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Inhibitory Effects of mesenchymal stem cell-derived from the amniotic membrane on breast cancer cells

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Abstract

Background: Mesenchymal stem cells (MSCs) are widely employed in the field of regenerative medicine due to their versatility and therapeutic potential. Notable developments in the stem cells role in the treatment of diseases have been achieved during past years. MSCs are showing increasing assurance in cancer treatment because of their unique features. Despite significant advances in the molecular basis of cancer and improvements in detection and treatment, cancer is still the major cause of death.
Objectives: In this study, the inhibitory effect of MSCs derived from amniotic membranes (AM-MSCs) on breast cancer cell lines (MCF7 and MDA-MB-468) was investigated.
Methods: MSCs were isolated from the human amniotic membrane (AM), and their identity was verified through specific tests. AM-MSCs were then co-cultured with MCF7 and MDA-MB-468 breast cancer cell lines. The apoptotic effect of AM-MSCs on these cells was assessed using Acridine Orange staining. The expression levels of Cyclin-dependent kinase 2 (CDK2), Bcl2, caspase3, and Vascular endothelial growth factor (VEGF) genes were measured via Real-time polymerase chain reaction (RT-PCR). Additionally, the impact of MSCs on the migration of the cancer cell lines was evaluated using a Scratch test.
Results: The results indicated that AM-MSCs induced apoptosis in both MDA-MB-468 and MCF7 breast cancer cell lines. RT-PCR analysis demonstrated a significant ($P<0.05$) decrease in the expression of CDK2 and VEGF genes, alongside an increase in the expression of BCL2 and caspase3 genes. The positive outcome of the scratch assay suggested that AM-MSCs inhibited the migration of cancer cells.
Conclusions: AM-MSCs exhibited anticancer effects against MDA-MB-468 and MCF7 breast cancer cells. Given their easy availability and adherence to medical ethical principles, AM-MSCs present a promising avenue for treating this type of cancer. Further research in this area is necessary and could be beneficial in the development of cancer treatments.

Keywords: Mesenchymal stem cells, breast cancer, co-culture, apoptosis

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1. Background

Cancer is identified as a multistage disease characterized by the transformation of a normal cell acquiring distinct abnormal features. These features encompass self-sufficiency in growth signals, evasion of apoptosis, resistance to anti-growth signals, unlimited replicative capacity, sustained angiogenesis, invasion of tissues and metastasis, reprogramming of energy metabolism, genome instability, evasion of immune response, and inflammation that promotes tumor growth (1). Malignancy is caused by various mutations in specific genes, such as proto-oncogenes and tumor suppressor genes, within a cell (2). Breast cancer (BC) is among the leading causes of mortality in women worldwide. Research on breast cancer has led to significant advancements in the understanding of the disease in recent decades, resulting in the development of more successful treatments. Currently, common treatments for breast cancer include surgery, radiotherapy, and chemotherapy (3). However, the challenges of metastatic spread and recurrence still remain for researchers. Cell therapy is a novel method in cancer treatment that can be safe with minimal or no toxicity to normal cells. Hematopoietic stem cells (HSCs) have been previously utilized as a well-established cellular therapy approach to rescue the bone marrow from hematologic malignancies. The emergence of medical strategies employing non-HSC stem cells offers similar alternatives for the treatment of other types of cancer (4).

Mesenchymal stem cells (MSCs), the most extensively

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researched stem cells, are increasingly employed in treating a variety of diseases, ranging from organ transplants to joint and cartilage disorders. Several characteristics make these cells suitable for human therapy, including their ease of retrieval from various adult tissues, preferential migration to sites of injury or inflammation, ability to promote repair through the release of bioactive factors, and capacity to modulate the immune system. Additionally, MSCs can be safely used from different donors as they do not provoke harmful immune responses in the host. In recent years, studies have investigated the effects of MSCs on cancer cells. (5) examined the effects of human umbilical cord mesenchymal stem cells (hUCMSCs) on breast cancer cells, indicating that hUCMSCs reduce tumor growth by arresting the cell cycle, inducing apoptosis in tumor cells, and repressing the activities of PI3K and AKT protein kinases. Furthermore, demonstrated the inhibitory effect of human bone marrow mesenchymal stem cells (hBMSCs) on human glioma U251 cells in a co-culture system by down-regulating the PI3K/AKT signaling pathway in cancer cells (6).

