Original Article

Time- and Concentration-Dependent Effects of the Stem Cells Derived from Human Exfoliated Deciduous Teeth on Osteosarcoma Cells

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Abstract

Background: Stem cells have been proposed to be one of the potent sources for treatment applications. Among diverse types of stem cells, stem cells derived from human exfoliated deciduous teeth (SHEDs) are known as the immature stem cell population, which are easily isolated, fast, and without ethical implications. SHEDs could induce pluripotent stem cells and show differentiation in chondrocytes, adipocytes, osteoblasts, neural cells, hepatocytes, myocytes, odontoblasts, and skin cells.

Materials and Methods: In the current study, we investigated the effects of SHED on osteosarcoma cells (Saos-II) following 3 and 5 days indirect coculture.

Results: Our results showed that indirect coculture of SHED with Saos-II cells could promote or inhibit Saos-II cells' growth in a concentration (the number of SHED vs. Saos-II cells) and time (days of indirect co-culture) dependent manner.

Conclusion: Our findings suggested that, indirectly, SHED co-culture with the Soas-II cells might functions as a tumor suppressor where a higher number of SHEDs are used in the culture in comparison with the one cultured in the absence of/or fewer SHED incubation.

Keywords: Coculture, mesenchymal stem cells, osteosarcoma, stem cells, deciduous teeth, tumor

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Introduction

According to previous studies, mesenchymal stem cells (MSCs) have been introduced as the multipotent stem cells occurring in the bone marrow (BM), which would be crucial for skeletal tissues, such as bones, cartilage, and the fats in the BM, to be rebuilt and repaired.[1-4] Beside BM-derived stem cells, stem cells from human exfoliated deciduous teeth (SHEDs) are another potential source for MSC transplantation and tissues engineering.[5] Numerous investigations have reported SHED to be superior to MSCs from other sources (BM, adipose tissue, and cartilage) for applications in regenerative medicine owing to several reasons including safe accessibility,[6] abundant source of postnatal stem cells,[7,8] higher proliferation rate,[9] and the ability to cause the nondental cell lineages.[10,11] SHDE has also raised a few number of ethical concerns since deciduous teeth (the source of these stem cells) have been frequently regarded as the medical wastes.[5,12]

Since cancer is believed to be a fierce health issue worldwide,[13] and given the aforementioned advantages of SHED, it could be investigated as a candidate for cancer treatment. However, the usage of these amazing stem

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cells for the treatment of various types of cancers has scarcely considered. For instance, we found just one study studies evaluating the effects of SHED on breast cancer cells. Nevertheless, many scientists have investigated the biology,[14] banking,[15] and regenerative ability[16] of SHED. Moreover, previous studies on MSCs and cancer cells have provided encouraging knowledge about both cell groups; for example, MSCs can move to the site of a tumor and surround it[17] or the tissue origin of MSCs may affect the tumor stem cells interaction.[18] These papers have yet failed to prove the effect of MSCs on tumor growth. Despite the efforts made, the function of MSCs in cancer is still contradictory.[19] It has been shown that MSC-derived prostaglandin E2 protected lymphoblastic leukemia cells from the p53 accumulation and subsequent apoptotic cell deaths across the DNA-damage mechanism.[20] Furthermore, previous researchers have observed that osteosarcoma cells can increase migratory capacity of cancer cells.[21] Several investigations have illustrated that MSCs can also suppress tumorigenesis.[13] According to a previous paper, MSCs suppressed A549 lung cancer cells and the human hepatocellular carcinoma cells migration and proliferation by suppressing the Wnt signaling pathways.[22] Moreover, MSCs suppress proliferation or rapid growth in glioblastoma cell[23] and hepatoma cell.[24]

Overall, since the effects of MSCs in cancer remain controversial and the contribution of SHED in the growth progression/regression of cancers have not been experimented, herein, we determined the impacts of SHED on osteosarcoma cells under indirect coculture conditions.

Materials and Methods

Materials

According to the research design, each chemical and medium was taken from Gibco (Grand Island, NY, USA) and Sigma Chemical Co. (St. Louis; MO: USA).

