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Original Article

Impact of Dental Pulp Stem Cell Secretome on Apoptosis, Proliferation and Drug Resistance of Head and Neck Squamous Cell Carcinoma Cell Lines: In vitro study

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Abstract

Background: Dental pulp mesenchymal stem cells' secretome (DPMSCs-sec) possess a variety of regenerative properties as well as therapeutic potential for cancer patients. However, since DPMSCs-sec has been scientifically demonstrated to promote prostate cancer cells, caution is advised. As a result, to ensure the safety of their application, it is crucial to determine whether these pro-carcinogenic criteria affect other cancer types. In this study, we explored the effects of DPMSCs-sec and stimulated DPMSCs-sec on the proliferation, apoptotic potential, and response to the chemotherapeutic drug (Taxotere) of head and neck cancer cells (HNO97). **Methods:** DPMSCs-sec was prepared. The cytotoxic and proapoptotic effects of DPMSCs-sec on HNO97 cells were evaluated using a cell proliferation assay and Annexin V-PI staining. **Results:** DPMSCs-sec did not increase proliferation of HNO97 cells, nor increased resistance to Taxotere. On the contrary, it induced apoptosis. Concomitant exposure of HNO97 cells to DPMSCs-sec and Taxotere showed significant increase to its cytotoxic effects. **Conclusions:** Our *in vitro* results revealed that DPMSCs-sec was not tumorigenic regarding proliferation, apoptosis, and drug resistance. Further animal studies are required to determine its impacts on further cancer-causing characteristics, such as stemness, migration, adhesion, invasion and metastasis.

Keywords: Mesenchymal stem cells, Secretome, Dental pulp stem cells, Conditioned medium, Oral cancer.

Introduction

Squamous cell carcinoma (SCC) of the head and neck is the sixth most common cancer worldwide according to the World Cancer Report. [1] Due to tumor spread and recurrence, oral SCC patients have a 5-year rate of survival with less than 60%. Studies on cancer prevention and novel treatment options should be prioritized to reduce cancer mortality.[2]

Cytokines produced by malignant cells exhibit bidirectional interaction with non-malignant cells within the tumor microenvironment, which play crucial role in tumor development. Consequently, it has a significant impact on cancer progression, and metastatic dissemination. [3]

Mesenchymal stem cells (MSCs), as a member of the tumor microenvironment and a cell with a high paracrine relationship action [4] release a range of bio-active substances that are protective, including cytokines, chemokines, interleukins, growth factors, adhesion molecule, exosomes, hormones, microvesicles, and more. [5]

Secreted molecules, defined as secretome otherwise known as conditioned medium, serves a prominent role in modulating crosstalk communications among cells and surrounding tissues. This piqued researchers' attention to the MSCs secretome, which has the potential to be employed in cell-free therapeutic modalities. Accordingly, a conditioned medium containing bioactive substances have anti-apoptotic, anti-

tumorigenic, anti-scarring, anti-inflammatory, angiogenic or immunomodulatory impacts on the recipient. [6,7]

Dental pulp derived MSC (DPMSCs) are more capable of differentiating, proliferating, and regenerating than other stem cells. These characteristics, taken together, enhance their ex-vivo proliferation and make them a desirable source of MSCs. Since the secretome is the primary effector of a DPMSCs (DPMSCs-sec), its clinical use as a primary rehabilitation tool for enhancing quality of life in the maxillofacial region by repairing damaged tissue, improve wound healing and alleviating any post-treatment fibrosis has been the topic of extensive research. [8,9,10]

Despite their wide range of applications, DPMSCs-sec should be used with caution since they have been associated with prostate cancer cell proliferation.[11] Accordingly, additional investigations on diverse cancer types are essential. [12]

The current research aimed to investigate the impact of DPMSCs-sec on head and neck squamous cell carcinoma cell line (HNO97) regarding their proliferative and apoptotic potential. In addition to detecting if DPMSCs-sec render HNO97 cell line more resistant to the chemotherapeutic agent "Taxotere".

Methods

Isolation and characterization of (DPMSCs)

After obtaining informed consent, healthy premolar teeth extracted for orthodontic reasons from five individuals (ages 18 to 25) served as the source of the DPMSCs. To expose the pulp cavity, the crowns of the extracted teeth were drilled in an aseptic manner. The pulp was extracted using broach. Within the sterile cell culture hood, the pulp was washed three times with phosphate-buffered saline. The pulp tissue was finely chopped into little pieces.

