Beni-Suef University Journal of Basic and Applied Sciences

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# The effect of cigarette and e-cigarette smoke on dental pulp stem cells proliferation capacity and differentiation [in vitro study]

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## Abstract

**Background** Mesenchymal stem cells (MSCs) have long been known for their ability to regenerate tissue. Cigarette smoking is one environmental risk factor that may impair the performance of MSCs. Electronic cigarettes have recently become a popular and widely accepted alternative to tobacco cigarettes due to their safety. The present study aims to analyze how smoke extracts of cigarette tobacco and electronic cigarettes affect the capability of dental pulp stem cell (DPSCs) proliferation and osteogenic differentiation. In this study, DPSCs were isolated from healthy impacted third molars of non-smokers, and two smoke extracts were made from tobacco powder and electronic cigarettes. Half maximal inhibitory concentration (IC50) was calculated at two time intervals (14 and 21 days), and its effect on the proliferation and osteogenic differentiation of the DPSCs was assessed.

**Results** The proliferation rate with the calculated IC50 of both smoke extracts was reduced compared to control cells. After 21 days of osteogenic induction, significantly fewer calcium deposits were visible among cells exposed to both smoke extracts. In addition, the expression of alkaline phosphatase and RANKL proteins was significantly reduced in differentiated DPSCs subjected to both smoke extracts.

**Conclusions** DPSCs exposed to both smoke extracts showed decreased cell viability and osteogenic differentiation potentiality compared to control cells. Smoking in any form has a detrimental effect on the proliferation and regenerative capacity of MSCs.

**Keywords** Dental pulp stem cells, Smoke extract, E-cigarettes, Osteogenic differentiation

## 1 Background

Mesenchymal stem cells (MSCs) have received widespread interest in recent decades due to their tremendous capability to repair and tissue regeneration [1]. Since they can self-renew and differentiate into several lineages in tissue engineering and cell therapy, MSCs have traditionally been regarded as valuable cell sources [2]. They are crucial in wound healing, bone remodeling, and tissue regeneration as they produce cytokines and growth factors that promote local cellular dynamics and release proangiogenic, chemotactic, and immunomodulatory factors [3, 4]. Dental pulp stem cells (DPSCs) are dental-derived mesenchymal stem cells originating from the neural crest cells. They are one of the critical sources in dental tissue engineering for regenerating the pulp–dentin complex [5]. DPSCs express mesenchymal cell surface protein markers and have a high proliferation rate. These multipotent cells can differentiate into various cell types [6]. Due to these characteristics, DPSCs are an excellent choice for applications in regenerative medicine [7]. Various environmental risk factors can impair

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stem cell viability and regeneration, including smoking cigarettes. It has been well documented that smoking has a detrimental impact on health, ranging from oral diseases to inflammation, abnormal cell differentiation, and cancer and tissue repair [8]. Moreover, components of smoke may decrease the amount and quality of stem cells found in tissue reservoirs [9]. However, the mechanism by which smoke impairs the regenerative capacity of human stem cells still needs to be fully understood. The adverse consequences of tobacco smoke substances on MSCs involve immediate impacts on the cells and their internal structures and changes in the cell's environment. So, excessive smoke exposure may cause damage to MSC populations of the oral cavity [10]. In addition, it has been shown that smoke-induced changes in the cell cycle might cause decreased cellular proliferation, stimulate oxidative stress, and cause the G0 cell cycle to stop [11]. In addition, high concentrations of reactive oxygen species (ROS) were observed in MSCs subjected to smoke extracts. This may be a factor contributing to the decreased cell differentiation ability [12].

Electronic cigarettes (E-cigarettes or E-cigs) have become a common substitute for traditional tobacco use. Their prevalence is currently 10.6% in Egypt [13]. Although E-cigs have advanced rapidly, the battery and heating coil used to aerosolize an e-liquid solution that includes solvents like propylene glycol and vegetable glycerin to carry nicotine for inhalation are still the essential components of these devices [14]. E-cigs are sometimes marketed as smoking cessation aids and safer replacements for conventional tobacco products; moreover, some studies suggest E-cig vaping has a lower disease burden than combustible cigarette smoking [15]. On the other hand, it has been reported that liquids used in E-cigs are cytotoxic, and heating e-liquids to high temperatures causes the release of cancer-causing carbonyl compounds like acetaldehyde, formaldehyde, and acrolein [16]. Additionally, exposure to E-cigs damages the epithelial barrier lining the airways. It intensifies respiratory syncytial virus infection, the primary cause of acute lower respiratory infection-related hospitalization in high-risk children and adults. This results in a leaky airway [17]. However, studies on the safety of E-cigs have not yet reached a clear consensus, and there is a lack of information on their long-term effects on health. Youth consumers find the E-cig liquid even more attractive due to the availability of various flavoring agents [18]. E-cig consumption raises the risk of nicotine addiction and their desire to smoke tobacco [19]. The dramatic rise in the popularity of E-cigs among adults, especially adolescents, has necessitated urgent research into the health effects of E-cig use [20]. So, this study aimed to explore and compare the effect of conventional cigarette and E-cig smoke extracts on the DPSCs proliferation and osteogenic differentiation capability.

## 2 Methodology

### 2.1 Sample processing and isolation of human DPSCs

A sample of ten ($n=10$) impacted permanent third teeth were extracted and collected. The sample size was determined by applying a G* power version of version 3.1, 3, 3.7. After sterilizing with 3% sodium hypochlorite solution for 2 min, the samples were immersed in (PBS) (pH 7.4) with a cocktail of antibiotics, including penicillin, streptomycin, and antimycotic, and 1% DMSO as a preservation media. A groove was made around the cervical line using a sterilized dental fissure burs with a high-speed handpiece under an abundant water supply. The roots were carefully separated from the tooth with a sterile scalpel and forceps, and then, the blood was removed by washing it in PBS. For bacterial decontamination, the tissue was submerged in a penicillin/streptomycin solution for 1 min before being rinsed in PBS again. A solution including 0.1 U/ml collagenase type II at 37 °C [Sigma-Aldrich] was used to digest the root tissue after it had been cut up into small pieces for 1 h. 1 ml Dulbecco's Modified Eagle's Medium (Gibco, Thermo Scientific, Germany) supplemented with Nutrient Mixture F-12 (DMEM/ F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Scientific) was used to inactivate collagenase (Invitrogen Life Technologies). This solution was centrifuged for 5 min at 1500*g*. After the supernatant was discarded, the pellet was resuspended in 1 ml DMEM/F12 with 10% FBS. Minced dental pulp fragment tissues were seeded into T25 flasks in DMEM/F12 medium containing 10% FBS, 1% penicillin G sodium (10.000 UI), streptomycin (10 mg), amphotericin B (25 g) (PSA) (Invitrogen Life Technologies). Flasks were incubated in a 5% $CO_2$ environment at 37 °C, and non-adherent cells were eliminated by changing the media every 2 days after single cells had adhered to the plastic surface. Plastic adherent cells were multiplied until they reached a confluence of about 80%. During this time, phase-contrast microscopy was employed to examine DPSC cells. Cells from passages up to 3rd passage were used in all experiments; the 3rd passage was utilized for further investigation [21].

