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Anticancerous Effect of Dental Pulp Stem Cell-Conditioned Media on Tongue Squamous Cell Carcinoma Cell Line

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Abstract

Background: The aim of the present study was to evaluate the impact of human dental pulp stem cell-conditioned medium (DPSC-CM) on apoptosis, proliferation, and angiogenesis in tongue squamous cell carcinoma cells. **Methods**: Dental pulp stem cells were derived from sound-extracted third molars and cultured under optimal conditions. After the isolated cells were characterized, the conditioned culture media were collected. HNO97 cells were treated with DPSC-CM for 24 hours or 48 hours and then compared to the control group. Analysis of cell apoptosis and proliferation was conducted using flow cytometry to measure the expression of Annexin and ki67. Cell cycle progression was studied, and the expression of VEGF was analyzed using RT–PCR to assess angiogenesis. **Results:** The number of apoptotic cells increased, with the greatest increase observed in HNO97 cells treated with DPSC-CM for 48 hours. Cell necrosis decreased as the treatment period increased. Flow cytometric analysis of ki67 expression demonstrated a reduction in the quantity of actively dividing cells. HNO97 cells were arrested at the G1/S phase. Rt-PCR evaluation of VEGF indicated a reduction in angiogenesis in HNO97 cells. **Conclusion:** DPSC-CM inhibited the proliferation and angiogenesis of HNO-97 cells, which became more pronounced as the treatment duration increased.

Keywords: Dental pulp stem cell, conditioned media, squamous cell carcinoma, angiogenesis

Introduction

Oral cancer is the 15th most common cause of death worldwide and has one of the greatest incidences. Oral squamous cell carcinoma comprises over 90% of all oral cancers [1]. Some of the risk factors for this condition include tobacco use, alcohol intake, exposure to UV radiation, and infection with viruses such as human papilloma virus [2]. The elevated mortality rate is partially attributed to delayed detection, as well as intricate, incapacitating, and inefficacious treatment methods [3,4]. Treatment modalities such as chemotherapy, radiotherapy, and surgery are associated with several adverse effects, including the possibility of recurrence, the development of secondary tumors, and difficulties in other organs such as the lungs and liver [5].

Mesenchymal stem cells (MSCs) possess regenerative and therapeutic properties and can be derived from several tissue sources, including adipose tissue, bone marrow, and dental tissues. Nevertheless, dental-derived stem cells are being increasingly recognized as a valuable source of mesenchymal stem cells since they are easily obtainable and do not cause ethical problems [6,7].

The therapeutic value of MSCs is not solely derived from their ability to differentiate into various cell lineages for tissue regeneration; it is also attributed to the paracrine factors they produce [8]. These factors that are secreted by MSCs into their immediate surroundings, or the medium in which they are cultured, are referred to as the secretome. The secretome of mesenchymal stem cells, also known as conditioned media (CM), can serve as a substitute for the mesenchymal stem cells themselves. MSCs can adjust and release paracrine factors and proteins in response to their surrounding environment, enabling them to exert a twofold effect on cancer cells. Therefore, they can either promote or inhibit the growth of cancer [9–12].

It has been proposed that the primary therapeutic impact of stem cell-based therapy is not caused by the cells themselves but rather by the factors produced by the cells [12–14]. Consequently,

there has been growing interest in mesenchymal stem cells as potential therapeutic agents. In fact, cell-free therapy is currently more common than cell-based therapy due to its ability to address the adverse effects associated with cell-based therapy, such as interactions with host cells, transmission of viral infections, and other complications[15]. One significant advantage of employing CM as a treatment modality for various disorders is the absence of biological components, which eliminates the chance of developing cancer, unlike other cell types such as induced pluripotent stem cells.

Various studies have characterized the CM of MSCs as a potential therapeutic agent against cancer cells; however, other studies have revealed that it can also function as a protumorigenic agent [16–18].

There is limited research examining the impact of CM administration on different cancer cells, with a particular lack of publications employing DPSC-CM. Thus, to explore novel treatment methods for oral cancer, we conducted a comprehensive analysis of the impact of dental pulp stem cell-conditioned media (DPSC-CM) on the apoptosis, proliferation, and angiogenesis of tongue squamous cell carcinoma cells. We hypothesized that the interaction between DPSC-CM and the microenvironment of cancer cells can lead to the suppression of cancer growth. Flow cytometry was used to quantify the expression of Annexin and the ki67 marker to analyze cellular apoptosis and proliferation. In addition, the cell cycle distribution was investigated, and the expression of vascular endothelial growth factor (VEGF) was analyzed to evaluate angiogenesis. Our results indicated that DPSC-CM inhibited the proliferation and angiogenesis of HNO-97 cells, which became more pronounced as the treatment duration increased.

Materials and Methods

The methods employed were conducted in compliance with the rules and guidelines outlined in the Declaration of Helsinki. All participants provided informed consent. The experimental protocol underwent assessment by the Ethical Committee of the Medical Research of the National Research Centre (Egypt), and was granted approval under the number 20040.