Tissue fragments were then digested for one hour at 37^0C in a solution containing 0.1 U/ml collagenase type II. One ml of Dulbecco's Modified Eagle's Medium supplemented with Nutrient Mixture F-12 (DMEM/F12) was supplemented with 10% heat-inactivated foetal bovine serum (FBS) which was used to inactivate collagenase. This solution was centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded and the pellet was resuspended in 1 ml DMEM/F12 with 10% FBS.

Dental pulp tissues that had been minced and broken down were put into T25 flasks together with 10% FBS, 1% penicillin G sodium (10.000 UI), streptomycin (10 mg), and amphotericin B (25 g) in DMEM/F12 media. Flasks were kept in an atmosphere with 5% CO_2 at 37°C. Once single cells had attached to the plastic surface, non-adherent cells were removed by replacing the media every two days. The plastic adherent cells were cultivated to a confluence of approximately 80%. Phase-contrast microscopy was utilized during this period to analyze DPMSCs, and cells from passages 1 through 5 were utilized in each experimental group. Given that asymmetrically dividing progenitor cells seem to ratify upon death, the use of early passages was done.

The CD90+ve, CD44+ve, CD73+ve and CD45-ve were used to stain the DPMSCs cells. Following data processing for flow cytometric analysis, cells were gated according to the staining of their monoclonal antibodies. [13]

Preparation of DPMSCs-sec

The fourth passage of cultivated DPMSCs was grown in DMEM media until 70% confluence was reached. After that, the cells were separated into two groups: intact DPMSCs and IFNγ-stimulated DPMSCs. The second group's DPMSCs were treated with IFNγ at a dosage of 20ng/ml after the growth media was withdrawn after 48 hours of incubation at 37°C in a 5% CO_2 environment. To remove any leftover media, the cell monolayers were twice washed in phosphate-buffered saline. The cells were then cultured in DMEM for 24 hours, and the DPMSCs-sec and IFNγ-stimulated DPMSCS secretome (stDPMSC-Sec) were separated. After being centrifuged for five minutes at 1800 rpm, the secretome underwent filtering through a 0.22 µm filter and kept at -80^{o}C until needed. An ultraviolet spectrophotometer was used to determine the secretome total protein concentration. [14]

Calculation of IC50 of Taxotere for HNO97 cells

Following the cell proliferation assay, the percentage of viability was identified, signifying the HNO97 cell's reaction to successive dosages of ***Taxotere*** (conc: 20 mg) including**,** 100, 1.0 ,0.1, 0.01 and 0.001µM of the drug, to provide a concentration of 20, 2.0, 0.2, 0.02 µg/mL and 0.002; respectively. The XY curve was plotted to illustrate the relation between the log dose of radiation (inhibitor) versus the normalized response. The best fit point was determined by linear regression analysis. Calculation of half

maximal stimulatory concentration (IC50): The IC50 was calculated **Fig. 1.**

Cell proliferation assay (MTT)

The cell proliferation assessment was performed using the ***Vybrant® MTT Cell Proliferation Assay Kit,*** cat no: M6494 ***(Thermo Fisher, Germany).*** After treating the HNO97 cells with 50% DPMSCs-sec, 50% stDPSCs-Sec, and 0.2µg/ml of Taxotere, 8×103 cells per well were seeded in 96-well culture plates and cultured in DMEM media for 48 hours at 37 °C with 5% CO_2. After that, 100µL of media was withdrawn and replaced with fresh media. Each well received twenty microliters of 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (1 mg/mL). For four hours, the plates were incubated at 37 °C with 5% CO_2. After extracting the MTT solution, 100 µL of sodium dodecyl sulphate and hydrochloric acid were introduced into the wells. Using a spectrophotometer, the optical density at 570 nm was measured to estimate the vitality of the cells.

Apoptosis assessment by flow cytometry using Annexin/PT labelling.