### 2.2 Flow cytometric characterization of isolated DPSCs

The isolated DPSCs were characterized using multiparametric flow cytometric analysis. The CD45- FITC, CD105-FITC, and CD90 FITC were used to stain the cells. The cells were suspended in PBS, and the count was adjusted to $1\times10^6$ cells/ml. The cell aspirate was discarded after 10 min of centrifugation at 800xg, and the cell pellet was washed twice with PBS. Each

antibody was applied directly to the cell pellet in 5 µL. Each antibody was employed in a separate tube to minimize significant autofluorescence signals from the larger number of cells. Cells were incubated at 4 °C for 45 min, washed, and resuspended in a binding buffer. Finally, Flow cytometric analysis was performed on the data, and cells were gated depending on their monoclonal antibody staining. The NAVIOS EX 10-color flow cytometer (Beckman Coulter Life Sciences, USA) and the Navios software SM: BE14548 were used to analyze flow cytometry data. The antibodies that stain the DPSCs are CD105 Monoclonal Antibody (MEM-226), FITC, cat no: MA1-19,594. CD90 (Thy-1.2) Monoclonal Antibody (30-H12), FITC, cat no: 11-0903-82 eBioscience™, CD45 Monoclonal Antibody (30-F11), FITC, eBioscience cat no: 11-0451-82, eBioscience™ (Thermo Fisher Scientific, USA).

### 2.3 Preparation of smoke extracts

Two forms of smoke were supplied for preparation: tobacco extract powder (TE-p) and electronic cigarette liquid vape (e-Cig-LV). According to the protocol, a 50-ml polypropylene conical tube with a silicone stopper was filled with 10 ml PBS; then, a 50-ml syringe was connected to the 3-way stopcock attached to the extension tubes. The cigarette was inserted into the 1000-µl pipette tip and attached to the silicone tubing, and the cigarette was lighted while pulling on the syringe plunger. The syringe plunger drained to the 50-ml mark in 10 s with a flow rate of 300 ml/min. In the conical tube, mainstream smoke was currently bubbling via PBS. Finally, the stopcock to the extension tubing was closed, the plunger was pushed back into the syringe, and the stopcock of the extension tubing was opened. The final two steps were repeated 8 to 10 times till the cigarette burns through the filter (typically 10 to 11 times per cigarette). The technique was repeated for a total of five cigarettes; the smoke from five cigarettes bubbles through ten milliliters of PBS. The extract was filtered through a 0.2 µm pore size membrane, yielding a solution that was 100% tobacco extract [22]. Electronic cigarette liquid was purchased at a 3% nicotine concentration. A commercially available bottle of cherry flavor (50% propylene glycol PG/50% vegetable glycerin VG) was available. E-cig devices generate aerosol (vapor) by applying a voltage to the heating coil within the e-liquid and aerosolizing to replicate the smoker's condition. Aerosol particles were trapped on glass fiber filter disks and then extracted in the culture media using a syringe. The extract was filtered through a 0.2-µm pore size membrane to final concentrations [23].

### 2.4 Calculation of IC50 of two smoke extracts (TE-P and e-Cig-LV) on DPSCs

DPSCs isolated from the wisdom teeth' roots were cultured in 96-well culture plates. Cells were cultured in DMEM/F12 supplemented with 10% FBS and 1% of penicillin G sodium (10.000 UI), streptomycin (10 mg), and amphotericin B (25 µg) (PSA). Cells were incubated in an atmosphere of 5% $CO_2$ at 37 °C for 24 h. On the next day, the media was changed, and the adherent cells were treated with serial concentrations of two different smoke extracts, including 100, 1.0, 0.1, 0.01, and 0.001 µM of each extract, named tobacco extract powder (TE-p) and e-cigarette extract liquid vape (e-Cig-LV). Two sets of the treated cells were cultured to be incubated in an atmosphere of 5% $CO_2$ for two time intervals, "14 days and 21 days" [23]. The cell growth and morphology were monitored by an inverted microscope every 48 h. At the end of each time interval, a cell proliferation assay was conducted by MTT assay. The % of viability was determined, representing the cytotoxic effect of serial doses of the smoke extracts. The XY curve was plotted to illustrate the relation between the log dose of inhibitor and the normalized response. The best-fit point was determined by linear regression analysis. Graph pad prism software 9 was used to calculate the IC50 [24]. *Cell proliferation assay* The MTT assay was performed to evaluate the proliferation activity of DPSCs control, and treated cells were seeded in duplicates onto 96-well culture plates. Cells were incubated with 10 µl of a 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) substrate solution (Thermo Fisher Scientific). The reaction was stopped by the addition of 100 µl of solubilizing solution (12 mM HCl, 346 mM SDS, and 5% isobutanol); the proliferation was then assessed, and the absorbance was measured at a wavelength of 450 nm by using a microplate reader [23].

### 2.5 Assessment of the smoke extracts on the proliferation potential of DPSCs

Following the calculation of IC50 for each tested smoke extract, the DPSCs were seeded in a 24-well culture plate 1 day before the experiment. An average of $1 \times 10^5$ DPSCs was seeded in 500 µL of DMEM/F12 containing 10% FBS, 1% of penicillin G sodium (10.000 UI), streptomycin (10 mg), and amphotericin B (25 µg) (PSA). Culture plates were incubated in an atmosphere of 5% $CO_2$ at 37 °C for 24 h. The next day, DPSCs cells were treated with the calculated IC50 of the smoke extracts at 14 days (TE-p: 0.008 and e-Cig-LV: 0.012). For the control cells, the carrier solvent was 0.1% DMSO; cells were incubated with 5% $CO_2$ for 14 days at 37 °C. In addition, DPSCs were co-cultured with the calculated

IC50 at 21 days of smoke extracts; the IC50 of TE-p and e-Cig-LV were 0.012 and 0.009, respectively. The cultured cells were examined microscopically every 48 h to evaluate the cell growth, and the media was changed every 48 h.

### 2.6 Assessment of the smoke extracts on the osteogenic differentiation potential of DPSCs

For osteogenic differentiation, cells were cultured in osteogenic culture media prepared by DMEM/F12 supplemented with 10% FBS, 0.01 μM dexamethasone, 50 μg/mL ascorbic acid, 10 mM sodium β-glycerophosphate, and 10,000 U/mL penicillin, and 10,000 U/mL streptomycin, in 24-well plates 24 h before the experiment. Culture plates were incubated in an atmosphere of 5% $CO_2$ for 24 h at 37 °C. The next day, when cells became adherent, the DPSCs were treated with the calculated IC50 of the smoke extracts at 14 days (TE-p: 0.008 and e-Cig-LV: 0.012). For the control cells, 0.1% DMSO was also used as carrier solvent; cells were then incubated for 14 days at 37 °C under 5% $CO_2$. In addition, DPSCs were co-cultured with the calculated IC50 at 21 days of smoke extracts; TE-p and e-Cig-LV are 0.012 and 0.009, respectively. The cultured cells were microscopically examined every 48 h to evaluate osteoblast differentiation. Images were captured with a LABOMED inverted microscope, USA, and the media was changed every 48 h. The validation of differentiation of DPSCs to osteoblasts was investigated by three tests, including (1) alkaline phosphatase assay, (2) expression of RANKL, and (3) Alizarin staining.