The amniotic membrane (AM) is known to be a readily accessible source of MSCs, the use of which does not pose ethical problems. Amniotic membrane-derived MSCs (AM-MSCs) have advantages, including differentiation, self-renewal, low immunogenicity, and anti-inflammatory properties, and have thus attracted significant attention (7-8). In recent years, some studies have examined the anticancer properties of AM-MSCs (9-10). In this study, the researchers aimed to investigate the effect of AM-MSCs on the growth of two breast cancer cell lines using the co-culture method. Additionally, the potential impact of AM-MSCs on cell cycle rate, angiogenesis, and apoptosis of breast cancer cells was examined by measuring the gene expression of VEGF, Caspase3, BCL2, and CDK2.

2. Materials and Methods

2.1. Cell lines preparation

Breast cancer cell lines MDA-MB-468 and MCS7 were obtained from “The Cell Bank of Pasteur Institute” in Tehran, Iran. In addition, the AM was prepared as an MSC source from a cesarean section of Milad Hospital (Iran, Tehran) with the certificate of pregnant women.

2.2. MSCs isolation

MSCs isolation was performed through method (11). The membrane tissue was washed with phosphate-buffered saline (PBS) and then cut into a few small pieces. To remove epithelial cells, the membrane pieces were trypsinized, and then the MSCs were released by adding collagenase and DNase.

2.3. Confirmation of MSCs

To verify the isolated MSCs, their surface markers were assessed using a flow cytometric method. First, the isolated MSCs were trypsinized and re-suspended in PBS. 1×105 cells were placed in 1.5 mL tubes and suspended in 10 µL of fluorescein isothiocyanate (FITC)-conjugated primary antibodies (Becton Dickinson, Franklin Lakes, NJ) and phycoerythrin (PE) as multicolor antibodies and isotype control antibodies for 1h on the ice bath. In the next step, the MSCs were washed with PBS solution and then analyzed using Flow MAX.

2.4. Osteoblast Differentiation

After 5 passages, the cells were utilized for differentiation into osteoblasts. Initially, 500 µL of the cells were seeded in a 6-well plate containing DMEM-Lg medium. Following a 2-day incubation period during which the cell number increased, 3 wells of the cell culture plate were exposed to osteogenic differentiation medium containing 50 µg mL-1 ascorbic acid, 5 mM β-glycerol phosphate, and 10 nM dexamethasone. The remaining 3 wells served as controls, with the addition of DMEM-Lg medium. The culture medium of each well was refreshed every 2 days. The differentiation of cells into osteoblasts was detected using 2% alizarin red staining after 14 days of treatment.

3. Adipocyte Differentiation

Similar to the osteoblast differentiation, cells were used for adipocyte differentiation after 5 passages. 500 µL of the centrifuged cells were seeded in a 6-well plate containing the DMEM-Lg medium. Following a 2-day incubation period and an increase in the number of cells, the adipocyte differentiation medium (50 µg mL-1 ascorbic acid, 60 mM indomethacin, and 10 nM dexamethasone) was added to 3 wells. The DMEM-Lg medium was added to the remaining 3 wells as the control cells. The cell culture media were renewed every 2 days for 14 days, and then the differentiated adipocyte cells were detected through oil red 2% staining.

4. Co-cultivation of MSCs and Cancer Cell Lines

To investigate the interaction between MSCs and breast cancer cell lines (MDA-MB-468 and MCF7), a co-cultivation system was established. An appropriate amount of MSCs were exposed to mitomycin C and then the trypsinized cells were transferred to a 25 cm2 flask. After 24 h of incubation, MDA-MB-468 and MCF7 cells were added to this flask. Dulbecco's Modified Eagle Medium (DMEM) was used as the culture medium for the co-cultivation (12).

5. Evaluation of Cell Death by AO/EB Fluorescent Staining

Following 72 hours of co-cultivation, the MDA-MB-468 and MCF7 cells were rinsed with PBS, and fixed in a 4% formaldehyde solution for 20 minutes. Subsequently, 25 µL of the cell suspensions were placed on glass slides along with 1 µL of a dual fluorescence staining the solution containing acridine orange (AO) and ethidium bromide (EB). Coverslips were then placed over the slides, and the apoptotic cell morphology was examined using a fluorescence microscope.

6. RNA Extraction

RNA extraction from the MDA-MB-468 and MCF7 cells co-cultured with MSCs was performed using the YTA Total RNA Purification Mini. Subsequently, an oligo-dT primer, buffer (×10) including Tris-HCl, KCl, MgCl2, and DTT was applied for cDNA synthesis. The quality of the RNA was checked using a Nanodrop, followed by the addition of reverse transcriptase enzyme to the deoxyribonucleotide triphosphate. Additionally, primers for target genes were designed for use in real-time polymerase chain reaction (RT-PCR) (Table 1).