HNO97 cells were treated with 50% DPMSCs-sec, 50% stDPSC-Sec, 0.2µg/ml of Taxotere, combined 50% DPSCs-sec with 0.2µg/ml of Taxotere and combined 50% stDPSC-Sec, with 0.2µg/ml of Taxotere. Additionally, negative control untreated cells were treated with the carrier solvent (0.1% DMSO), and treated cells were retrieved 72 hours after transfection. After trypsinization, the collected cells were twice washed with phosphate-buffered saline before being stained with FITC-Annexin V and Propidium iodide. The Dead Cell Apoptosis Kit (Invitrogen, cat. no. V13242) was utilized to detect apoptotic cells utilizing Annexin V FITC and Propidium iodide for Flow Cytometry. Monoclonal antibodies detected the externalization of phosphatidylserine in apoptotic cells and the presence of dead cells using recombinant annexin V coupled to green, fluorescent FITC dye and propidium iodide, respectively.

Statistical Methods & analysis for data:

The statistically significant difference between the means of more than two research group comparisons was evaluated using the ANOVA test. A post-hoc test was employed to investigate variations across various groups. P-value: level of significance, $p>0.05$: non-significant, $p<0.01$: high significant, $p<0.05$: statistically significant.

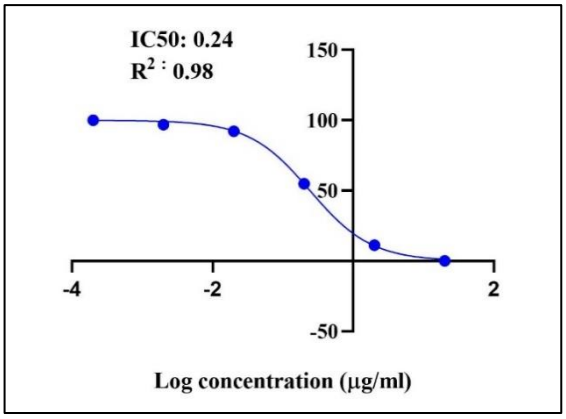

Fig. (1): Linear regression curve illustrating the log dose of Taxotere versus the normalized response in HNO97 cells. IC50: half maximum cytotoxic effect, CI: confidence interval.

Results

Cell viability

Assessment of cytotoxic effect of DPMSCs-sec, stDPMSC-sec and Taxotere in comparison to untreated cells showed that, the percentage of cell viability for HNO97 cells treated with stDPMSC-sec and Taxotere were significantly different from untreated cells (negative control), (p-value of <0.0001). **Fig.2.** However, no significant difference was observed between HNO97 cells treated with 50% DPMSCs-sec, when compared to untreated cells (p=0.177).

The cytotoxic effect of DPMSC-sec and stDPMSC-sec were compared to Taxotere, the obtained results revealed a high significant difference between the studied groups ($p<0.0001$). **Table 1** and **Fig. 2.**

On the other hand, high significant difference was detected between the proliferation potential of HNO97 cells treated with DPMSCs-sec/Taxotere, stDPMSC- sec/Taxotere compared to the untreated cells ($p<0.0001$). in **Table 2** and **Fig.3.**

Apoptotic potential

Regarding dead cells (apoptosis & necrosis), the obtained results revealed a high significant difference between HNO97 cells treated with DPMSCs-sec/Taxotere (apoptosis: 27.5%, necrosis: 19.3%) compared to (12% apoptosis and 14% necrosis) in cells treated with DPMSCs-sec. On the other hand, 21% and 12% of dead cells were detected respectively in HNO97 cells treated with Taxotere.**Fig.4(A)**

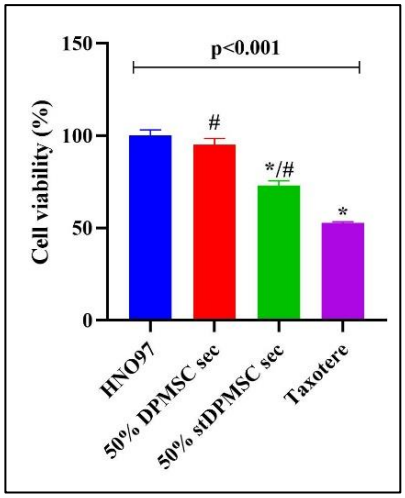

Fig. (2): Bar chart graph showed high significant difference (p<0.0001) between the % of cell viability of HNO97 cells treated with DPMSCs-sec,stDPMSC-sec and Taxotere, *: significant difference compared to HNO97 untreated cells, #: significant difference compared to cells treated with Taxotere.