#### *2.6.1 Assessment of alkaline phosphatase (ALP) activity in osteogenic differentiated DPSCs*

At the end of the incubation time, the DPSCs were harvested using 0.25% trypsin EDTA (Gibco, Thermo Scientific). The cells were washed twice with PBS and suspended in DMEM/F12 culture media. The ALP is an essential marker enzyme of the initial phases of osteogenic differentiation. The ALP activity was measured in differentiated DPSCs using an ALP assay kit (Sigma) with p-nitrophenyl phosphate as substrate. In a 96-well microtiter plate, 100 μL of each p-nitrophenol standard and 50 μL of each test sample were added. The AMP-substrate buffer was then applied to each test sample. Following 37 °C incubation, the absorbance of the sample was immediately determined at 405 nm by spectrophotometry (ELx 800) using Bio-Tek instruments (Winooski, Vermont, United States). A standard curve of the absorbance vs. concentration was generated and used to calculate the ALP (U/L) activity.

#### *2.6.2 Immunofluorescence staining of RANKL expression in co-cultured DPSCs*

To assess the inhibitory effect of the smoke extracts on the differentiation of DPSCs to osteoblasts, the DPSCs were cultured in DMEM/F12 media treated with IC50 of two smoke extracts at two time intervals. The cells were cultured for 24 h on coverslips until they became adherent. The next day, the DPSCs were treated with the calculated IC50 of the smoke extracts at 14 days (TE-p: 0.008 and e-Cig-LV: 0.012). For the control cells, 0.1% DMSO was also used as carrier solvent; cells were then incubated for 14 days in an atmosphere of 5% $CO_2$ at 37 °C. In addition, DPSCs were co-cultured with the calculated IC50 at 21 days of smoke extracts; the IC50 of TE-p and e-Cig-LV is 0.012 and 0.009, respectively. The cultured cells were examined microscopically every 48 h to evaluate the cell growth, and the media was changed every 48 h. At the end of each time interval, a polyclonal antibody was used to analyze the expression of RANKL in the cells. The cells were fixed with a 4% concentration of warm formaldehyde. The cells were then immunostained with Rabbit Anti-RANKL, MA5-35120 primary antibodies (Invitrogen; Thermo Fisher Scientific, Hilden; Germany) and incubated overnight at 4 °C. Goat Anti-Rabbit IgG H&L Secondary Antibody-Alexa Flour 488 (Invitrogen; Thermo Fisher Scientific, Hilden) was used to incubate the cells after PBS was used to wash them. The slide was coated with Prolong Gold Antifade Reagent (Abcam, Cambridge, UK) and mounted at room temperature overnight. The samples were tested immediately or kept at 4 °C with light protection for long-term storage. LABOMED fluorescence microscope LX400, cat no: 9126000, was used for the microscopic investigation. The intensity of immunofluorescence staining was scored according to a four-tier system: 0, no staining; 1+, weak; 2+, moderate; and 3+, strong. In brief, the H-score of each sample was calculated as the sum of each intensity (0–3) multiplied by the percentage of positive cells (0–100%). The score ranged from 0 to 300. The median value of the H-score was calculated.

#### *2.6.3 Assessment of calcium deposition in osteogenic differentiated DPSCs*

The cells were seeded on coverslips in 6-well culture plates in different media at 37 °C for 24 h and in an incubator with 5% $CO_2$. The next day, the DPSCs were treated with calculated IC50 of the smoke extracts at 14 days (TE-p: 0.008 and e-Cig-LV: 0.012). For the control cells, a carrier solvent of 0.1% DMSO was used; cells were then incubated at 37 °C for 14 days, with a $CO_2$ concentration

of 5%. In addition, DPSCs were co-cultured with the calculated IC50 at 21 days of smoke extracts; the IC50 of TE-p and e-Cig-LV are 0.012 and 0.009, respectively. Following the incubation period, the culture medium was discarded, and the cells were washed three times with 1×PBS. Subsequently, the cells were fixed for 15 min at room temperature with 4% formaldehyde, followed by a three-time wash with distilled water. 1 mL of 40 mM Alizarin stain (ARS) was added to each coverslip and incubated for 30 min at room temperature with shaking. Finally, the coverslips were washed with distilled water five times and examined under a LABOMED microscope. The amount of calcium deposited in differentiated cells was assessed by calculating the percentage % of positive cells (red stained) and the color intensity, which reflects the intracellular Ca++ deposits.

### 2.7 Statistical analysis

Using GraphPad Prism Software 8.4.2 [San Diego, US], the data collected were coded, tabulated, and displayed on a PC. Data were checked for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Collected data showed parametric distribution. To determine the statistical significance of the difference between the means of more than two study groups, the data were analyzed using the one-way Analysis of Variances (ANOVA) test. The post hoc Tukey HSD test was performed to determine the equality of at least three group means, $p$ value: significance level, $p > 0.05$: non-significant, $p < 0.01$: highly significant, and $p < 0.05$: statistically significant. Mean, standard deviation (± SD), and range are used for parametric numerical data.

### 2.8 Ethics statement

Recruitment and collection of teeth samples: Patients were enrolled from dental clinics at Ahram Canadian University, Egypt. Inclusion criteria for teeth selection were adult, non-smoking donors provided DPSCs of normal, sound, impacted third molars. Before the teeth were extracted, each donor provided informed consent. The Research Ethics Committee of the Faculty of Oral and Dental Medicine, Ahram Canadian University, approved the study, with approval number (IRB) IRB00012891#21. The study's methodology was based on the principles outlined in the Helsinki Declaration.