Isolation of stem cells

SHED isolation was performed as proposed by Samiei *et al.* and Alipoue *et al*.[25,26] Briefly, normal exfoliated deciduous teeth were collected from 6- to 9-year-old children. Afterward, the extracted pulp tissue was digested in collagenase Type I (Sigma Aldrich) at 37°C for 1 h. The obtained single-cell suspension was passed through a 40 μm filter, cultured in the Roswell Park Memorial Institute (RPMI) 1640 medium complemented with fetal bovine serum (FBS) (10%), and streptomycin and penicillin (2%) at 37°C with 5% CO_2 atmosphere. Following 70%–90% confluency, we trypsinized these cells and gathered them for further passages. The SHED from passage 4 was used for indirect coculture assay in this study.

Preparation of osteosarcoma cell suspensions

The human osteosarcoma cell lines (Saos-II) were provided by the Iranian Institute of Pasteur (IPI) (Tehran, Iran). Subsequently, we suspended Saos-II cells in the RPMI 1640 Medium complemented with 100 mg/mL streptomycin (Roche) and 10% FBS and Saos-II cell suspension was adjusted at 10^6 Cells/ml.[27]

Indirect co-culture

Figure 1 depicts a brief outline of the steps of the indirect (transwell) coculture assay[26] and a timeline, indicating the order of all the performed experiments under sterile conditions. For indirect assay, ThinCert flat-bottom 24 well cell culture plates (Greiner Bio One, 662640) were used. In these plates, each well is divided into two chambers by a polyethylene terephthalate (pet) capillary pore membrane. The size of pore diameter is 0.4 μm; thus, it allows to exchange biological compound but not cells between two chambers. SHED and Saos-II cells were cultured in upper and lower chambers, respectively, meanwhile separated by a permeable membrane. According to the assay, approximately 2×10^4 or 2×10^5 cells/well of Saos-II cells were seeded in the lower chambers. To evaluate the impacts of SHED on Saos-II cells, we recruited four experimental groups, in which a fixed number of Saos-II cells were cocultured with different SHED concentrations (e.g., 2×10^5 cells/well of Saos-II cells with 0, 2×10^4, 4×10^4, or 2×10^5 cells/well of SHED; as a result, the cocultures with different Saos-II: SHED rates [1:0 (control), 10:1, 5:1, and 1:1] were obtained). Indirect cocultures were performed in triplicate. Saos-II cells in each experimental group were then employed for apoptosis assay, cell viability assay, and cell cycle determination after 3 or 5 days of culture in transwell plates.

Apoptosis assay

The percentage of the apoptotic Saos-II cells 3 and 5 days post indirect coculture was assessed by Annexin V-FITC Apoptosis detection kit (BMS500FI/300CE, eBioscience) by the protocol provided by the manufacturer. Saos-II cells from transwell cocultures were harvested, washed, and suspended in binding buffer (provided by the kit). Subsequently, the cells were incubated with AnnexinV-FITC (5 μL) for 20 min in room temperature. Then, the cells were washed and suspended in binding buffer and were added by propidium iodide (PI) (10 μL) and immediately run with flow cytometry (FACSCalibur flow cytometer [BD]). The data were analyzed by FlowJo software version 7.6.1. (Tree Star, inc). In this method, FITC-Annexin adheres to apoptotic cells and makes them green-fluorescent; necrotic cells take up PI and stain orange-fluorescent. The cells that simultaneously adhere to FITC-Annexin and take up PI are late apoptotic. These experiments were performed in triplicates.