7. Real-Time PCR

The target genes were expressed in co-cultivated breast cancer cells using RT-PCR technology (CFX96™ RT-PCR Detection System, Bio Rad), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene and SYBER Green used to determine DNA concentration. The ΔCT method was utilized to normalize the genes.

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Table 1. The sequence of primers used in this study.

Genes	Primers' sequence	Amplicon size (bp)
BCL2	Forward5'- ATCGCCCTGTGGATGACTGAGT Reverse 5'-GCCAGGAGAAATCAAACAGAGGC	110
CDK2	Forward 5'- CGCTCCTTTCAGACCGCTGTTT Reverse 5'- CCATCTCCTCTATGACTGACAGC	114
Caspase3	Forward 5'- TCCTTATCCTTTGACGCTACTTA Reverse 5'- AACCACCAACCAACCATATCG	110
VEGF	Forward 5'- TTGCCTTGCTGCTCTACCTCCA Reverse 5'- GATGGCAGTAGCTGCGCTGATA	115
GAPDH	Forward 5'- TTGTACCCTTGTGCCTCGCTCA Reverse 5'- AGATCAGCCGGCGTTTGGAGT	128

8. Cell migration investigation

To evaluate the inhibitory effect of AM-MSCs on breast cancer cell migration, a scratch assay was conducted. AM-MSCs were first exposed to a culture medium containing mitomycin C for 1 hour in a 6- well plate and then co-cultured with breast cancer cells for 72 hours. After this, the cells were washed with PBS buffer, and a DMEM-rich culture medium containing 10% FBS was added to the plates. Subsequently, a scratch was made in the bottom of the wells, and breast cancer cell migration status was examined after 3 days.

9. Statistical analysis

The statistical analysis was conducted using analysis of variance (ANOVA) with SPSS software version 16 (version 16, Chicago, IL, USA). The T-test was utilized to compare two independent groups, while the One-way ANOVA test was used for comparisons involving more than two groups. Results with a P value of less than 0.05 were considered statistically significant**.**

10. Results

10.1. Growth characterization and Surface markers of AM-MSCs

The growth characteristics and surface marker profile of AM-MSCs are displayed in Fig 1. The AM-MSCs exhibited typical adherent growth, with a large rounded nucleus and elongated cellular processes, and maintained a robust mitotic capacity. Furthermore, the AM-MSCs were isolated and their surface antigen expression was analyzed using flow cytometry. The analysis revealed that the AM-MSCs were positive for mesenchymal lineage markers, such as CD73 (97.2%) and CD29 (99.8%), and negative for hematopoietic cell antigens, including CD34 (0.983%) and CD45 (0.519%) (Fig. 2).

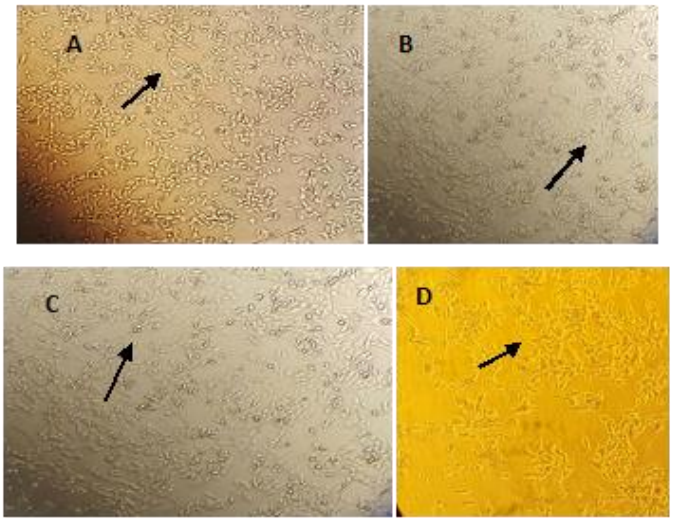


Figure 1. Morphology of amniotic membrane stem cell. A: AM-MSC cells in passage 2, B: AM-MSC cells in passage 3, C: AM-MSC cells in passage 5, D: AM-MSC cells in passage 6. These images show AM-MSC spindle cells, they are part of the adhesive cells. These images were taken by a reverse microscope with 40x magnification. The dots in the figure indicate spindle-shaped mesenchymal cells.