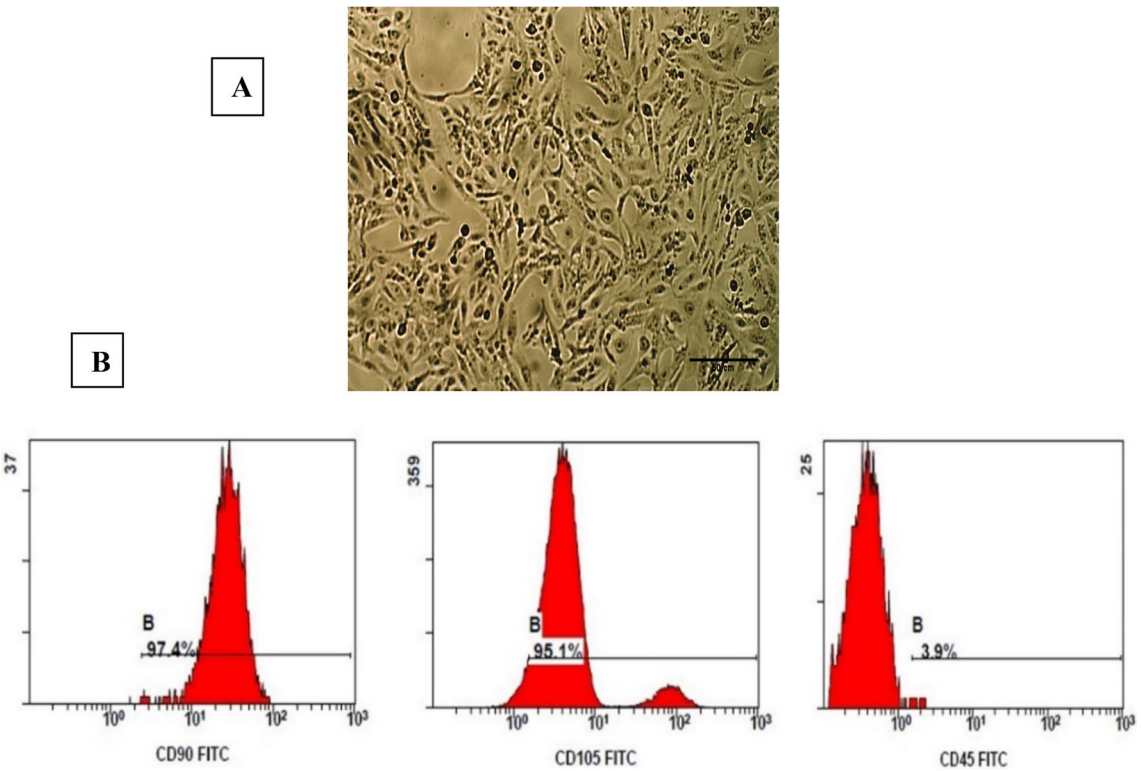


**Fig. 1** **A** Spindle-shaped cultured DPSCs that showed 75% confluence in 14 days. The magnification [10X] (Scale bar = 50 µm) **B** showed representative histograms of the flow cytometric analysis of DPSCs displaying the surface marker expression (CD90, CD105, and CD45). DPSCs positively expressed stem cell markers CD90 and CD105 while negatively expressed CD45

## 3 Results

### 3.1 Morphological assessment and flow cytometric analysis

DPSCs adhered to plastic and multiplied effectively, reaching 75% confluence on day 14 and a consistent spindle-shaped morphology on day 21 for all cells (Fig. 1A).

Multiparametric analysis was used to characterize DPSCs, as shown in Fig. 1B: a representative FCM dot plot displaying the gating technique for MSCs. The isolated DPSCs were stained by multiparametric analysis employing three distinct markers, as detected by flow cytometric pictures (CD90, CD105, and CD45). The findings demonstrated a bright expression of CD90 (97.4%), and 95.1% of cells showed a bright expression of CD105. The results reflect pure MSCs isolation (CD90+/CD105+). The negative expression of CD45 (3.9%) confirms that the pure MSCs are of non-hematopoietic origin.

### 3.2 Comparative analysis between the IC50 of the two smoke extracts

For each tested smoke extract, serial doses including 100, 1.0, 0.1, 0.01, and 0.001 μM were used to treat the isolated DPSCs at 14- and 21-day exposure. Each dose was performed in triplicates, and then, the cell viability (%) was calculated for each after normalization to the average absorbance of the untreated DPSCs cells. Furthermore, the IC50 was calculated for each extract.

From the MTT assay of the DPSCs, the IC50 values were determined: for TE-p at 14 days = 0.008, 95%CI 0.007–0.01 at 21 days IC50 = 0.007, 95%CI 0.005–0.01. For e-Cig-LV at 14 days = IC50 = 0.012, 95%CI 0.009–0.013 and at 21 days IC50 = 0.009, 95%CI 0.007–0.01. There was a high linear regression coefficient between the two variables (normalized response of the DPSCs and log dose of TE-p (Table 1a, Fig. 2A) and e-Cig-LV (Table 1b, Fig. 2B) after 14 and 21 days. The statistical results showed a mild significant difference between the calculated IC50 of TE-p and e-Cig-Lv after 14 days of treatment of DPSCs, the t-value: 3.1, $p = 0.036$. In addition, the IC50 of TE-p significantly differed from e-Cig-LV when treated with the DPSCs for 21 days ($t = 3.4$, $p = 0.027$). Throughout the experiment periods, the proliferative rate of treated DPSCs was lower than that of untreated cells, demonstrating that the two smoke extracts reduced the proliferative ability (Fig. 2C, D).

### 3.3 Analysis of the effect of IC50 of the two smoke extracts on the proliferation potential of DPSCs

The calculated IC50 concentration of the two smoke extracts impairs the proliferation potential of DPSCs at 14 and 21 days. Images for DPSCs cultured in media treated with TE-p and e-Cig-LV smoke extracts showed few scattered colonies with spindle morphology, in contrast with dense colonies with normal spindle morphology observed in untreated control DPSCs (Fig. 2E).

**Table 1** Log dose of (a) TE-p, (b) e-Cig-LV versus the normalized response in DPSCs after 21 days of treatment

| log(inhibitor) versus normalized response | |
|---|---|
| *(a)* | |
| Best-fit values | |
| LogIC50 | −2.2 |
| IC50 | 0.007 |
| 95% CI (confidence interval) | |
| LogIC50 | −2.26 to −1.99 |
| IC50 | −0.005 to −0.01 |
| R squared | 0.95 |
| *(b)* | |
| Best-fit values | |
| LogIC50 | −2.04 |
| IC50 | 0.009 |
| 95% CI (confidence interval) | |
| LogIC50 | −2.14 to −1.94 |
| IC50 | 0.007 to −0.01 |
| R squared | 0.98 |

### 3.4 Osteogenic differentiation potential of DPSCs

The differentiated DPSCs were observed regularly for morphological changes; the cells started to change their spindle shape into round-shaped cells, giving rise to rounded aggregates. The differentiated DPSCs exposed to smoke extracts (TE-p and e-Cig-Lv) showed few scattered colonies with differentiated osteogenic cells at 14 and 21 days, in contrast with dense colonies of cells observed in untreated control DPSCs cultured in DMEM (Fig. 3A).

#### *3.4.1 Comparative analysis of the alkaline phosphatase (ALP) level*

The isolated DPSCs were exposed to calculated IC50 of TP-p and e-Cig-LV for two exposure times, 14 and 21 days. At the end of incubation, the cells were harvested, and the level of ALP enzyme activity was measured. The results were compared between the cellular ALP activity of DPSCs exposed to the two smoke extracts and the un-exposed cells. Results revealed a significant difference between the three tested groups [control untreated DPSCs, TE-p group, and e-Cig-LV group ($F = 19.08$, $p = 0.002$, $R^2 = 0.86$) after 14 days of exposure. The results showed that cells exposed to an IC50 dose of TE-p significantly decreased ALP