Table 1. The percentage of HNO97 cell viability cultivated in DPMSCs-sec, stDPMSC in comparison to Taxotere and untreated cells.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
HNO97 vs. 50% DPMS-sec	4.990	-1.965 to 11.94	ns	0.1777
HNO97 vs. 50% stDPMSC-sec	27.14	20.19 to 34.09	****	<0.0001
HNO97 vs. Taxotere	47.36	40.41 to 54.31	****	<0.0001
50% DPMSC-sec vs. 50% stDPMSC-sec	22.15	15.20 to 29.10	****	<0.0001
50% DPMSC-sec vs. Taxotere	42.37	35.42 to 49.32	****	<0.0001
50% stDPMS-sec vs. Taxotere	20.22	13.27 to 27.17	****	<0.0001

CI: confidence interval, ns: non-significant difference, *: mild significant difference (p<0.05), ****<0.0001: marked significant difference (p<0.0001), ***<0.001: moderate significant difference (p<0.001).

The percentage of apoptosis was compared between the four studied groups. The obtained results showed no significant difference between the DPMSCs-sec and Taxotere as regard their potential to induce apoptosis. However, a highly significant difference between the cells treated with combined DPMSC-sec/Taxotere, DPMSC-sec and Taxotere were detected **Table 3** and **Fig. 4(B)**. Conversely, the obtained results showed no significant difference between the percentage of necrotic cells in the same groups (p=0.9). Which implies the DPMSCs-sec potential to enhance apoptosis in HNO97 cells. **Fig.4(C)**

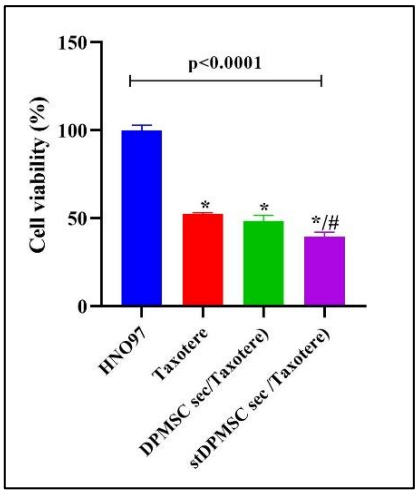

Fig. (3): Bar chart graph showed high significant difference (p<0.0001) between the % of cell viability of HNO97 cells treated with combined DPMSCs-sec /Taxotere, stDPMSC sec/Taxotere, *: significant difference compared to HNO97 untreated cells, #: significant difference compared to cells treated with Taxotere.

Table 2. Percentage of HNO97 cell viability of combined DPMSCs-sec/Taxotere, stDPMSC-sec/Taxotere compared to Taxotere and untreated cells.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Summary	Adjusted P Value
HNO97 vs. DPMSC-sec/Taxotere)	51.52	44.64 to 58.41	****	<0.0001
HNO97 vs. stDPMSC-sec /Taxotere)	60.47	53.59 to 67.36	****	<0.0001
Taxotere vs. DPMSC-sec/Taxotere)	4.163	-2.71 to 11.05	ns	0.2862
Taxotere vs. stDPMSC-sec /Taxotere)	13.11	6.231 to 20.00	**	0.0013
DPMSC-sec/Taxotere) vs. stDPMSC-sec /Taxotere)	8.950	2.068 to 15.83	*	0.0134

CI: confidence interval, ns: non-significant difference, *: mild significant difference (p<0.05), ****<0.0001: marked significant difference (p<0.0001), ***<0.001: moderate significant difference (p<0.001).

Table 3. Percentage of HNO97 apoptotic cells in combined DPMSCs-sec/Taxotere, DPMSCs-sec and Taxotere compared to untreated cells.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Summary	Adjusted P Value
HNO97 vs. Taxotere	-6.300	-10.99 to -1.608	*	0.0112
HNO97 vs. DPMSC-sec	-4.900	-9.592 to -0.208	*	0.0410
HNO97 vs. DPMSC-sec/Taxotere)	-20.40	-25.09 to -15.71	****	<0.0001
Taxotere vs. DPMSC-sec	1.400	-3.292 to 6.092	ns	0.7772
Taxotere vs. DPMSC-sec/Taxotere)	-14.10	-18.79 to -9.408	****	<0.0001
DPMSC-sec vs. DPMSC-sec/Taxotere)	-15.50	-20.19 to -10.81	****	<0.0001

CI: confidence interval, ns: non-significant difference, *: mild significant difference (p<0.05), ****<0.0001: marked significant difference (p<0.0001), ***<0.001: moderate significant difference (p<0.001).