1) Isolation and Characterization of Human Dental Pulp Stem Cells:

Human dental pulp stem cells (DPSC) were collected from sound impacted third molars for extraction according to previous protocols [19]. The immunophenotype of isolated DPSC was analyzed by flow cytometry using anti-human antibodies directed toward the mesenchymal cell markers CD 90 and CD105 and the hematopoietic marker CD45 [20]

2) HNO-97 cell culture:

The human tongue squamous cell carcinoma cell line (HNO-97) was obtained from Nawah-Scientific Research Institute (Al-Mokattam, Cairo, Egypt). Cells were acquired from the American Type Culture Collection (ATCC) in the form of a frozen vial. HNO-97 cells were cultured in complete culture medium in 6-well plates. The cell monolayers were washed with 3 ml of PBS. Trypsin/EDTA solution (2.0 to 3.0 ml) was added to a 6-well plate, and the cells were observed under an inverted microscope until a cell layer was dispersed (usually within 3 to 5 minutes). Then, 6.0 to 8.0 ml of complete growth medium was added to deactivate the action of trypsin. The cell suspension was then transferred to a centrifuge tube with culture medium and cells from step 1 and centrifuged at approximately 125 ×g for 5 min to obtain a cell pellet. Cultures were incubated at 37°C for 24 hours in a CO_2 incubator. [21]

3) Conditioned media preparation (CM)

For the preparation of DPSC-CM, DPSC were seeded in 100 mm cell culture dishes and grown to approximately 80% confluence. The cells were then washed with PBS three times and incubated with freshly prepared serum-free DMEM containing penicillin, streptomycin and amphotericin B for 48 hours at 37°C in 5% CO2. Subsequently, only the supernatant was collected, centrifuged at 4°C at 3,000 × g for 3 minutes, followed by 5 minutes at 1,500 g to remove cell debris, filtered through a 0.2-μm filter to obtain the final CM, and then stored in aliquots at -80°C as DPSC-CM [22].

4) Experimental design and grouping

HNO-97 cells were grouped according to the duration of culture in DPSC-conditioned media (CM). The letter X was used to identify the control group, the letter A was used for HNO-97 cells treated with DPSC CM for 24 hours, and the letter C was used for HNO-97 cells treated with DPSC CM for 48 hours. **Group X:** The control group (untreated HNO-97) was cultured in complete culture medium consisting of DMEM supplemented with 10% FBS in combination with antibiotics and antimycotics. **Group A included** HNO-97 cells treated with DPSC CM for 24 hours. **Group C:** HNO-97 cells treated with DPSC CM for 48 hours

5) Coculture of HNO-97 cells with DPSC-CM

HNO-97 cells were treated with a mixture of complete medium containing DMEM supplemented with 10% FBS, in addition to penicillin, streptomycin, amphotericin and DPSC-conditioned medium (100% concentration) (1:1) (HNO-97 cells: DPSC-CM) in a sterile humidified incubator with 5% CO2 at 37°C for 24 and 48 h [23]

6) Morphological characterization of HNO-97 cells:

For histological assessment of cells from each group, HNO-97 cells were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus, CX41) for morphological assessment of cellular changes after treatment with CM.

7) Assessment of apoptosis, cell cycle progression and proliferation by flow cytometry (FACS):

- **Assessment of apoptosis by the Annexin V-FITC Assay:**

Cells were collected (1-5 x 10^5) by centrifugation. Subsequently, the cells were resuspended in 500 µl of 1X binding buffer. Then, 5 µl of annexin V-FITC and 5 µl of 50 mg/ml propidium iodide (PI) were added. The cells were incubated at room temperature for 5 min in the dark. Annexin V-FITC binding was analyzed using a FITC signal detector, and PI staining was performed with a phycoerythrin emission signal detector; the results are expressed as a dot plot or histogram.

- **Assessment of proliferation by Ki-67 assay:**

70% ethanol was prepared and chilled at -20°C. The cells were prepared and washed 2× with PBS by centrifugation at 350×g for 5 minutes. The supernatant was subsequently removed. The cells were incubated at -20°C for 1 hour. The cells were washed 3× with BioLegend cell staining buffer and then resuspended at a concentration of 0.5-10 × 10/ml. One hundred microliters of cell suspension were mixed with a fluorochrome-conjugated Ki-67 antibody and incubated at room temperature in the dark for 30 minutes. The cells were washed 2× with BioLegend Cell Staining Buffer and then resuspended in 0.5 ml of cell staining buffer. A FITC signal detector was used to analyze Ki67 binding by flow cytometry, and a phycoerythrin emission signal detector was used to analyze PI staining.

- **Cell cycle analysis:**

Cell cycle analysis of HNO-97 cultured in DPSC-CM was performed according to previous protocols [24]. Briefly, cells were harvested in the appropriate manner, washed twice in cold PBS and fixed in 70% ethanol for 30 min at 4°C. The cells were then washed 2× in PBS, followed by careful centrifugation to avoid cell loss when the supernatant was discarded, especially after the ethanol was spun out. Cells were treated with ribonuclease. Then, 50 µl of a 100 µg/ml stock of RNase was added. Then, 200 µl of PI was added (from 50 µg/ml stock solution).