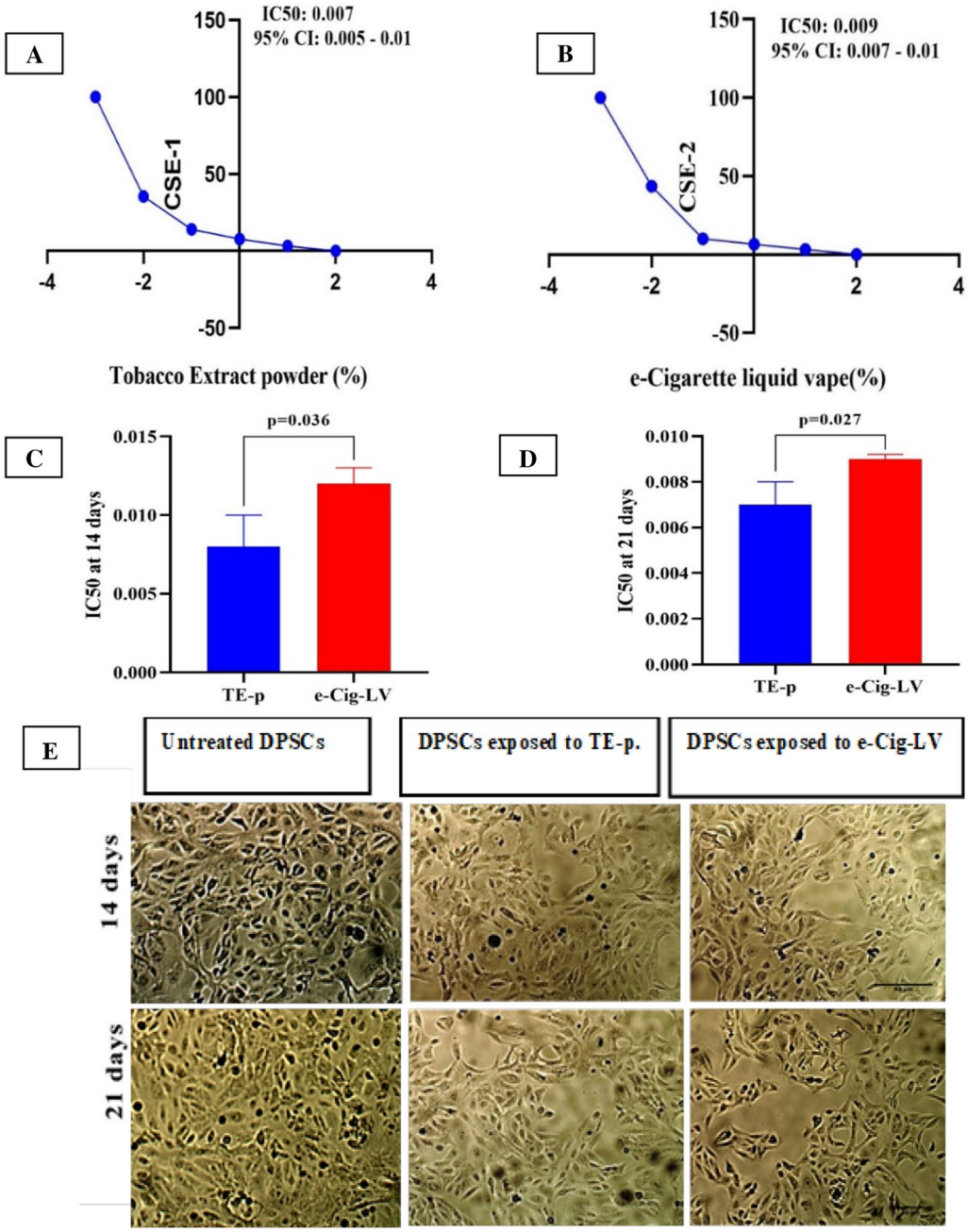


**Fig. 2** **A** Linear regression curves were determined using the MTT assay of the DPSCs treated with the log dose of TE-p. **B** e-Cig-LV versus the normalized response in DPSCs cells after treatment for 21 days. There was a high linear regression coefficient between the response of the control untreated DPSCs and the log dose of TE-p and e-Cig-LV. *IC50* half maximum cytotoxic effect, *CI* confidence interval. **C** The Bar chart showed a mild significant difference between the calculated IC50 of TE-p and e-Cig-LV on DPSCs after 14 days of exposure ($p = 0.036$), $t = 3.09$, and the $dF = 4$. **D** The bar chart showed a mild significant difference between the calculated IC50 of TE-p and e-Cig-LV on DPSCs after 21 days of exposure ($p = 0.027$), $t = 3.4$. Data are presented as mean and SD. **E** DPSCs were cultured for 14 and 21 days in DMEM media treated with TE-p and e-Cig-LV smoke extracts. The cell viability was compared to untreated cells co-cultured in DMEM. Images for DPSCs cultured in media treated with smoke extracts showed few scattered colonies, in contrast with dense colonies of cells observed in control untreated cells. The magnification [10X] (Scale bar = 50 µm)

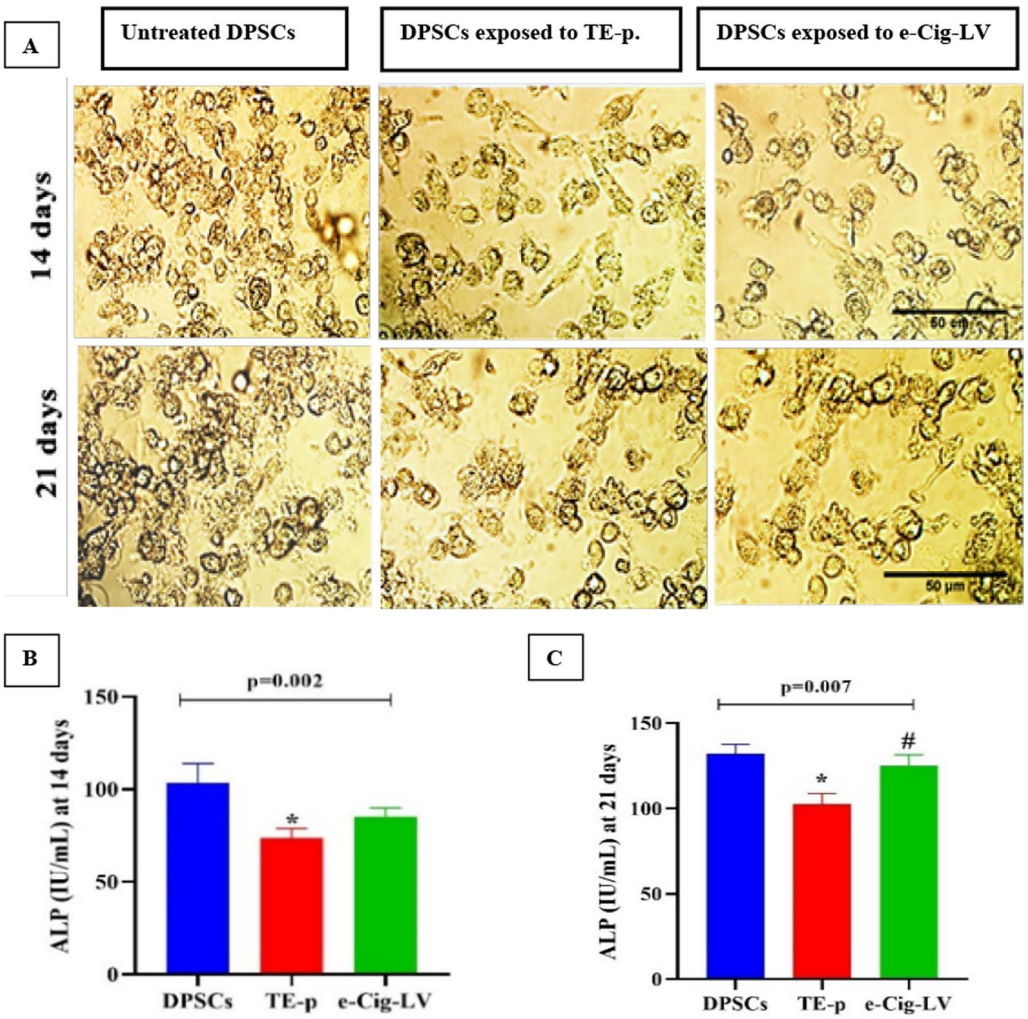


**Fig. 3** **A** DPSCs treated with TE-p and e-Cig-Lv smoke extracts showed few scattered colonies with osteogenic induction at 14 and 21 days; the smoke extracts have a bimodal effect on the proliferation and osteogenic differentiation of DPSCs in contrast to dense colonies of cells observed in untreated control DPSCs cultured in DMEM. The magnification [10X] (Scale bar = 50 µm). **B** Bar chart showed a mild significant difference between the ALP activity of TE-p, e-Cig-LV, and control DPSCs after 14 days of exposure ($p = 0.002$). Data were presented as mean and SD *: significant difference compared to control DPSCs. **C** Bar chart showed a mild significant difference between the ALP activity of TE-p, e-Cig-LV, and DPSCs after 21 days of exposure ($p = 0.007$). Data were presented as mean and SD *: significant difference compared to control DPSCs, #: significant difference compared to DPSCs exposed to TE-p extract