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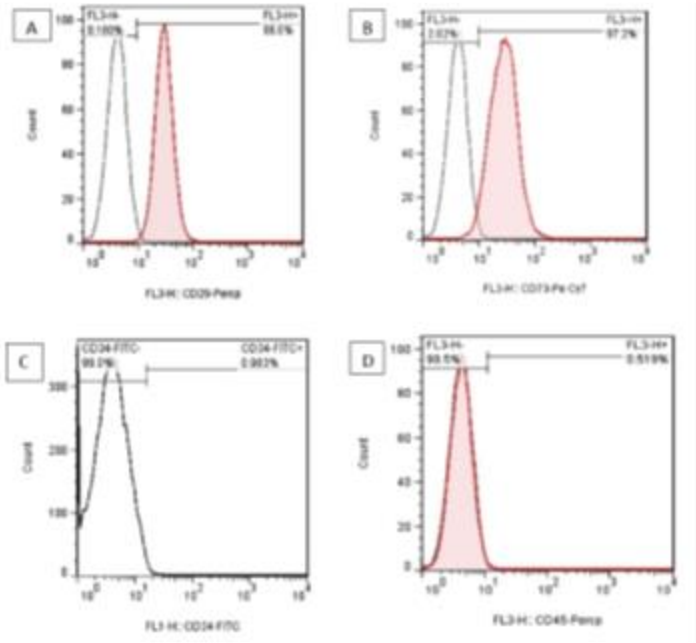


Figure 2. Expression of surface markers, Sections A –D show the expressed markers CD 29, CD 73, CD 34, and CD 45 respectively.

10.2. Osteogenic and adipogenic differentiation from AM-MSCs

The differentiation potential of passage 5 AM-MSCs cultured in osteogenic and adipogenic induction media. The results indicate that AM-MSCs cultured in these specialized media differentiated into osteogenic and adipogenic cells, respectively, whereas AM-MSCs cultured in the basic medium did not exhibit any differentiation (Fig. 3). After 14 days, the osteogenic and adipogenic cells were identified through Alizarin Red and Oil-Red-O staining, respectively. The Alizarin Red staining could represent the osteogenic differentiation of AM-MSCs, as it indicates the presence of calcium deposition. Similarly, the Oil-Red-O staining could reveal the adipogenic differentiation of AM-MSCs, as it demonstrates the presence of red lipid vesicles.

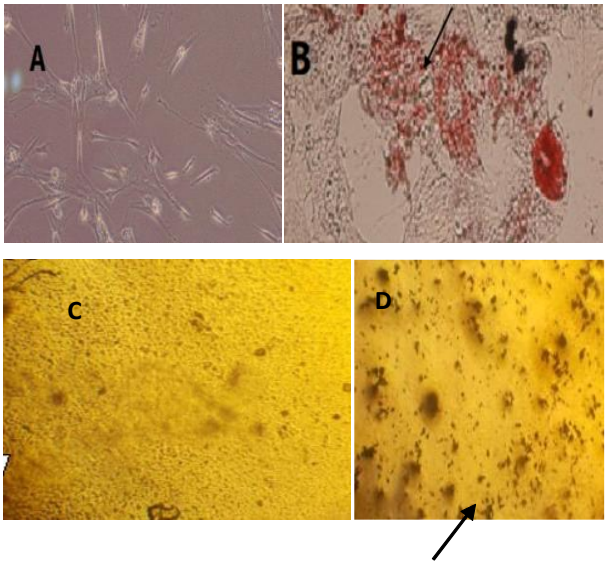


Figure 3. Osteogenic and adipogenic differentiation from AM-MSCs and A-AM-MSCs were considered as control cells. B- Osteogenic differentiated from AM-MSCs were stained by Alizarin Red. Red points represented calcium deposition crystals. C- AM-MSCs were considered as control cells. D- Adipogenic differentiated from AM-MSCs was stained by Oil-Red-O. Red points represented lipid vesicles. These images were taken by a reverse microscope with 40x magnification.

10.3. Cell morphology of MDA-MB-468 and MCF7

The MDA-MB-468 cell line exhibits an epithelial-like appearance, characterized by an oval or polygonal shape, and a tendency to form adhesive clusters (Figure 4A). In contrast, the MCF7 cell line, a well-known breast cancer cell line, is composed of elongated epithelial structures organized into large, spindle-shaped clusters. These large clusters often consist of multiple cells attached, although individual cells may also be observed (Figure 4B).