3-(4,5-Dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide assays

In this section, we carried out 3-(4,5-dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide (MTT) assay to determine the effect of different SHED concentrations on Saos-II cells viability after 3 and 5 days from indirect coculture according to the previously reported instruction. Seeding the Saos-II cells was done into 24 well plates (2×10^4 cells/well). In addition, dissolution of 5 mg of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide was performed in 1 ml of PBS. After

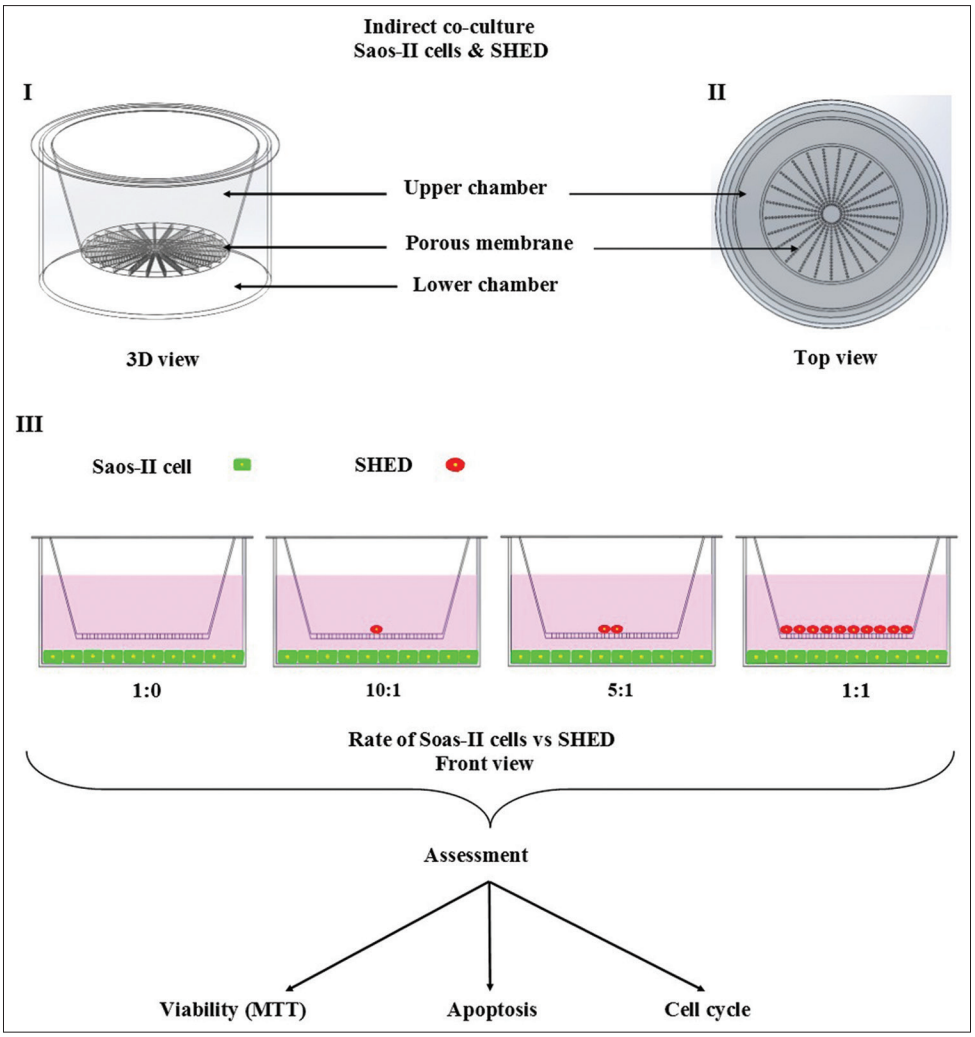

Figure 1: Schematic representation of different stem cells derived from human exfoliated deciduous teeth concentrations and osteosarcoma (Saos-II) cell lines cocultured for 3 and 5 days. (I) Three dimensional, (II) top, and (III) lateral views of coculture system

washing the cells with PBS, we poured the stock solution (40 µl) into the culture media (400 µl). Incubation of these plates was performed at a temperature of 37°C for 4 h, and aspiration of the medium was performed. Afterward, we poured 400 µl of dimethyl sulfoxide for extracting the MTT formazan (2 h). A Microplate reader (Hiperion MPR 4+, Germany) was utilized for detecting the absorption of all the wells at the wave length of 540 nm. In addition, equation: % cell viability = (treated cell OD − treated blank OD)/(untreated cell OD − untreated blank OD) ×100 was used for determining the percentage of the cells viability.[28]