enzyme activity than control cells ($p = 0.006$). However, no significant difference was noticed between the DPSCs exposed to IC50 of e-Cig-LV ($p > 0.05$). Similar results were seen after 21 days of exposure to the two smoke extracts ($F = 12.38$, $p = 0.007$, $R^2 = 0.80$). Meanwhile, a mild significant difference was detected in the ALP activity between the DPSCs exposed to TE-p ($p = 0.005$), and no significant difference was noticed between the ALP activity in DPSCs exposed to IC50 of e-Cig-VL compared to control cells ($p > 0.05$) (Fig. 3B, C).

#### *3.4.2 Comparative analysis of the level of RANKL protein expression*

Immunofluorescence labeling with specific polyclonal antibodies against the RANKL protein was used to evaluate the stained DPSCs. For detection, an Alexa Fluor 488 anti-rabbit IgG secondary antibody was utilized. An immunofluorescence microscope assessed the fluorescence intensity (LABOMED). There is a reduction in the number and intensity (yellow arrows) of RANKL-positive cells in cells co-cultured with two smoke extracts at 14 or 21 days compared to the untreated DPSCs. Discrete cells with a homogeneous faint expression of RANKL

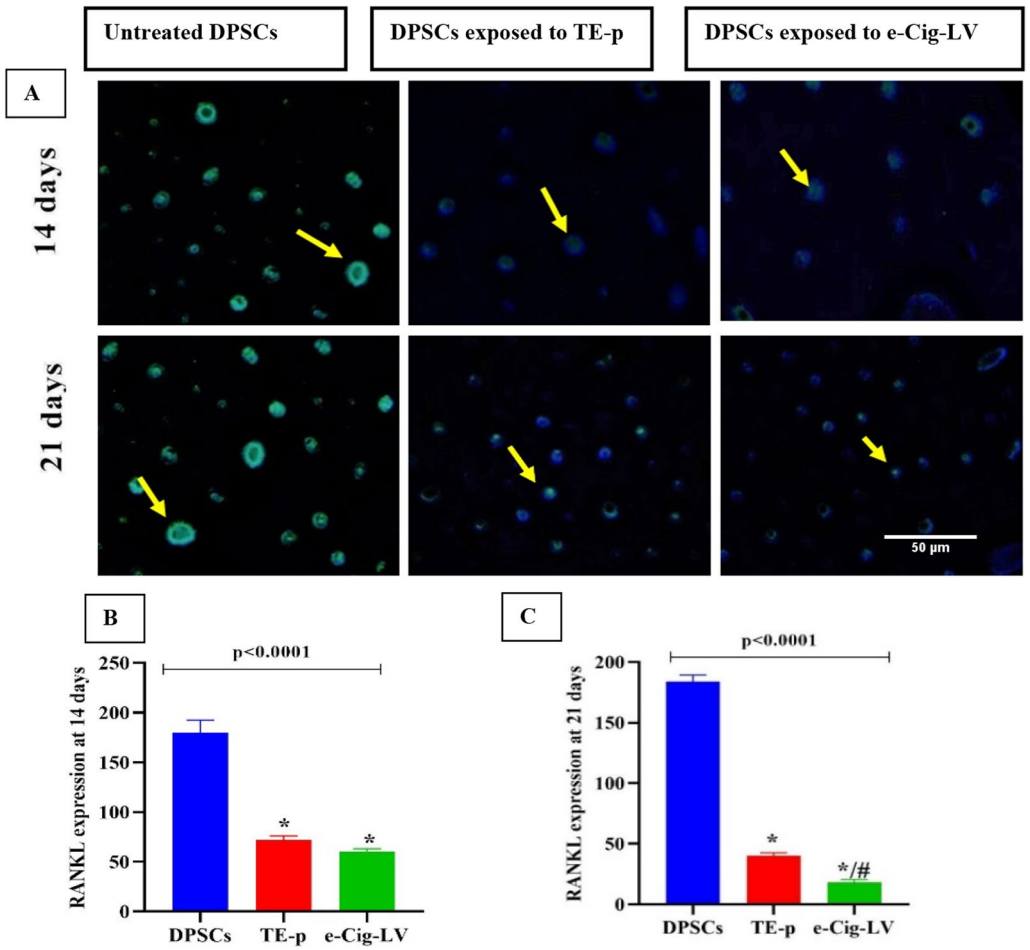


**Fig. 4** **A** Expression of RANKL protein in osteogenic differentiated DPSCs by immunofluorescence at 14 or 21 days showed discrete cells with homogeneous faint weak (1+) expression with dim fluorescence intensity found in DPSCs cultured with smoke extracts with a reduction in both the number and intensity [yellow arrows] compared to the untreated control cells which showed increased cells with strong (3+) expression intensity of RANKL protein. The magnification [10X] (scale bar-50 µm). **B** Bar chart showed a significant difference between the RANKL protein expression of TE-p, e-Cig-LV, and un-exposed DPSCs on DPSCs after 14 days of exposure ($p < 0.0001$). Data were presented as mean and SD; no significant difference existed between the RANKL protein expression of TE-p, e-Cig-LV ($p > 0.05$). **C** Bar chart showed a significant difference between the RANKL protein expression of TE-p, e-Cig-LV, and control DPSCs after 21 days of exposure ($p < 0.0001$). *: significant difference compared to un-exposed DPSCs, #: significant difference compared to DPSCs exposed to TE-p extract

with dim fluorescence intensity (+) were found in DPSCs cultured with two smoke extracts compared to the control untreated cells. The 4′,6-diamidino-2-phenylindole stain (DAPI) fluorescent (blue) was used as the nuclear counterpart stain (Fig. 4A). The statistically obtained results revealed a highly significant difference between the RANKL protein expression (H-score) after exposure to IC50 of both smoke extracts for 14 and 21 days ($p < 0.0001$). As obtained by the post hoc Tukey HSD test, exposure to TE-p or e-Cig-LV significantly differed in the RANKL expression compared to unexposed cells. However, no significant differential effect on RANKL expression on DPSCs was detected between the two smoke extracts ($p > 0.05$) after 14 days of exposure. On the other hand, a significant decrease was detected between the RANKL expression in DPSCs exposed to e-Cig-LV compared to cells exposed to TE-p extract after 21 days ($p < 0.01$). Data are presented in Fig. 4B, C.