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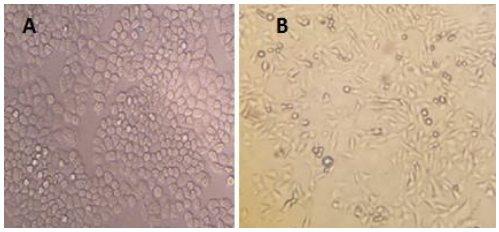


Figure 4. The morphology of breast cancer cell lines MDA-MB-468 (A) and MCF7 (B)in passage 1. These images were taken with a reverse microscope at 40% magnification.

10.4. Co- Culture of AM-MSC with MDA-MB-468 and MCF7

The experiment involving the co-culture of adipose-derived mesenchymal stem cells (AM-MSC) with two breast cancer cell lines, MDA-MB-468 and MCF7 which after a 24-hour incubation period, the breast cancer cells were added to the flask containing the AM-MSC cells (Fig 5 and 6). It was observed that, following 72-hours, the cancer cells were positioned adjacent to the AM-MSC cells, and the growth and proliferation of the breast cancer cells in both cell lines were reduced. Microscopic images were captured at a magnification of 100x to document these findings.

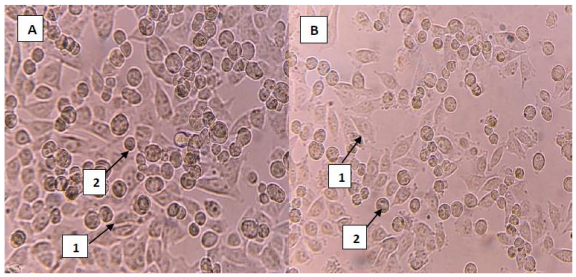


Figure 5. A: AM-MSC and MCF7 on the first day. B: AM-MSC and MCF7 on the third day (72 hours). These images were taken with a reverse microscope and 100 x magnifications. Number 1 indicates AM-MSC cell and number 2 indicates MCF7 cell.

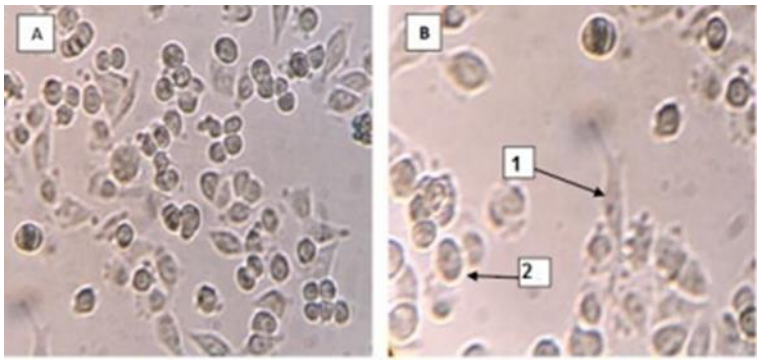


Figure 6. A: AM-MSC and MDA-MB-468 on the first day. B: AM-MSC and MDA-MB-468 on the third day (72 hours). These images were taken with a reverse microscope and 100 x magnifications. Number 1 indicates AM-MSC cell and number 2 indicates MDA-MB-468 cell.

10.5. Evaluation of cell death by AO/EB fluorescent staining

Acridine orange and ethidium bromide (AO/EB) fluorescent staining was employed to assess cell death, specifically to evaluate the viability of cancer cells following co-culture with AM-MSCs. The results indicated that the co-culture of AM-MSC led to an increase in apoptosis in the MCF7 and MDA-MB-468 cell lines, as observed under a fluorescence microscope (Figures 7 and 8). The findings demonstrated that the co-culture with AM-MSC induced apoptosis in these cancer cell lines.

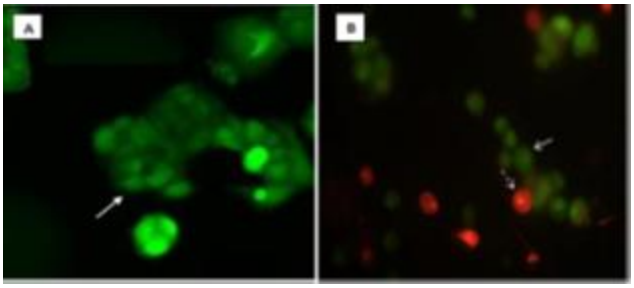


Fig 7. Evaluation of cell death results by acridine orange staining. A: control cancer cells after 3 days. And B: MCF7 cancer cells co-cultured with AM-MSC cells after 3 days. Orange regions indicate apoptosis of the MCF7 cell line and green areas represent living cells (100 x magnifications).