Cell cycle analysis

Cell cycle distributions of Saos-II cells were analyzed through flow cytometry 5 days post indirect coculture. For this purpose, after harvesting cell and washing once with PBS, a monodispersed cell suspension of approximately 2 × 10^6 cells/ml was prepared by aspirating several times. Then, the cells were fixed by ice-cold 70% ethanol (4.5 mL to every 0.5 mL of the cell suspension) and incubated for at least 2 h in 4°C. Centrifugation of the fixed cells was also done at 3000 rpm for 5 min at a temperature of 4°C, and they were washed again with PBS. Finally, a solution of 10 µg/ml PI (sigma: P4170) with 100 µg/mL DNase-free RNase A and 0.1% of triton X-100 in PBS was employed for staining the nuclei. After 10 min incubation at 37°C, the solutions were passed through 30 µm filters and transferred to flow cytometry tubes. Following the assessment of the cells with flow cytometry, the cells' distribution in various stages of the cell-cycle were analyzed through FlowJo software softwarethe.[29]

Results

Apoptosis assay

Effects of indirect coculture of different stem cells derived from human exfoliated deciduous teeth concentration on Saos-II cells apoptosis after 3 days

As shown in Figure 2, flow cytometry results indicated that the highest rates of early (78.1%) and late (4.19%) apoptosis after 3 days indirect coculture were observed in Saos-II: SHED ratio 5:1 group compared to the other groups. In addition, the

percentage of early apoptosis of Saos-II cells in 1:1 group increased (56.7%) – approximately twice – compared to that of the control (1:0) group (25.7%).

Effects of indirect coculture of different stem cells derived from human exfoliated deciduous teeth concentrations on Saos-II cells apoptosis after 5 days

According to the results, 5 days post indirect coculture, in Saos-II: SHED ratio 5:1 group, the percentage of viable cells (68.4%) decreased and the highest rate of necrosis (26.5%) was observed compared to the other groups. Moreover, in 10:1 group, indirect coculture after 5 days reduced the necrotic cells (10.1%) compared with the control group (21.5%) [Figure 3]. The indirect coculture had no effects on the total apoptotic cells in 1:1 group after 5 days although the pattern of apoptosis was different.

Effects of indirect co-culture of different stem cells derived from human exfoliated deciduous teeth concentrations on Saos-II cells apoptosis after 3 days versus those after 5 days

At 1:0 ratio of Saos-II: SHED, the indirect coculture after 5 days showed decreased early apoptosis (2.39%) but increased necrosis (21.5%) compared to day 3 (25.7% and 0.34%, respectively) [Figures 2 and 3]. In 5:1 group, after 5 days, less early (2.98%) and late apoptosis (2.17%) as well as more necrosis (26.5%) were observed compared to those on day 3 (78.1%, 4.19% and 0.87%, respectively). In 1:1 group, after 5 days, early apoptosis decreased (9.82%), whereas late apoptosis (2.04%) and necrosis (8.48%) increased compared to those on day 3 (56.7%, 0.46, and 0.38%, respectively).