Discussion

The amount of therapeutically promising proteins found in the MSC secretome increases the utility of MSCs in cell-free therapy.[15] Comparing this cutting-edge study to cell-based applications, there are several noteworthy advantages: (A) Since secretome preparation can be generated in large quantities from MSCs populations, it is more economical. (b) Secretome can be kept for a long time without the use of harmful cryopreservatives. (c) Assessment of secretome for safety and efficacy will be easier. [16] (d) MSCs and gastrointestinal epithelial cells can fuse together, creating a cell type that is more likely to develop cancer. While there were no cell-cell interactions when MSCs secretome was used. [17]

Likewise, Do and colleagues [18] demonstrated in their study that when exposed to released substances, adipose tissue-derived MSCs changed into tumor-associated fibroblasts from lung carcinoma cell lines. Also, Shin and coworkers showed similar results in adipose tissue derived MSCs that was crucial in tumorigenesis by promoting adhesion and, proliferation of cancer cells. Therefore, a superior approach is utilizing MSCs secretome rather than MSCs themselves. [19] Conversely, in accordance with a systematic review of the literature published in 2021 [20], which revealed that specific cancer cell lines were susceptible to the pro-carcinogenic effects of the MSCs-secretome, comprising pro-carcinogenic impact of DPMSCs-sec on the prostate cancer lines [11]. Thus, its administration to cancer patients may raise the chance of the condition progressing by enhancing one or more carcinogenic qualities. Considering this, our research examined the paracrine effect of DPMSCs-sec on oral cancer cell line regarding apoptosis, proliferation and drug resistance.

Our results showed that despite the fact that DPMSCs-sec did not promote tumor cell proliferation, it did trigger apoptosis. It also didn't increase resistance to Taxotere. On the contrary, combination of DPMSCs-sec with Taxotere enhanced its proapoptotic action.