8) Real-time quantitative polymerase chain reaction (qRT–PCR) analysis of VEGF expression:

Real-time quantitative polymerase chain reaction analysis of the differential expression of the VEGF marker was conducted using the following primers: 5'-TTGCCTTGCTGCTCTACCTCCA-3' and 5'GATGGCAGTAGCTGCGCTGATA-3'. Samples were run twice. The data were analyzed with a biosystem with software version 3.1 (StepOne™, USA) using the automatic cycle threshold (Ct) setting. The ΔΔCT method was used to calculate the relative expression (RE) of the genes, with GAPDH serving as the internal control. qRT–PCR experiments were performed in triplicate. The data are presented as the average values ± standard errors of the means.

Results

1) Successful Isolation of dental pulp stem cells (DPSC)

DPSC were successfully isolated and displayed the characteristic morphological appearance of mesenchymal stem cells. Scattered spindle-shaped cells appeared three days after isolation, followed by colony formation at approximately day 5. The cells increased in number and reached 70% confluence after one week of isolation. The cells were subcultured until the third passage **(Figure 1)**.

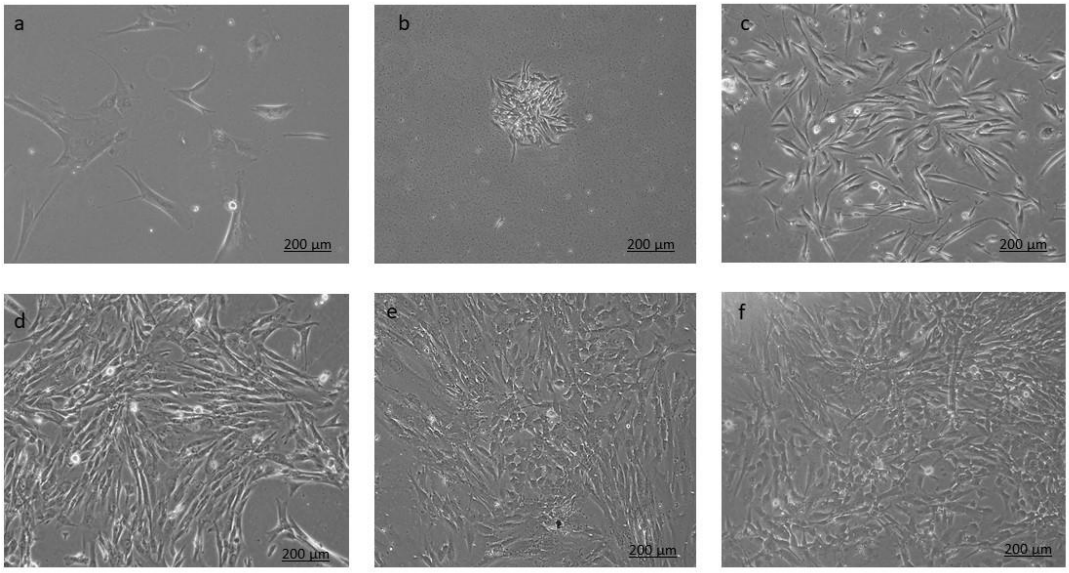

Figure (1) Photomicrograph illustrating the culturing of DPSC. **a)** Single scattered DPSC illustrating stellate and fibroblast-like appearances 3 days after isolation. **b)** Single colonies started to form after five days of culture**. c)** The number of cells started to gradually increase. **d)** Multiple colonies started to form. **e)** These colonies fused together. **f)** Cells reached 70% confluence. **(Magnification 20×) (Leica inverted microscope, Germany).**

2) Isolated DPSC express mesenchymal stem cell-specific markers

FACS analysis of isolated stem cells revealed negative expression of CD45 (4.76%), which confirmed that the cells did not express hematopoietic markers. FAC analysis of isolated stem cells revealed positive CD90 and CD105 expression (86.89% and 78.78%, respectively), which confirmed that the stem cell population was mesenchymal stem cells **(Figure 2).**

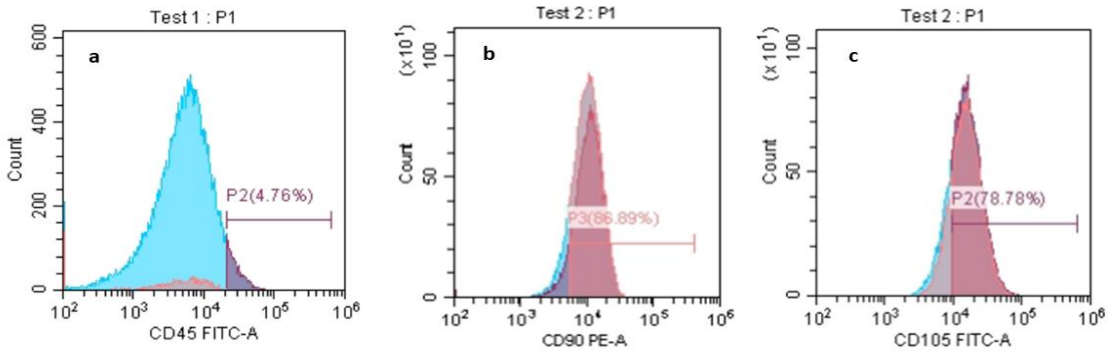

Figure (2) Characterization of isolated DPSC via flow cytometric analysis (FACs). Isolated stem cells showing negative expression of the hematopoietic marker CD45 (a) and positive expression of CD90 (b) and CD105 (c).