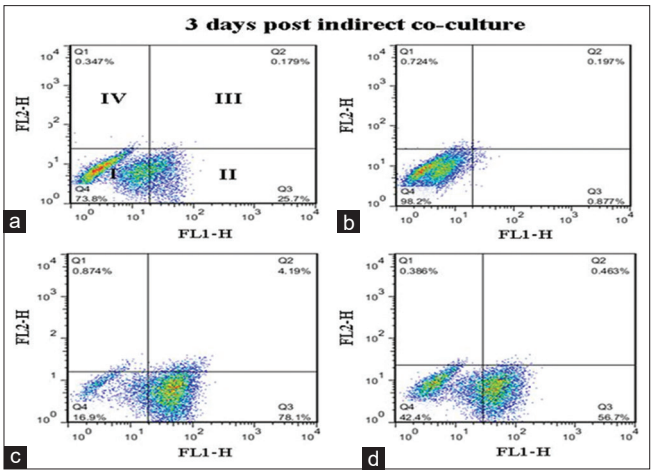

Figure 2: Flow cytometric analyses representing the effect of indirect coculture of different stem cells derived from human exfoliated deciduous teeth concentrations on the percentage of apoptotic osteosarcoma (Saos-II) cells after 3 days. (a-d) Saos-II cells: stem cells derived from human exfoliated deciduous teeth 1:0 (a), 10:1 (b), 5:1 (c), and 1:1 (d) ratios. Quadrant (I-IV): The viable control cells were negative for PI and annexin-V staining (i), apoptotic become cells green through annexin-V binding whereas are negative for PI staining (II) late apoptotic cells that are double annexin-V and PI positive (III), necrotic cells, which are annexin-V negative, and PI positive stained (IV)

3-(4,5-Dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide assay

Effects of indirect coculture of different stem cells derived from human exfoliated deciduous teeth concentrations on Saos-II cells viability after 3 days

The MTT results showed that indirect coculture of SHED with Saos-II cells after 3 days could significantly decrease the proliferation and survival rate of 5:1 (57.52%) group compared to the other groups [Figure 4]. The total Saos-II cells yield rate also showed no significant differences between group 1:0 and 10:1 (100% and 99.95%); however, they are higher in viability than 1:1 group (83.49%).

Effects of indirect coculture of different stem cells derived from human exfoliated deciduous teeth concentrations on Saos-II cells viability after 5 days

As illustrated in Figure 5, when Saos-II and SHED were indirectly cocultured for 5 days in 5:1 group, it was observed that viable Saos-II cells (79%) were significantly fewer than those of the other groups.

Effects of indirect co-culture of different stem cells derived from human exfoliated deciduous teeth concentrations on Saos-II cells viability after 3 days versus 5 days

Figure 6 represents the overall viability rate of Saos-II cells assessed at two different times after indirect coculture with different concentrations of SHED. Accordingly, we did not observe any differences between two time periods of the

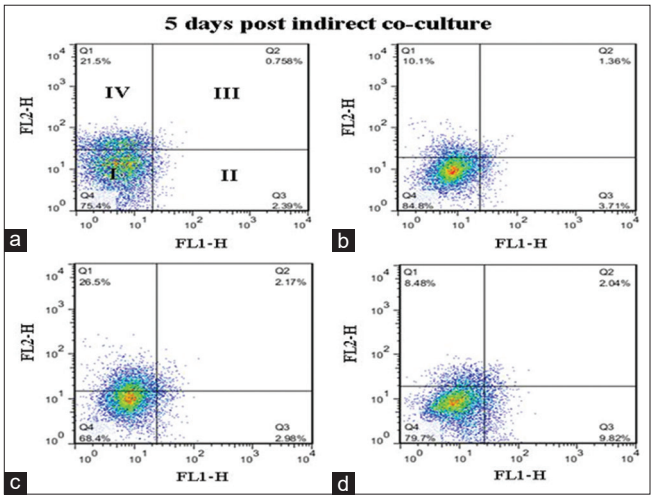

Figure 3: Flow cytometric analyses representing the effect of indirect coculture of different stem cells derived from human exfoliated deciduous teeth concentrations on the percentage of the apoptotic osteosarcoma (Saos-II) cells after 5 days. (a-d) Saos-II cells: stem cells derived from human exfoliated deciduous teeth 1:0 (a), 10:1 (b), 5:1 (c), and 1:1 (d) ratios. Quadrant (I-IV): The viable control cells have been shown to be negative for PI staining and annexin-V binding (i), apoptotic cells become green through annexin-V binding, whereas are negative for PI staining (II), the late apoptotic cells that are double PI and annexin-V positive (III), necrotic cells, which are PI positive but negative for annexin-V binding (IV)

indirect coculture on the proportions of viable Saos-II cells in 10:1 (99.95% ± 3.1% and 93.55% ± 1.6% for 3 and 5 days, respectively) and 1:1 (83.49% ± 0.8% and 90.6% ± 2.6% for 3 and 5 days, respectively) groups. In 5:1 group, however, we observed a obvious difference between the 3 and 5 days of indirect coculture regarding the viability of Saos-II cells, namely, the impact of indirect coculture could be seen more evidently where was carried out for 3 days (57.52% ± 1.8%) compared to 5 days (79.0% ± 4.0%).