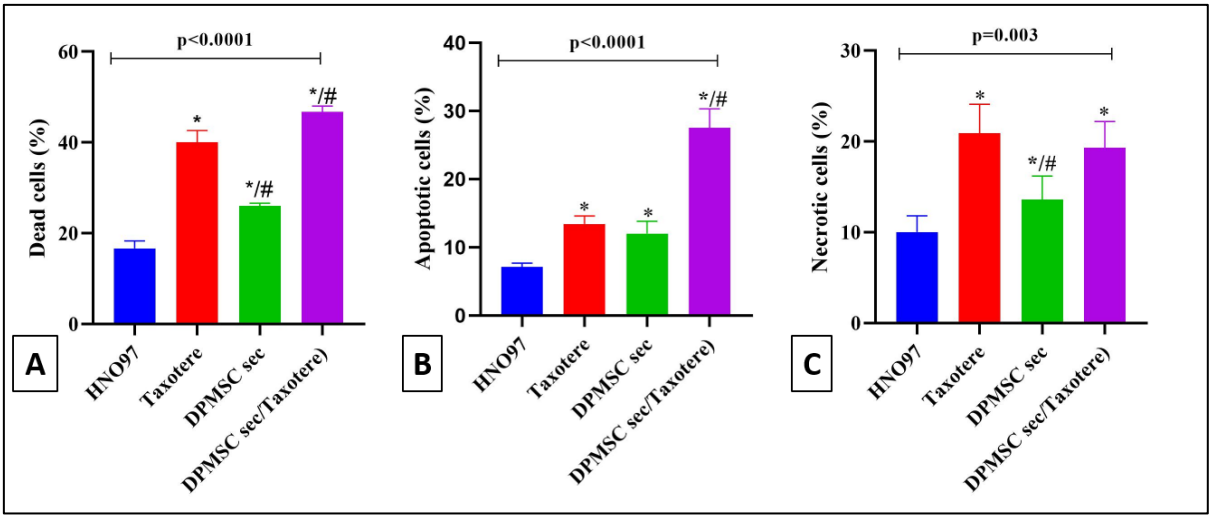

Fig (4): Bar chart graph showed **(A)** high significant difference (p<0.0001) between the % of dead HNO97 cells treated with combined DPMSC-sec/Taxotere, DPMSC-sec and Taxotere *: significant difference compared to HNO97 untreated cells, #: significant difference compared to cells treated with Taxotere. **(B)** high significant difference (p<0.0001) between the % of apoptotic HNO97 cells treated with combined DPMSC-sec /Taxotere, DPMSC-sec and Taxotere *: significant difference compared to HNO97 untreated cells, #: significant difference compared to cells treated with Taxotere. **(C)** no significant difference between the percentage of necrotic cells in cells treated with Taxotere and cells treated with combined DPMSCs-sec/Taxotere.

Another research also done in 2021 on oral cancer [22], stated that: based on a Ki-67 assay, DPMSCs-sec increased proliferation at low doses. While proliferation was suppressed at greater concentrations. They also stated that there were notable pro-carcinogenic effects, such as the potential for invasion, migration, and drug resistance, despite anti-carcinogenic benefits, which included the reduction of cell proliferation and enhancement of apoptosis. Further compatible results were shown by Ataei et al. using MSCs secretome in oral cancer therapy. [23]

Controversial results between different studies were explained by Bhandi, Kahtani, et al. [24]; who declared that cytokine profiles and growth factors of DPMSCs are age dependent. Where they performed an age-based analysis of the DPMSCs-sec. According to their study, pro-inflammatory cytokines increase with age while growth factors deplete. Consequently, the DPMSCs-sec's regeneration potential may be subsided, besides age, the DPMSC's cell culture circumstances have an impact on its secretome profile and capacity for regeneration. Researchers in the forementioned study were able to alter the DPMSCs-sec profile by inducing hypoxia by cobalt-chromium therapy to increase its regeneration capacity.

Numerous research investigated MSCs that have been genetically altered to produce an anticancer effect. [25,26] However, genetic transformation may produce some challenges such as insertional mutagenesis. [27] Consequently, we advocated activating MSCs by including IFNγ in their culture media. IFNγ, an inflammatory cytokine with an anti-tumor defensive response secreted by Th1 cells, functions in a paracrine manner alone or in combination with other cytokines to inhibit tumor cell line proliferation by activating a number of anti-proliferative and tumoricidal pathways. [28]

Hence, evidence proved that IFNγ is proven to modify functions of MSCs.[29] Therefore, we also proposed that it might stimulate the secretome's anticancer activity. To the best of our knowledge, to date, no study has been conducted to use stDPMSCs-sec in oral squamous cell carcinoma. Whereas our study showed that treating oral squamous cell carcinoma cell line with stDPMSCs-sec had significant difference regarding cell viability and apoptosis especially when used in combination with Taxotere compared to untreated cells as well as non-stimulated DPMSCs-sec groups.

Based on these findings, we made a hypothesis that DPMSCs-sec efficacy depends on the amounts of the growth factors and cytokine profile, which in turn depends on the age, type, and culture environment, which could be adjusted to meet our demands. Besides, it also can explain controversy in results as pro and anti-carcinogenic effect of DPMSCs-sec.

Conclusions:

DPMSCs-sec did not increase proliferation of HNO97 cells, nor increased resistance to Taxotere. On the contrary, it induced apoptosis. Concomitant exposure of HNO97 cells to DPMSCs-sec and Taxotere showed significant increase to its cytotoxic effects. These findings can be helpful in evaluating the safety of utilizing DPMSCs-sec in patients with oral cancer. However, additional research is imperative to employ *in-vivo* models for assessing the immunomodulation and tumor microenvironment impacts on DPMSCs-sec and vice versa. Correspondingly, investigate altering the DPMSCs' secretory profile to reduce the production of pro-inflammatory cytokines and hence the pro-carcinogenic effect and augment anti-carcinogenic effect.

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Conflict of Interest:

The authors declare no conflict of interest.

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Ethics:

This study had been accepted by the Ethics Committee of Faculty of Oral and Dental Medicine, Ahram Canadian University (IRB00012891#20).

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