3) Changes in the morphology of HNO-97 cells after culture in Dental Pulp Stem Cells-Conditioned Media (DPSC-CM)

Morphological observation of HNO-97 after H&E staining revealed signs of nuclear hyperchromatism, an increased nuclear/cytoplasmic ratio, nuclear and cellular pleomorphism and abnormal mitotic figures in the control group. (**Figure 3a).** However, HNO-97 cells treated with DPSC-conditioned media for 24 hours demonstrated signs of nuclear hyperchromatism, increased nuclear/cytoplasmic ratios, and increased nuclear and cellular pleomorphism. Some cells revealed signs of apoptosis, such as shrunken cells and nuclei, peripheral chromatin condensation and apoptotic bodies. The other cells were necrotic and swollen with ruptured cell membranes. Clear areas free of cells were also evident **(Figure 3b).** Finally, HNO-97 cells treated with DPSC-conditioned media for 48 hours exhibited an increase in the number of apoptotic cells, as indicated by signs of apoptosis, such as cellular and nuclear shrinkage, irregular cell membranes, peripheral chromatin condensation, membrane blebbing and apoptotic body formation. Some necrotic cells were also detected **(Figure 3c).**

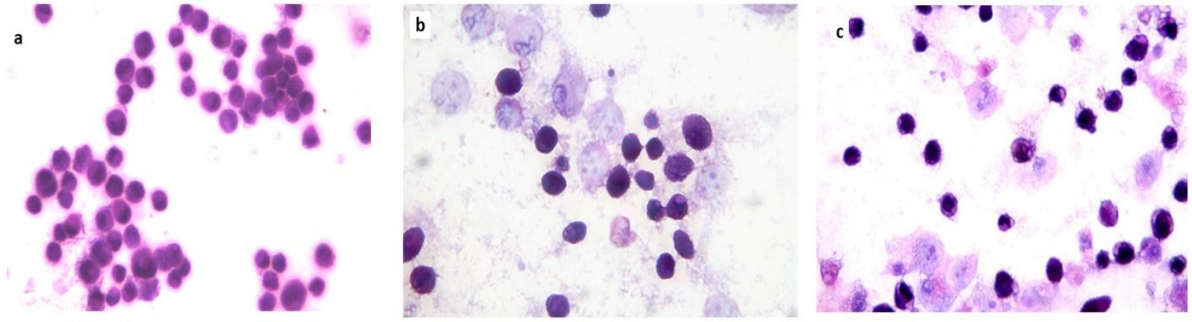

Figure (3) Morphology of HNO-97 by H&E. (a) Photomicrograph of HNO-97 cells in the control group showing increased mitotic activity, an increased nuclear/cytoplasmic ratio, anisocytosis and anisonucleosis, and abnormal mitosis. **(b)** HNO-97 cancer cells in group A showing apoptotic cells with shrunken nuclei.

Necrotic swollen cells with ruptured cell membranes. **(c)** HNO-97 cancer cells in group C showing apoptotic shrunken cells with pyknotic nuclei. Peripheral condensation of chromatin and apoptotic bodies. Membrane blebbing and apoptotic body. Necrotic swollen cells with ruptured cell membranes. Large clear acellular areas are also evident (H and E, 100X oil).

4) Assessment of apoptosis, cell cycle progression and proliferation by flow cytometry (FACS):

a) DPSC-CM demonstrate a cytotoxic effect on HNO-97 cells

The expression of Annexin V in the different studied groups was assessed by flow cytometry to compare the proportion of apoptotic cells in each group. The HNO-97 cancer cells in the control group had the lowest percentage of apoptotic cells, with a total of 3.11% (early: 0.91% and late: 0.13%). In group A, 29.53% (early: 19.51% and late: 6.44%) of the HNO-97 cells were apoptotic. The HNO-97 cancer cells in group C had the greatest percentage of apoptotic cells, with a total of 36.47% (early: 23.81% and late: 9.94%) (**Figure 4).** There was a highly statistically significant difference between the studied groups (P value <0.001). The significantly highest Annexin V expression level was noted in group C (early, late, total), (23.98, 9.94, 36.47), followed by group A (19.55, 6.44, 29.53), while the significantly lowest Annexin V expression level was noted in control group X (0.91, 0.13, 3.11). One-way ANOVA revealed highly significant differences between groups A and X, between groups C and X (P value <0.001) and between groups C and A (P value <0.001) (**Figure 5**).