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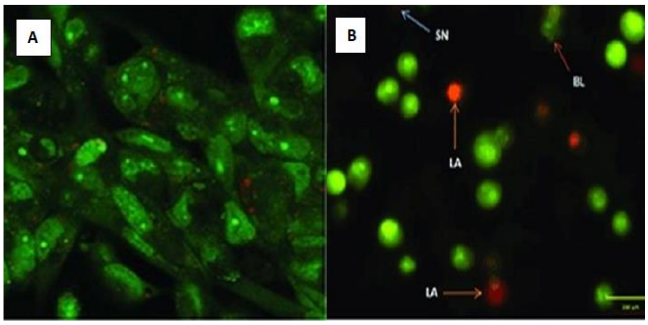


Figure 8. Evaluation of cell death results by acridine orange staining. A: control cancer cells after 3 days. And B: MDA-MB-468 cancer cells co-cultured with AM-MSC cells after 3 days. Orange regions indicate apoptosis of the MDA-MB-468 cell line and green area represents living cells (100 x magnifications).

10.6. VEGF, Caspase 3, CDK2, BCL2 gene expression by RT-PCR analysis

The quantitative evaluation of RNA was performed using a Nanodrop device, and the cDNA was then utilized for the RT-PCR analysis to assess the expression of various genes, including VEGF, Caspase 3, CDK2, and BCL2, in co-culture experiments involving MCF7 and MDA-MB-468 cells with AM-MSCs. The results were evaluated statistically (Fig. 9 and 10).

The findings indicate that the expression level of the caspase-3 gene was markedly elevated in the co-culture of MCF7 cells with AM-MSCs, with a nine-fold increase compared to the control cells ($p<0.05$). Conversely, the expression level of VEGF was decreased in the co-culture of MCF7 cells with AM-MSCs, in comparison to the control cells ($p<0.05$).

Furthermore, the results demonstrated that in the co-culture of MDA-MB-468 cells with AM-MSCs, the gene expression level of BCL2 increased 6.3-fold compared to the control cells ($p<0.05$), while the gene expression level of CDK2 decreased considerably (Fig 10).

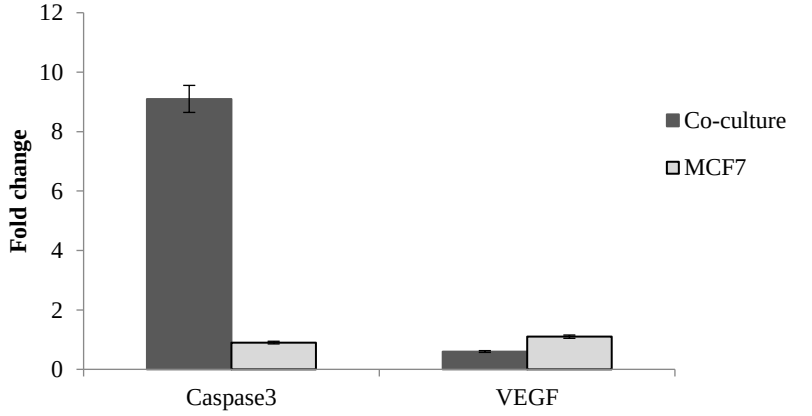


Figure 9. Real-time PCR was performed to measure the mRNA level of Caspase 3 and VEGF in co-culture cells of MCF7 with AM-MSCs compared with the MCF7 cells (Control), all data are presented as the means ± SD ($P < 0.05$).

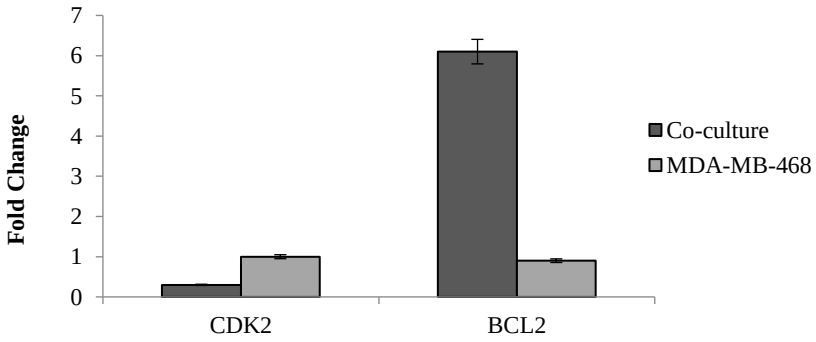


Figure 10. Real-time PCR was performed to measure the mRNA level of co-culture cells of MDA-MB-468 with AM-MSCs compared with the MDA-MB-468 cells (Control), all data are presented as the means ± SD ($P < 0.05$).