Cell cycle analysis

Cell cycle analysis showed that S phase cell distributions were similar between the groups, but the cell population in G2/M phase significantly increased in 5:1 group compared with that in 10:1 and 1:1 groups following indirect coculture of Saos-II cells with SHED after 5 days [Figure 7]. These results indicated that coculture of Saos-II cells with SHED at 5:1 ratio statistically enhanced in the G2/M phase cells, then declined in G0/G1 phase cells significantly.

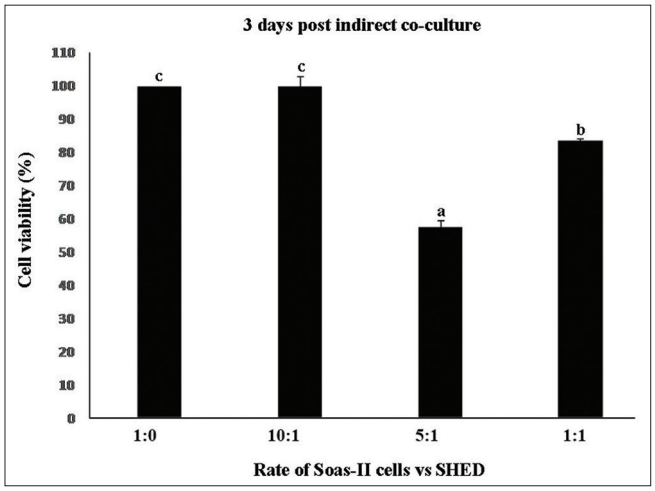

Figure 4: Comparison of 3-(4,5-dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide assay between four groups with different cell ratios of Saos-II cells to stem cells derived from human exfoliated deciduous teeth (a fixed number of Saos-II cells with different number of stem cells derived from human exfoliated deciduous teeth) after 3 days of indirect coculture. Different letters above columns show statistically significant differences among the groups. In the other word, columns with different letters vary significantly (Data are presented as mean ± SD, a vs. b vs. c $P < 0.05$)

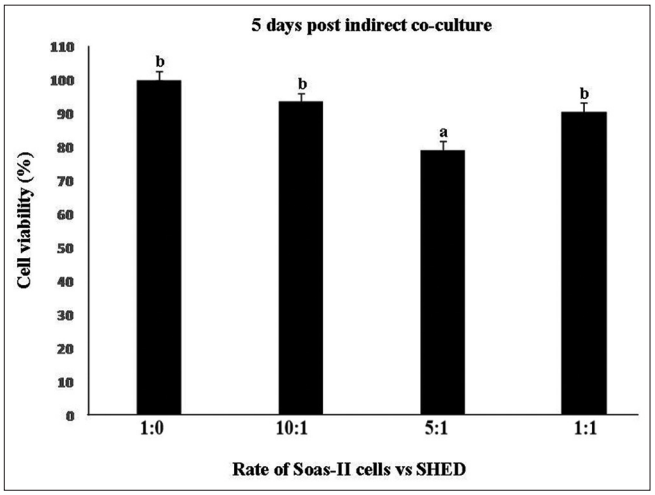

Figure 5: Comparison of 3-(4,5-dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide assay between four groups with different cell ratios of Saos-II cells to stem cells derived from human exfoliated deciduous teeth (a fixed number of Saos-II cells with different number of stem cells derived from human exfoliated deciduous teeth) after 5 days of indirect coculture. Different letters above columns show statistically significant differences among the groups. In the other word, columns with different letters vary significantly (Data are presented as mean ± SD, a vs. b $P < 0.05$)