Moreover, the number of necrotic cells in the different studied groups was measured by flow cytometry to compare the proportion of necrotic cells in each group. HNO-97 cells in the control group had the lowest percentage of necrotic cells (2.07%). Compared with those in group X, the number of necrotic HNO-97 cells in group A increased (3.58%). HNO-97 cells in group C showed a decrease in the number of necrotic cells (2.72%) **(Figure 4).** There was a highly statistically significant difference between the studied groups (P value <0.001). The most significant difference in the number of necrotic cells was noted in group A (3.58), followed by group C, and the least significant difference in the number of necrotic cells was in group X (2.07). One-way ANOVA revealed highly significant differences between groups X and A (P value <0.001), between groups X and C (P value 0.051) and between groups A and C (P value 0.009). **(Figure 5)**

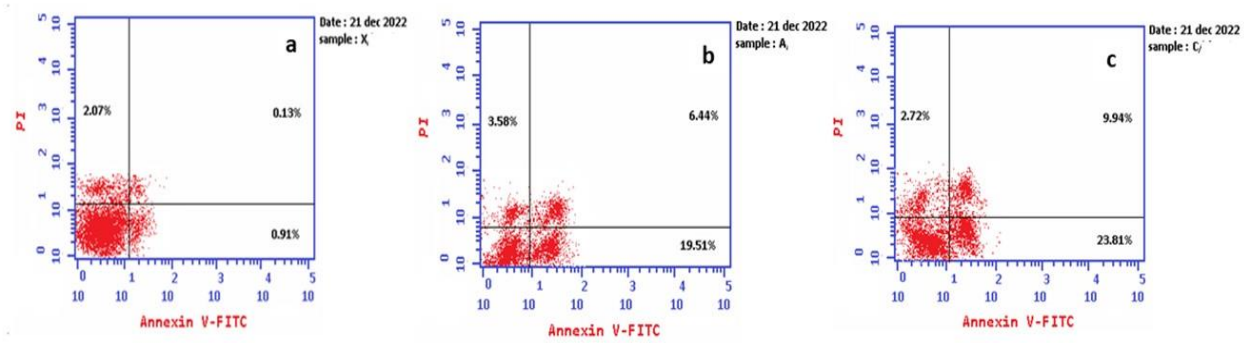

Figure (4) Expression of Annexin V by flow cytometry in the different studied groups. Unviable cells are represented in the Q1 quadrant. Cells that underwent late apoptosis or necrosis are represented in the Q2 quadrant. Viable cells are represented in the Q3 quadrant. Cells undergoing early apoptosis are represented in quadrant Q4. a. Dot plot flow cytometric analysis of apoptotic/necrotic cells in the control group (X) using FITC-annexin V/PI. b. Dot plot flow cytometric analysis of apoptotic/necrotic cells in group A using FITC-annexin V/PI. c. Dot plot flow cytometric analysis of apoptotic/necrotic cells in group C using FITC-annexin V/PI.

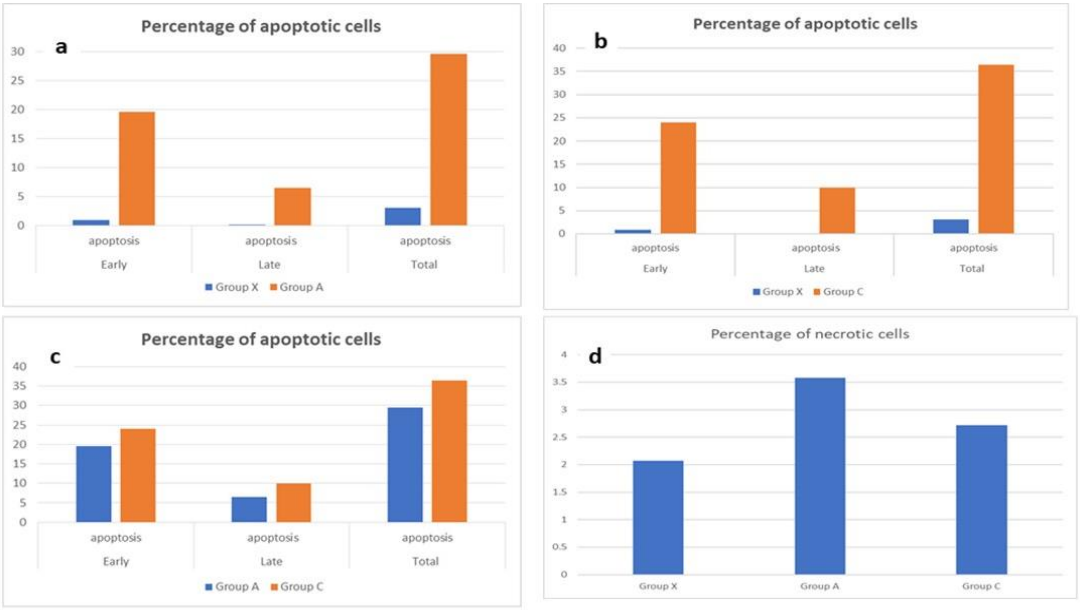

Figure (5) Statistical analysis of the percentages of apoptotic and necrotic cells after treatment with CM for different durations. (a) Bar chart comparing the percentages of apoptotic cells (early, late, and total) in group X and group A. (b) Percentages of apoptotic cells (early, late, and total) in group X and group C. (c) Comparison of the percentages of apoptotic cells (early, late, and total) in group A and group C. d. Bar chart showing the mean percentages of necrotic cells in groups X, A and C.

b) Cell cycle analysis:

Cell cycle analysis revealed that the majority of the cells were in G0/G1 phase, followed by G1/S phase, and a small number of cells were in G2/M phase in both group A and group C, which indicated cell cycle arrest at G1/S phase in both groups A and C at both durations **(Figure 6A).** There was a highly statistically significant difference between the 3 studied groups (P value <0.001) in the G0/G1 phase. The significantly highest cell expression level was noted in group C (61.08), followed by group A (57.06), while the significantly lowest cell expression level was noted in group X (55.17). One-way ANOVA revealed highly significant differences between groups A and X (P value <0.001), between groups C and X (P value <0.001) and between groups A and C (P value <0.001) (**Figure 6B).** There was a highly statistically significant difference between the 3 studied groups (P value 0.001) in the S phase. The significantly highest cell

expression level was noted in group C (33.26), followed by group A (32.77), while the significantly lowest cell expression level was noted in group X (29.05). One-way ANOVA revealed a highly significant difference between group X, group A, and group C (P value 0.001) (**Figure 6B).** There was a highly statistically significant difference between the 3 studied groups (P value <0.001) in the G2/M phase. The significantly highest cell expression level was noted in group X (15.78), followed by group A (10.17), while the significantly lowest cell expression level was noted in group C (5.66). One-way ANOVA revealed a highly significant difference between group X, group A, and group C (P value <0.001) (**Figure 6B).**

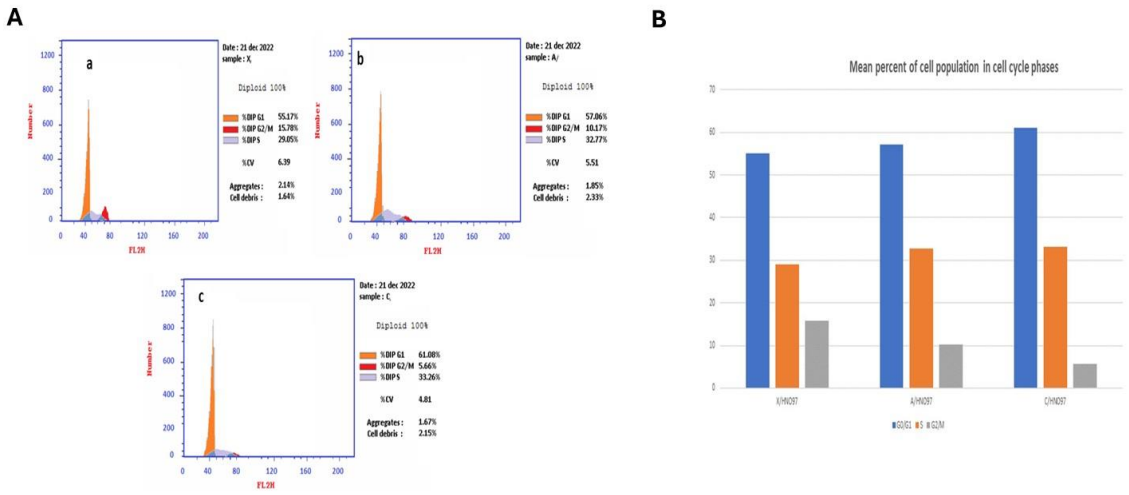

Figure (6A) Cell cycle analysis of HNO-97 after culture in CM for different durations. Chart illustrating different cell cycle phases in group X (a), group A (b) and group C (c). **Figure (6B)**: Bar charts comparing the mean percentages of the cell population in distinct phases of the cell cycle between groups.

c) DPSC-CM inhibits the proliferation of HNO-97 cells

The expression level of Ki67 in the different studied groups was measured by flow cytometry to compare the proportion of proliferative cells in each group. Compared with those in groups A and C, Ki67 expression was greater in HNO-97 cells in the control group (62.41%). Compared with those in group X, Ki67 expression was lower in HNO-97 cancer cells in group A (54.12%).

Compared with those in group C, Ki67 expression was lower in HNO-97 cancer cells in group C (48.33%) (Figure 7). There were highly significant differences among the studied groups (P value <0.001). In the control group, X had the highest expression of the Ki67 marker (62.41), followed by group A (54.12), while group C had the lowest expression (48.33). One-way ANOVA revealed highly significant differences between groups A and X, between groups C and X (P value <0.001) and between group C and group A (P value <0.001) **(Figure 7).**