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10.7. Scratch assay

The scratch assay was utilized to investigate the impact of AM-MSC on the migratory behavior of MDA-MB-468 and MCF7 cells following their co-culture. The results of the scratch test revealed that MDA-MB-468 and MCF7 cells co-cultured with AM-MSC did not migrate into the vacant space between cells, whereas the control MDA-MB-468 and MCF7 cells had migrated to fill the wound after 3 days. This finding suggests that AM-MSCs inhibited the migration of MDA-MB-468 and MCF7 cells (Fig.11).

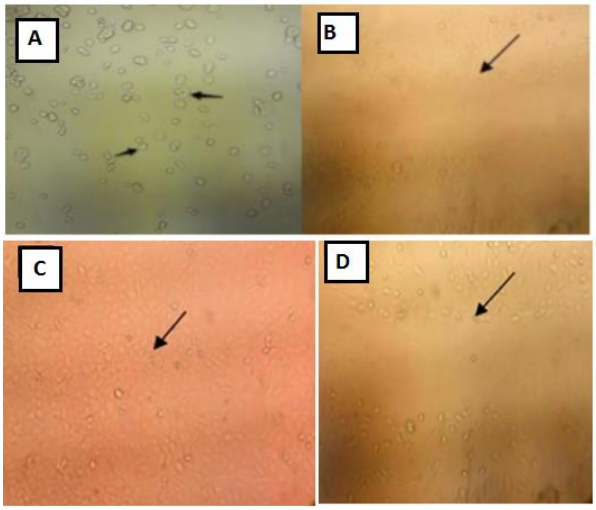


Figure 11. After co-cultured AM-MSC with MDA-MB-468 and MCF7, the scratch test was used to evaluate the effect of AM-MSC on the cell line MDA-MB-468 and MCF7 migration. A: MCF7 after 3 days filled the wound. B: the co-culture of MCF7 cells with AM-MSC did not migrate on the wounding. C: MDA-MB-468 after 3 days filled the wound. D: co-culture of MDA-MB-468 cell with AM-MSC did not migrate on the wounding (Images are taken at 100x magnification).

11. Discussion

Breast cancer represents the most common form of cancer affecting women globally (13). Tumor angiogenesis and neovascularization are crucial processes that arise during the development and metastasis of tumors, involving the formation of new blood vessels to support tumor growth and spread. (14). Despite advancements in cancer survival rates, several traditional treatments require replacement with novel and innovative approaches. MSCs exhibit intrinsic antitumor properties through their ability to inhibit cell proliferation, suppress angiogenesis, and induce apoptosis. (15). Previous studies indicate that MSCs can resensitize and induce apoptosis in cancer cells by releasing antitumor paracrine factors. These factors stimulate the release of chemoattractants from the tumor, contributing to the suppression of cancer cell growth and survival. Furthermore, MSCs can secrete interleukin and interferons, such as IL-18 and IL-15. These cytokines play crucial roles in inhibiting proliferation and cell division in tumor cells. Additionally, they can activate the immune system's response against tumor cells (16-17). While MSC therapy is regarded as a promising approach for various biological activities, disease treatment, and cancer, the role of MSCs as a tumor cell supporter or suppressor remains inconclusive, potentially due to factors such as tumor type, animal host, the dose or timing of MSC injection, and their activation status (18).

Given the significance of this issue, the present study investigated the impact of AM-MSCs on the growth of two breast cancer cell lines, MDA-MB-468 and MCF7, using a co-culture method. Additionally, the potential impact of AM-MSCs on cell cycle, angiogenesis, and apoptosis of breast cancer cells was evaluated by measuring the gene expression of VEGF, Caspase3, BCL2, and CDK2. Several researchers have demonstrated the presence of distinct surface antigens, such as CD29, CD44, CD90, and CD105, on MSCs derived from umbilical cord blood, bone marrow, and adipose tissue studies (19-20). In this investigation, the identification of AM-MSCs was conducted using flow cytometry, which demonstrated their differentiation into osteoblasts and adipocytes, and the expression of characteristic surface antigens, such as CD73 and CD105.

