):254.
29. El-Sayed KM, Paris S, Graetz C, et al. Isolation and characterisation of human gingival margin-derived STRO-1/MACS(+) and MACS(-) cell populations. *Indian J Orthop Surg*. 2014;7(2):80-88.
30. Sivandzade F, Bhalerao A, Cucullo L. Analysis of the mitochondrial membrane potential using the cationic JC-1 dye as a sensitive fluorescent probe. *Bio-protocol*. 2019;9(1):e3128.
31. NEPM Toxic equivalence factors for PAHs as per NEPM Schedule B1, Table 1A. World Health Organization (WHO). Accessed 29th August 2021. Retrieved from https://www.alsglobal.com/-/media/als/resources/services-and-products/environmental/enviromails-au/enviromail-59---bap-teq-and-other-summed-nepm-parameters---re-release-july-2013.pdf
32. Fawzy El-Sayed KM, Hein D, Dörfer CE. Retinol/inflammation affect stemness and differentiation potential of gingival stem/progenitor cells via Wnt/β-catenin. *J Periodontal Res*. 2019;54(4):413-423.
33. Tomasello L, Mauceri R, Coppola A, et al. Mesenchymal stem cells derived from inflamed dental pulpal and gingival tissue: a potential application for bone formation. *Stem Cell Res Ther*. 2017;8(1):179.
34. Semlali A, Chakir J, Goulet JP, Chmielewski W, Rouabhia M. Whole cigarette smoke promotes human gingival epithelial cell apoptosis and inhibits cell repair processes. *J Periodontal Res*. 2011;46(5):533-541.
35. Siggins RW, Hossain F, Rehman T, Melvan JN, Zhang P, Welsh DA. Cigarette smoke alters the hematopoietic stem cell niche. *Medical Sciences*. 2014;2(1):37-50.
36. Kim CW, Go RE, Hwang KA, Jeung EB, Choi SJ, Choi KC. Apoptotic effects of cigarette smoke extracts on mouse embryonic stem cells via oxidative stress. *Environ Toxicol*. 2019;34(6):689-698.
37. Romagna G, Allifranchini E, Bocchietto E, Todeschi S, Esposito M, Farsalinos KE. Cytotoxicity evaluation of electronic cigarette vapor extract on cultured mammalian fibroblasts (ClearStream-LIFE): comparison with tobacco cigarette smoke extract. *Inhalation Toxicol*. 2013;25(6):354-361.
38. Soundara Rajan T, Giacoppo S, Scionti D, et al. Cannabidiol activates neuronal precursor genes in human gingival mesenchymal stromal cells. *J Cell Biochem*. 2017;118(6):1531-1546.
39. Gowran A, McKayed K, Campbell VA. The cannabinoid receptor type 1 is essential for mesenchymal stem cell survival and differentiation: implications for bone health. *Stem Cells Int*. 2013;2013(2):66.
40. Miller HP, Bonawitz SC, Ostrovsky O. The effects of delta-9-tetrahydrocannabinol (THC) on inflammation: a review. *Cell Immunol*. 2020;352:104111.
41. Sazmand M, Mehrabani D, Hosseini SE, Amini M. The effect of hydroalcoholic extract of *Cannabis sativa* on morphology and growth of bone marrow mesenchymal stem cells in rat. *Electron J Gen Med*. 2018;15:em32. https://doi.org/10.29333/ejgm/86195
42. Aguiar JA, Huff RD, Tse W, et al. Transcriptomic and barrier responses of human airway epithelial cells exposed to cannabis smoke. *Physiol Rep*. 2019;7(20):e14249.
43. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol*. 2008;8(4):279-289.
44. Sundar IK, Javed F, Romanos GE, Rahman I. E-cigarettes and flavorings induce inflammatory and pro-senescence responses in oral epithelial cells and periodontal fibroblasts. *Oncotarget*. 2016;7(47):77196.
45. Sakamuru S, Attene-Ramos MS, Xia M. Mitochondrial membrane potential assay. In: Zhu H, Xia M, eds. *High-Throughput Screening Assays in Toxicology*. Methods in Molecular Biology Vol 1473. New York, NY: Humana Press; 2016:17-22.
46. Sakamuru S, Li X, Attene-Ramos MS, et al. Application of a homogenous membrane potential assay to assess mitochondrial function. *Physiol Genomics*. 2012;44(9):495-503.
47. Clapp PW, Lavrich KS, van Heusden CA, Lazarowski ER, Carson JL, Jaspers I. Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function. *Am J Physiol Lung Cel Mol Physiol*. 2019;316(3):L470-L486.
48. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer*. 2009;9(6):400-414. https://doi.org/10.1038/nrc2657
49. Weber AM, Ryan AJ. ATM and ATR as therapeutic targets in cancer. *Pharmacol Ther*. 2015;149:124-138. https://doi.org/10.1016/j.pharmthera.2014.12.001
50. Bigarella CL, Liang R, Ghaffari S. Stem cells and the impact of ROS signaling. *Development*. 2014;141(22):4206-4218.
51. Barlow C, Dennery PA, Shigenaga MK, et al. Loss of the ataxia–telangiectasia gene product causes oxidative damage in target organs. *Proc Natl Acad Sci USA*. 1999;96(17):9915-9919.
52. Alves MG, Carta CF, de Barros PP, Issa JS, Nunes FD, Almeida JD. Repair genes expression profile of MLH1, MSH2 and ATM in the normal oral mucosa of chronic smokers. *Arch Oral Biol*. 2017;73:60-65. https://doi.org/10.1016/j.archoralbio.2016.09.006
53. Villeneuve NF, Sun Z, Chen W, Zhang DD. Nrf2 and p21 regulate the fine balance between life and death by controlling ROS levels. *Cell Cycle*. 2009;8(20):3255.
54. Ahmad T, Sundar IK, Lerner CA, et al. Impaired mitophagy leads to cigarette smoke stress-induced cellular senescence: implications for chronic obstructive pulmonary disease. *FASEB J*. 2015;29(7):2912-2929.
55. Yao H, Sundar IK, Gorbunova V, Rahman I. P21-PARP-1 pathway is involved in cigarette smoke-induced lung DNA damage and cellular senescence. *PLoS One*. 2013;8(11):e80007.

56. Tsai C-C, Su P-F, Huang Y-F, Yew T-L, Hung S-C. Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Mol Cell*. 2012;47(2):169-182.
57. Niwa H. Molecular mechanism to maintain stem cell renewal of ES cells. *Cell Struct Funct*. 2001;26(3):137-148.
58. Suchánek J, Browne KZ, Nasry SA, et al. Characteristics of human natal stem cells cultured in allogeneic medium. *Braz Dent J*. 2018;29(5):427-434.
59. Assadollahi V, Mohammadi E, Fathi F, et al. Effects of cigarette smoke condensate on proliferation and pluripotency gene expression in mouse embryonic stem cells. *J Cell Biochem*. 2019;120(3):4071-4080.
60. Zeineddine D, Abou Hammoud A, Mortada M, Boeuf H. The Oct4 protein: more than a magic stemness marker. *Am J Stem Cells*. 2014;3(2):74.

## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**How to cite this article:** El-Mouelhy ATM, Nasry SA, Abou El-Dahab O, Sabry D, Fawzy El-Sayed K. In vitro evaluation of the effect of the electronic cigarette aerosol, *Cannabis* smoke, and conventional cigarette smoke on the properties of gingival fibroblasts/gingival mesenchymal stem cells. *J Periodont Res*. 2022;57:104–114. https://doi.org/10.1111/jre.12943