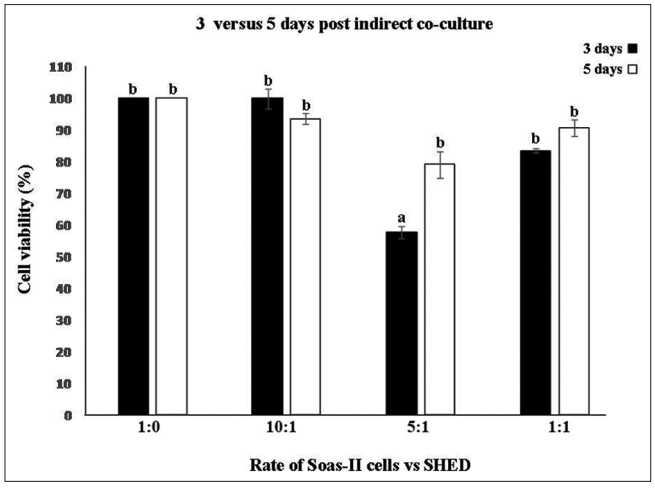

Figure 6: Impact of the post indirect coculture intervals on viability of osteosarcoma (Saos-II) cells indirectly cocultured with different concentration of stem cells derived from human exfoliated deciduous teeth for 3 and 5 days. Different letters above columns show statistically significant differences among the groups. In the other word, columns with different letters vary significantly (Data are presented as mean ± SD, a vs. b $P < 0.05$)

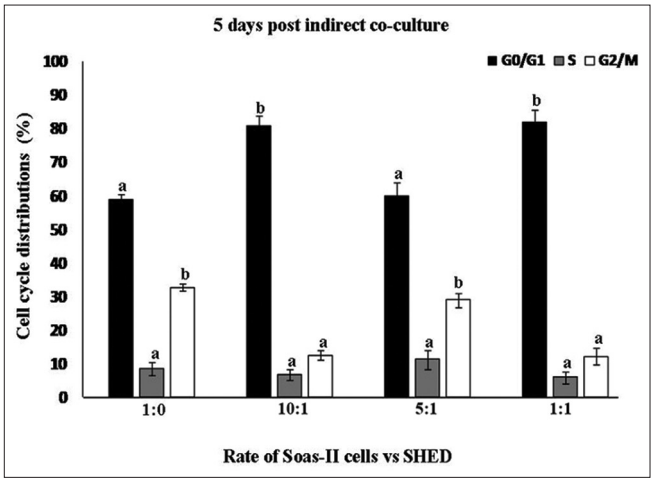

Figure 7: Analysis of the relative proportion of osteosarcoma (Saos-II) cells indirectly co-cultured with different concentrations of stem cells derived from human exfoliated deciduous teeth after 5 days in each cell-cycle phase. Different letters above columns show statistically significant differences among the groups. In the other word, columns with different letters vary significantly (Data are presented as mean ± SD, a vs. b $P < 0.05$)

Discussion

To date, we have seen different results regarding the promotion or inhibition impact of MSCs on tumorigenesis.[17-19,30] We herein provided evidence that the indirect coculture of Saos-II cells with SHED could promote or inhibit Saos-II cells growth in a concentration window (the number of SHED vs. Saos-II cells) and time (days from indirect co-culture) dependent manner. 3 and 5 days after indirect co-culture of Saos-II cells and SHED in 10:1 group, early and late apoptosis and necrosis were less than those of the controls. It is known that diverse growth factors, fibroblast growth factor, epidermal growth factor, as well as platelet-derived growth factor, chemokines, and inflammatory cytokine are secreted by human MSCs (hMSCs).[31] The expression of several counter-receptors and receptors on hMSCs for cell-cell and cell to matrix interaction have been demonstrated by previous studies.[32,33] Hence, indirect coculture of SHED with Soas-II cells may work as a tumor promoters in cases that a fewer number of SHEDs were used in the culture in comparison with those cultured in the absence of SHED co-incubations. In contrast, although Soas-II cells apoptosis increased when being treated with higher proportion SHED compared to the control (1:0) group after 3 days from indirect coculture, the highest apoptosis rate belonged to 1/5 group. Moreover, our cell cycle analyses indicated that coculture of Saos-II cells and SHED with 1/5 ratio significantly enhanced in G2/M phase cells; meanwhile, in 10:1 and 1:1 groups, it was significantly enhanced in the G0/G1 phase cells. These findings revealed that SHED, in a certain ratio, results in apoptosis of Soas-II cell through the specific stages of the cell cycle arrest. However, the effects and underlying mechanisms of different ratios of SHED in the induction of apoptosis of Soas-II cell are unknown.