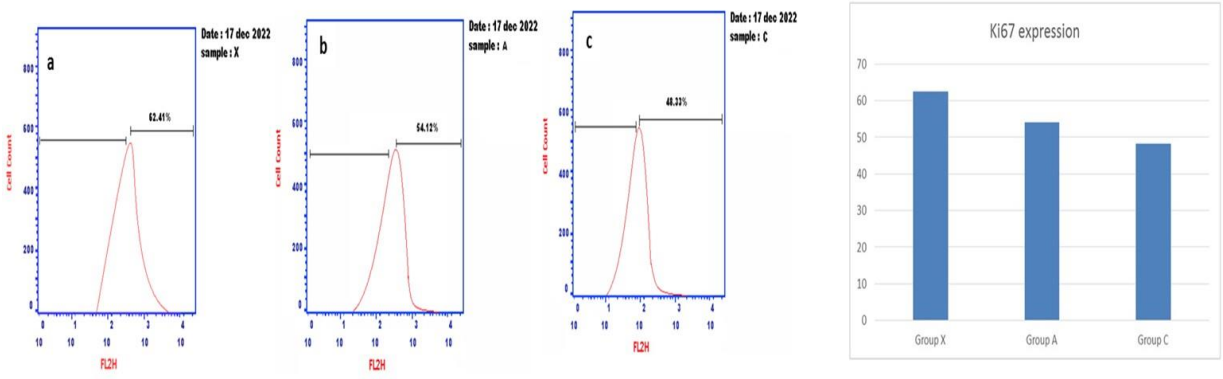

Figure (7) Proliferation assessment via Ki67 expression (left) and statistical analysis of Ki67 expression in the tested groups (right). FAC analysis diagram showing Ki67 marker expression in the control group (a), group A (b) and group C. (c) Bar chart showing the mean expression of Ki67 in groups A and C and control group X (right).

5) DPSC-CM suppress angiogenesis in HNO-97 cells

The VEGF expression level in each group was assessed by RT–PCR. Compared with those in group A and group C, the VEGF marker expression in HNO-97 cells in the control group was greater (fold change of 1). Compared with that in group X, the VEGF expression in HNO-97 cancer cells in group A decreased (fold change of 0.591)**.** HNO-97 cells in group C exhibited the lowest VEGF expression (fold change of 0.272) **(Figure 8).** There were highly significant differences among the studied groups (P value <0.001). The control group X had the highest mean VEGF level (0.998), followed by group A (0.592), while group C had the lowest VEGF expression (0.270). One-way ANOVA revealed highly significant differences in VEGF levels between group A and the control group (X), between group C and the control group (X) (P value <0.001) and between group A and group C (P value <0.001) **(Figure 8).**

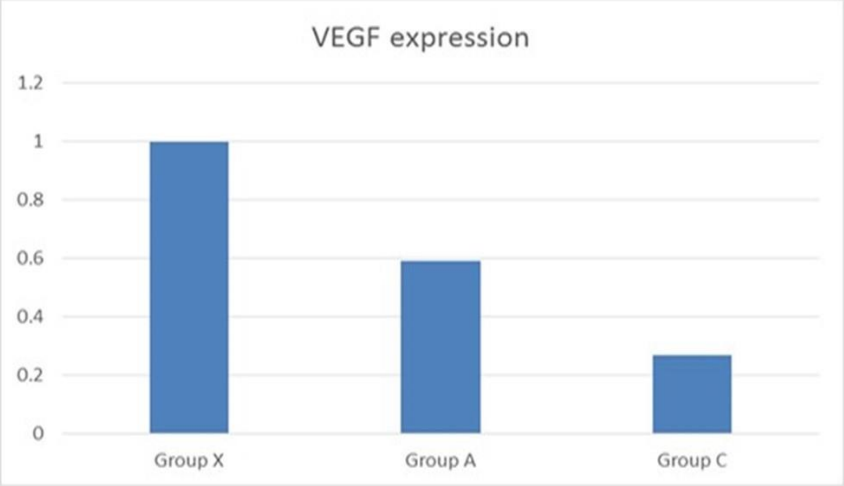

Figure (8) RT–PCR for assessment of angiogenesis via VEGF expression. Bar chart showing the mean expression of VEGF in groups A and C compared to that in the control group (X).

Discussion

Oral squamous cell carcinoma (OSCC) is a type of cancer that has a low chance of survival and a high probability of occurrence. They make up approximately 90% of all oral cancers. Although there have been significant advancements in treatment approaches, the 5-year survival rate remains low [25]. Stem cells are becoming increasingly prominent in the field of regenerative medicine because of their capacity to undergo differentiation into various cell types. However, it has now been determined that the regenerative action of these cells is attributed to their paracrine activity rather than the cells themselves. This paracrine activity can be achieved through utilizing cell-conditioned media [26]. Conditioned medium has various advantages over stem cells, including easier generation, freeze drying, manufacturing, packaging, and transportation. Furthermore, since it lacks cells, there is no requirement to ensure compatibility between the donor and recipient to prevent rejection issues [27]

Thus, this study aimed to investigate the effect of DPSC-CM on tongue squamous cell carcinoma cell lines of different durations and evaluate its ability to induce apoptosis and inhibit proliferation and angiogenesis.

Our study employed flow cytometry to investigate the Annexin V binding capacity of treated cells for the purpose of detecting apoptosis. Apoptosis is an important and crucial process that occurs in healthy and abnormal conditions, and this process results in abnormal or unhealthy cells in the body. Annexin V is an important protein implicated in apoptosis, and dysregulation of its expression and function occurs in several types of cancers; thus, it can be used as a biomarker or indicator during cancer therapy [28]. After administering DPSC-CM to HNO-97 cells for two different time intervals, flow cytometry analysis revealed an increase in the overall number of apoptotic cells as the duration increased, as indicated by Annexin V staining. The highest outcomes were observed in the group that was evaluated for 48 hours. The results also demonstrated that

DSPC-CM caused early apoptosis, with the maximum percentage observed in group C (23.98%) after 48 hours and the lowest percentage in the control group (0.91%). These results were consistent with those of **Gomes et al. (2018), Mirabdollahi et al. (2019), Raj et al. (2021), Nikkhah et al. (2021)** and **Opo et al. (2023)** [29–33]