#### *3.4.3 Calcium deposition in differentiated DPSCs using Alizarin red stain (ARS)*

The amount of calcium deposited in differentiated cells was evaluated by calculating the % of positive cells (red stained) and the color intensity, which reflected the intracellular Ca++ deposits (blue arrows). However, moderate reductions in the intensity of the stain (orange) color were detected in cells co-cultured in both smoke extracts. The untreated control DPSCs showed an intense bright

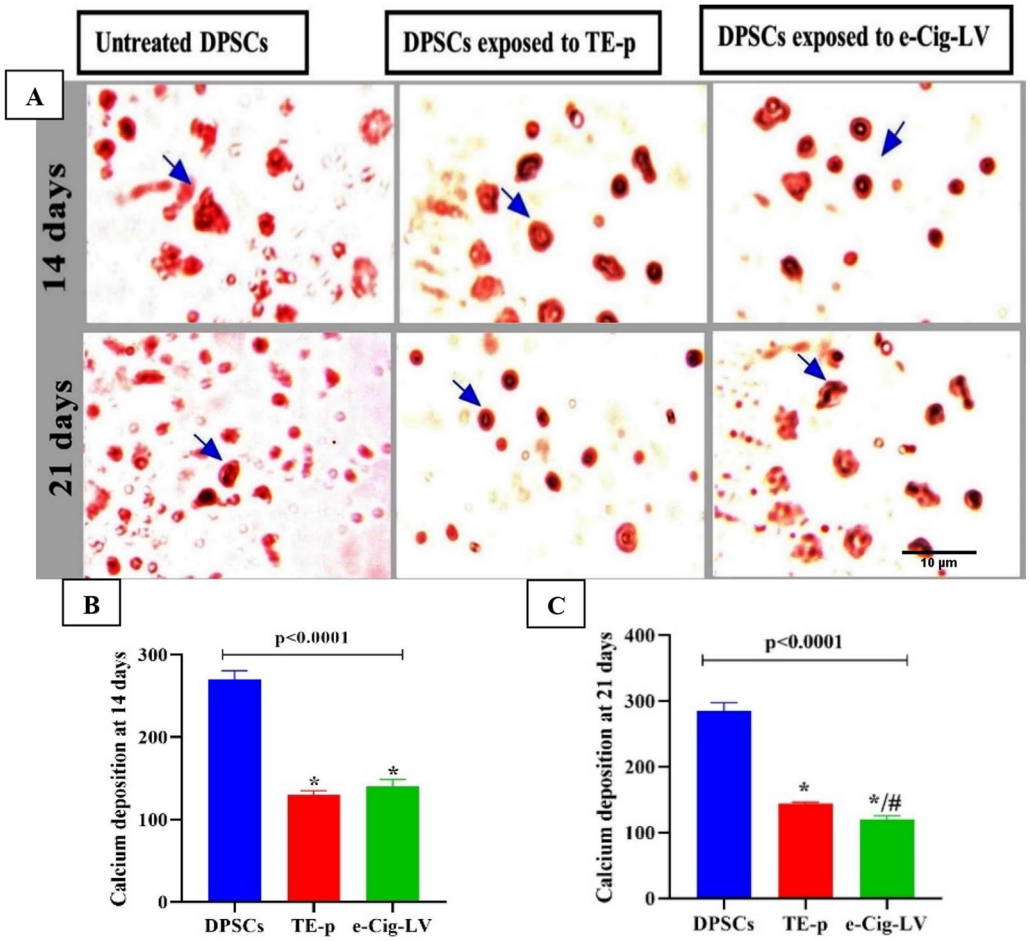


**Fig. 5** **A** Alizarin staining of DPSCs on the untreated and co-cultured (TE-p and e-Cig-LV) groups displayed the amount of calcium deposited in differentiated cells (blue arrows). A moderate reduction in stain intensity (orange color) was detected in DPSCs cultured with smoke extract groups, indicating a reduction in calcium deposition. At the same time, the untreated control DPSCs showed an intense bright stain (red) with an increased percentage of stained cells, indicating an increase in calcium deposition. The magnification (40x) (scale bar = 10 µm). **B** Bar chart showed a significant difference between the calcium deposition of TE-p, e-Cig-LV, and control DPSCs after 14 days of exposure ($p < 0.0001$). **C** Bar chart showed a significant difference between the calcium deposition of TE-p, e-Cig-LV, and control DPSCs after 21 days of exposure ($p < 0.0001$). *: significant difference compared to control DPSCs, #: significant difference compared to DPSCs exposed to TE-p extract

stain (red) with an increased percentage of stained cells. The magnification is 40x (Fig. 5A).

Statistical results showed a significant difference between the control untreated and exposed cells at the two exposure times ($p < 0.0001$). No significant difference was observed in the cellular calcium between cells exposed to IC50 of TE-p and e-Cig-LV ($p > 0.05$); meanwhile, each smoke extract showed a significant reduction in calcium level after 14 days of exposure compared to control cells ($p < 0.001$). On the other hand, a significant decrease in calcium level was associated with cells exposed to e-Cig-LV at 21 days compared to cells exposed to TE-p ($p = 0.02$).

So, in DPSCs treated with both smoke extracts, the Alizarin red staining was markedly decreased. This indicates that both TE-p and e-Cig-LV significantly impair differentiation progress (Fig. 5B, C).

## 4 Discussion

Currently, conventional cigarettes have well-known adverse effects, and there is a lack of comprehensive evidence on the effects of E-cig on human health. Furthermore, several in vitro studies found that E-cig vaping may be less harmful than traditional cigarette smoking. All of that encourages smokers to turn to so-called safer alternatives [25, 26]. This study aimed to explore and compare the effect of TE-p and e-Cig-LV smoke extracts on the DPSCs proliferation and differentiation potential. The results showed that exposure of cells to both smoke extracts altered cell proliferation and differentiation

capacity. These adverse effects were no or less significantly different between the two smoke extracts; these findings agreed with studies that reported the effect of E-cig vapor smoke on the changes in proliferation rate, migration, and apoptosis of different stem cells [23, 27].

The flow cytometric analysis showed that different markers had been used to identify DPSCs. The cells exhibited positive stem cell markers CD90 and CD105 while negatively expressing the hematopoietic cell marker CD45. These findings agreed with the prior studies [28, 29] that demonstrated that DPSCs expressed significant levels of MSC markers such as CD29, CD90, and CD105 while expressing deficient levels of hematopoietic cell markers such as CD14, CD34, and CD45. The current study revealed that cultured DPSCs for 14 and 21 days treated with two smoke extracts (TE-p and e-Cig-LV) showed few scattered colonies, in contrast with dense colonies of cells observed in control untreated DPSCs. The proliferation rate of DPSCs in the control group was significantly higher than that of the treated groups, according to the analysis of cell proliferation. This outcome was in line with another study [30], which found that the non-smoker groups' DPSCs mean absorbance rates were higher than those of the smoker groups ($p=0.0001$). Also, Zeng et al. in vitro study [31] stated that the exposed human cord MSCs to nicotine at concentrations ranging from 0.5 to 1.5mg/ml (3–9 mM) led to dose-dependent reductions in proliferation and increased apoptosis ($p<0.05$) at all concentrations. Furthermore, according to another study, [9] smokers' periodontal ligament stem cells (PDLSC) proliferated at a rate that was 2.53 times lower than that of non-smokers. They illustrated that smoking lowers the quantity of blood circulating $CD34^{+}$ progenitor cells. A decline in cell proliferation rates could be the root of this decreased level.