Given the distinct molecular profiles of breast cancer, researchers have focused on breast cancer cell lines to investigate whether the molecular characteristics observed in breast carcinomas are accurately represented in these cell line models of the disease (21). In this study, the MCF7 and MDA-MB-468 cell lines were selected. The findings indicated that the expression of the Caspase3 gene was elevated in the co-culture of MCF7 and AM-MSC cells compared to the control group ($p<0.05$). This result aligns with prior studies demonstrating that human MSCs can suppress the growth of human MCF-7 breast cancer cells in vitro by activating the caspase-3 pathway and inducing apoptosis (22-23). Similarly, Aziz et al. observed a significant decrease in the survival rate of ovarian cancer cells after co-culturing with human amniotic fluid mesenchymal stem cells (hAFSCs), accompanied by upregulation of P53, P21, and apoptosis-related genes (10).

The paracrine role of MSCs is a critical mechanism involved in the regulation of cancer, mediated through various factors such as growth factors and cytokines. VEGF and its receptors Flk-1/KDR RTK play a crucial role in the angiogenesis observed in various tumors, making the VEGF pathway a prime target in anticancer therapy efforts (24-25). Downregulation of factors like VEGF, PDGF-AA, PDGF-BB, and CXCL16 has been reported after the co-culturing of cancer cells with MSCs, suggesting the anti-migration and anti-angiogenesis effects of MSCs (8).

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Specifically, in the co-culture of SPC-A-1 cells with hAMSCs experiments, the expression of VEGF was reduced in MCF7 breast cancer cells. when co-cultured with AM-MSCs compared to control cells (26). In this study, the expression level of VEGF was decreased in the co-culture of MCF7 cells with AM-MSCs compared to the control cells as well ($p<0.05$). Additionally, in co-culture of MDA-MB-468 breast cancer cells with AM-MSCs, the gene expression of the anti-apoptotic protein BCL2 which serves as a critical prognostic marker in clinical breast cancer assessment was 6. 3 fold increased, while the expression of the cell cycle regulator CDK2 was notably decreased compared to the control cells ($p<0.05$). During the growth and involution of the mammary gland, key apoptosis-inducing BCL2 family proteins like Bax, Bad, and Bcl-w are elevated, with Bcl-2 playing a regulatory role in Bax levels. The balance between Bax and Bcl-2 levels is crucial for normal breast development (27).

Moreover, CDK2 is a gene that encodes a member of the serine/threonine protein kinase family, essential for regulating the cell cycle by facilitating the transition from the G1 to S phase (28). Chao et al. reported that human umbilical cord mesenchymal stem cells (HUMSCs) play a role in breast cancer tumor formation. They conducted a co-culture study using MDA-MB-231 breast cancer cells and HUMSCs (29).

Tian et al. examined how hMSCs affect the A549 lung cancer cell line and the Eca-109 esophageal cancer cell line. They reported that in cultured cells, the expression of the CDK2 is reduced (30). Similarly, our research confirmed that hMSCs inhibit cell migration and invasion, arrest tumor cells in the G1 phase of the cell cycle, and induce tumor cell apoptosis in vitro using co-culture methods. Additionally, umbilical cord-derived MSCs were observed to diminish growth and promote apoptosis in HepG2 cells by reducing AFP, Bcl2, and Survivin expression (31). Conversely, adipose tissue-derived MSCs were found to enhance the progression of colorectal cancer (HCT116 cell line) through AMPK/mTOR-mediated NF-κB activation, promoting cell survival by downregulating p21, p16, p53, and BAX, and increasing Bcl2 protein expression (32). Nevertheless, according to various studies, stem cells may be considered as a potential future treatment for diverse cancer types

12. Conclusion

In conclusion, MSC therapy is a promising approach for the treatment of various cancers, as the paracrine factors secreted by MSCs can modulate key cellular processes involved in tumor progression, such as angiogenesis, cell cycle regulation, and apoptosis. Further research in this field is essential to fully understand the mechanisms underlying the anticancer effects of MSCs. Moreover, exploring their potential clinical applications could lead to novel therapeutic strategies for treating cancer.

Disclosure of conflicting interests

The authors declare that there is no conflict of interest to disclose.

Author contributions

Akhlaghi and Hosseinzadeh carried out the experiment (Both have an equal share in writing the article). Barikrow was involved in planning and supervising the work.

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