During this study, when indirectly cocultured Soas-II cells with SHED were left intact for extended durations (5 days), MTT analyses also confirmed that viability was remarkably affected by indirect coculture in 1/5 group. Nonetheless, the observed impact was clearer in the cases that indirect cocultures were accomplished for 3 days compared to 5 days. Therefore, indirect coculture of Saos-II cells and SHED with a certain ratio may largely lead to several events in Saos-II cells, causing death of 92.16% of the cells.

The obtained outcomes are in accordance with earlier investigations, in which MSCs inhibited the growth of tumors through induction of apoptosis and cell cycle arrest.[34,35] Previous results have demonstrated that inhibition effect of microvesicles extracted from human umbilical cord Wharton's jelly MSC on the growth of the bladder tumor cells can be mediated through the apoptosis induction and cell cycle arrest.[36] Furthermore, our results are in line with those reported by Long *et al*. determining the impact of MSCs extracted from the BM on the growth of Hela and HepG2 cells through transwell intraction with the ratio of 1/10, 1:1, and 2:1 hMSCs versus the cancer cells.[37] They showed that, in case of adding higher proportions of hMSCs, cell growth declined and vice versa.

In contrast, using coinjection of BM-derived MSCs with the Lewis lung carcinoma cells, Carnet *et al*. (2015), in a study on mice reported the involvement of BM-MSCs in the invasive features of the tumor cells. They found juxtacrine-mediated interaction of the BM-MSCs with the cancer cells.[38] Other reports have indicated that coculturing the MSCs with a variety of ovarian and breast cancer cells could enhance the proliferation or rapid growth of the cancer cells.[39] Although the mechanisms considered for secretion and interactions of MSCs with the cancer cells for these controversial observations still remain unclear, it appears that MSC origin is responsible for the tumor cell growth inhibition versus the promotion effect of MSCs.[18] Moreover, proproliferative effects of SHED on cell growth might be attributed to the cooperative induction between SHED and Soas-II cells. Put differently, higher proportions of SHED may obviously refer to the growth inhibitory impact rather than the proliferation or rapid growth simulation; this suggested that inhibitory activity of bioactive molecules is predominant when greater volumes of SHED are added in 5:1 group compared to 10:1 group. It was reported that low and high amounts of hMSCs exhibit stimulatory and inhibitory effect on T cell, respectively.[40]

We illustrated either inhibitory or stimulatory impact of SHED on the growth of cancer cells (Soas-II cell) dependent on the experimental conditions (the cell ratio and time of the co-cultures). In fact, such notable features of SHED might be associated with the competition of produced cytokines with different (even opposing) properties in the culture, which can culminate in the inhibitory or proliferative activity on indirectly adjusted cells (here osteosarcoma cell). Nevertheless, further studies are needed on SHED interactions with tumor cells.

Conclusion

Our findings suggested that, indirectly, SHED co-culture with the Soas-II cells might functions as a tumor suppressor where a higher number of SHEDs are used in the culture in comparison with the one cultured in the absence of/or fewer SHED incubation. However, further studies are needed on SHED interactions with tumor cells.

Declarations

Ethics Approval and Consent to Participate This article does not contain any studies with human participants performed by any of the authors. All participants signed informed consent forms approved by the Ethical Committee of Isfahan University of Medical Sciences (189025).

Ethics approval and consent to participate

This work was approved by Isfahan University of medical sciences research ethical committee with code number of (189025).

Availability of data and materials

There is no data to be deposited into a website. All data was included in manuscript.

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Conflicts of interest

None of the authors has any conflicts of interest to disclose and all authors support submission to this journal.

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