Furthermore, Yu et al. study [20] findings show that E-cig smoke extract substantially affects cell proliferation and causes a tenfold rise in cell death compared to the control group. They illustrated those dangerous substances in the vapor from E-cigs, and the emissions from heated e-liquid are recognized as DNA-destructive agents. In addition, Jiang et al. study [32] revealed that nicotine exerts an anti-proliferative effect as a result of p53-dependent cell cycle arrest in the G2/M phase mediated by p16 in PDLSCs, indicating that nicotine dramatically induces an increase in the ratio of cells in the G2/M phase and inhibits the cell division, which leads to the reduction in G0/G1 cells ratio, a quiescent phase of the cell cycle. According to Guo and Hecht's review [33], most in vitro investigations showed that E-cig liquid or vapor can cause cytotoxicity, oxidative stress, and DNA damage in various oral cell types or established oral cell lines under different circumstances.

During the healing process, one approach for repair at the injury site is the differentiation of stem cells into tissue-specific cells [34]. It has been studied and known that DPSCs can differentiate into osteoblasts. The current study assessed ALP and RANKL protein expression in differentiated osteoblasts after exposure to two smoke extracts, in addition the assessment of calcium deposition in differentiated DPSCs cells using ARS.

The study results indicated that cells exposed to a dose of IC50 TE-p and e-Cig-LV significantly decreased ALP activity levels compared to control cells, with a statistically significant difference in ALP activity across the three experimental groups. The findings of Zhou et al. [35] corroborated these findings study, which found that $10^{-4}$ mol/L nicotine exposure to PDLSCs significantly suppresses the expression of regulating osteogenic genes such as ALP, BSP, Runx2, and OCN compared to the untreated group with a statistical significance ($p<0.05$). Additionally, the qualitative ALP staining data showed that nicotine significantly decreased PDLSCs' capacity to generate ALP during the early stages of osteoblast differentiation compared to the normal control group. Also, Ng et al. [9] found that smokers' PDLSCs had a significantly lower ALP production than those of non-smokers after 14 days of in vitro osteogenic differentiation.

RANKL is crucial for bone remodeling because it balances the roles of osteoblasts and osteoclasts in the production and resorption of bone. The study findings revealed that DPSCs exposed to TE-p or e-Cig-LV showed a marked significant reduction in RANKL expression compared to control cells. In addition, a significant difference was detected between the RANKL expression in DPSCs exposed to e-Cig-LV and that of cells exposed to TE-p extract. These results agreed with Sreekumar et al. [36] study found that when human MSCs were treated with cigarette smoke extract (CSE), they dramatically inhibited the production of RANKL, which may affect the overall stability of the bone. In addition, CSE may contribute to delayed bone healing by inhibiting early osteogenic differentiation of MSCs and altering the balance of mature osteoblasts and osteoclasts, thereby influencing bone remodeling processes in fractured bones. Also, the findings of Ng et al. [9] study revealed that nicotine exposure significantly reduces the osteogenic differentiation potentials of PDLSC through altered microRNA expression, where microRNAs play an important role in monitoring these functional alterations related to cigarette smoking. Additionally, smoking may have a negative genetic impact on human alveolar bone marrow MSCs by reducing their proliferation and osteogenesis differentiation, according to research by Zhao et al. [37]. Smoking was related to the diminished biofunction of these cells and was linked to bone healing

and a lower incidence of osseointegration and implant success rate.

Moreover, Alizarin red staining detected a significant reduction in calcium levels in exposed DPSCs to the two smoke extracts after 14 days of exposure compared to control cells. Cells treated with e-Cig-LV for 21 days showed significantly decreased calcium levels compared to cells exposed to TE-p. Consistent with the current findings, Ng et al. [9] found that Alizarin red staining also showed a significant decrease in the calcium deposition in smokers' PDLSCs, indicating that osteogenic cell differentiation was markedly suppressed by cigarette smoking. Also, a study by Zhou et al. [35] reported that Alizarin red staining revealed that nicotine significantly inhibited PDLSCs' capacity to form mineralized nodules and explained that nicotine suppressed PDLSC osteogenic differentiation by activating the Alpha7 nicotinic acetylcholine receptor and led to activating the wnt/β-catenin signaling pathway.

Shaito et al. [23] demonstrated that MSCs of bone marrow exposed to smoke extracts from cigarettes and E-cigs significantly decreased ALP activity and Alizarin red staining. These results showed that smoke extracts significantly obstruct differentiation progress beyond ALP expression.

Zhang et al. [38] evaluated that overproducing ROS causes this inhibition of osteogenic differentiation of the smoke-treated bone marrow MSCs group. Furthermore, data collected by different studies [23, 38] suggested that the decrease in osteogenic differentiation may be caused by Cx43, the primary connexin present in MSC and involved directly in osteogenic remodeling. Therefore, the deficiency of Cx43 after being subjected to cigarette and E-cig smoke extracts is linked to the reduction in the potential of osteogenic differentiation and the disruption of cell communications (junctional gap complexes), closely related to the modulation of repair mechanisms. It has been proved by several studies that gap junctions are essential for preventing oxidative stress-induced cell death. As such, exposure to cigarettes and E-cigs may compromise tissue repair [39].

The limitations of the current study include, first, examination of several concentrations of the used extracts, as well as the use of a larger sample size and more prolonged periods of observation, which would have allowed for further investigation into the cell's morphology, characteristics, and differentiation to provide a better understanding of the cells' response to these chemicals. Second, several techniques are used to generate E-cig aerosols in different studies and may affect the results; the gas chromatography-mass spectrometry (GC–MS) test is recommended for future studies to analyze the components of E-cigs after heating. Finally, in vitro studies can never fully replicate the complex biodynamic systems seen in the human body. So, utilizing positive groups in which DPSCs were taken from teeth of both conventional and E. cigs smokers could have increased the credibility of the results.

## 5 Conclusions

In conclusion, the present research indicates that E-cigs may not be as safe as their marketing suggests. Based on the results of our in vitro study, we found that the effects of E-cigs impair the proliferation and differentiation of DPSCs and, subsequently, their therapeutic potential as combustible cigarettes. Further studies are required to establish conclusively the long-term impacts of E-cig usage.

#### Abbreviation

| | |
|---|---|
| MSCs | Mesenchymal stem cells |
| DPSCs | Dental pulp stem cells |
| IC50 | Half maximal inhibitory concentration |
| E-cigs | Electronic cigarettes |
| ROS | Reactive oxygen species |
| FBS | Fetal bovine serum |
| TE-p | Tobacco extract powder |
| e-Cig-LV | Electronic cigarette liquid vape |
| ALP | Alkaline phosphatase |
| RANKL | Receptor activator of nuclear factor kappa-**B** ligand |
| ARS | Alizarin red stain |

#### Acknowledgements

Not applicable.

#### Author contributions

DR performed the research design and interpretation of the data, drafted the manuscript, and revised the manuscript. MA performed the measurements, collected the data, participated in the research design, and revised the manuscript. AN evaluated the statistical analysis and revised the manuscript. All authors read and approved the final manuscript.

#### Funding

Self-funded research.

#### Availability of data and materials

All data generated or analyzed during this study are reported in this published article.

### Declarations

#### Ethics approval and consent to participate

The study protocol and informed consent form were reviewed and approved by the ethical and research committee at the Faculty of Oral and Dental Medicine, Ahram Canadian University, with approval number (IRB)IRB00012891#21. The study was conducted following the principles of the Declaration of Helsinki, and donors signed the informed consent before sample collection.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no conflicts of interest.

Received: 26 October 2023 Accepted: 17 April 2024
Published online: 26 April 2024

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