We further investigated the impact of DPSC-CM on cell necrosis using flow cytometric analysis. Our results indicated an increase in the number of necrotic cells in group A cells that were exposed to DPSC-CM for 24 hours compared to that in the control group. Compared with group A, group C, which was treated with DPSC-CM for 48 hours, exhibited a significant reduction in the number of necrotic cells. This finding indicates that the duration of DPSC-CM treatment led to a decrease in necrosis. The results of our study were the same as those of **Gomes et al. (2018) and Mirabdollahi et al. (2019)**[29,30]

The cell cycle is controlled by a group of proteins that are either positive or negative regulators, such as cyclins, P53, P21 and P16. During cancer, disruption of these regulators leads to abnormal proliferation and cell division [34–37]. In our cell cycle analysis, we found that the greatest number of cells were in the G0/G1 and S phases, while the lowest number were in the G2/M phase across all groups. Additionally, as the duration increased, the number of cells in the G0/G1 and S phases also increased, peaking after 48 hours. Conversely, the number of cells in the G2/M phase decreased with increasing duration, reaching its lowest point at 48 hours. This indicates a decrease in the number of cells undergoing mitosis as time progresses. The results of our study were consistent with those of **Gomes et al. (2018), Nikkhah et al. (2021)** and **Opo et al. (2023)**[29,32,33]. We also analyzed the effects of DPSC-CM on HNO-97 cells using ki67 analysis as an indicator of proliferation. Ki67 is a protein that is expressed at variable levels throughout different cell cycle phases. During cancer, ki67 expression is altered, and ki67 is highly expressed; thus, ki67 is used as a biomarker and indicator for cancer treatment [38,39]. We observed a decrease in both the

proliferation and quantity of cancer cells as the length of treatment increased. When comparing the three groups (X, A, and C), it was found that group C had the lowest average percentage (48.33%), whereas group X had the greatest average percentage (62.41%). This was in line with the findings of **Gomes et al. (2018)**, **Mirabdollahi et al. (2019)**, **Raj et al. (2021)** and **Nikkhah et al. (2021)** [29,31,32]. In contrast, our findings contradict those of Hanyu et al. (2019), who conducted an in vivo study evaluating the impact of DPSC-CM on xenograft squamous cell carcinoma. In their study, DPSC-CM (0.2 ml) were subcutaneously injected into the intraperitoneal cavity of nude mice, and the production of tumor growth factors in the culture medium was measured over time using an enzyme-linked immunosorbent assay. Their results indicated that there was no significant difference in proliferation between the DPSC-CM and control groups [40]. The difference in results can be attributed to the difference in the experimental procedures. Our study was conducted in vitro, while the other study was conducted in vivo. Additionally, in our study, the cancer cell line was cocultured with DPSC-CM, whereas in another study, DPSC-CM was injected into the peritoneal cavity rather than directly into cancer cells [41]. Angiogenesis plays a key role in cancer, and disruption of angiogenesis promotes cancer propagation. VEGF is a key player in angiogenesis and is used as an indicator of the effect of cancer drugs [42,43]. The RT–PCR analysis conducted in the present study demonstrated that the expression of VEGF decreased as the duration of DPSC-CM treatment increased, with the lowest levels observed after 48 hours. The findings of our study were consistent with the results reported by Pakravan et al. (2017) and Gomes et al. (2018) [29,44]. Again, our findings contradicted those of Hnayu et al. (2019). They conducted a study where they implanted oral squamous cell carcinoma cell lines (HSC-2, HSC-3, and OSC-19) subcutaneously in the dorsal part of nude mice. The DPSCs-CM were then injected into the peritoneal cavity of the mice. The results demonstrated an increase in the protein expression of VEGF, indicating an increase in angiogenesis [40]. This study was conducted in vivo, which may be

attributed to the effect of the cancer environment on angiogenesis. In contrast, our investigation was conducted in vitro, which could explain the disparity in the findings [41].

Finally, our findings indicate that DPSC-CM could play a role in promoting apoptosis and inhibiting cell proliferation and angiogenesis in HNO-97 cells, with the effects becoming more pronounced over time. These findings can serve as a foundation for future investigations into the use of DPSC-CM as a potential treatment for oral cancer. **Conclusion** The results of the present research indicate that DPSC-CM exhibits a cytotoxic effect on HNO-97 cells, which becomes more pronounced with longer treatment duration, as evidenced by the occurrence of apoptosis. Moreover, conditioned medium from dental pulp stem cells (DPSC-CM) inhibited the proliferation and angiogenesis of HNO-97 cells. This effect appears to be more pronounced when the length of treatment is prolonged. Additional future research is necessary to investigate the effects of applying varying concentrations of DPSC-CM at different time intervals and on several types of oral cancer cell lines.

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Ethics declarations

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All methods were performed in accordance with the guidelines and regulations of the Declaration of Helsinki. Informed consent was obtained from all participants. The experimental protocol was reviewed by the Ethical Committee of the Medical Research of the National Research Centre (Egypt), and approval was granted under the number 20040.

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Data availability:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.