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# Antiproliferative and apoptotic effects of conditioned medium released from human amniotic epithelial stem cells on breast and cervical cancer cells

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## Abstract

**Introduction:** Human amniotic membrane (hAM) and its cells have been proposed for several clinical applications, including cancer therapy. However, reports on the anticancer effects of human amniotic epithelial stem cells-conditioned media (hAECs-CM) are limited. This work aims to evaluate the anticancer effects of hAECs-CM on cervical cancer and breast cancer cell lines in vitro.

**Methods:** Human term placentas were gained from uncomplicated Cesarean sections from healthy donor women. After amnion peeling from the chorion, its epithelial stem cells were isolated and cultured, and its conditioned medium (CM) was collected for experiments. MTT assay was performed to assess cancer cells viability. Migration rate of cancer cells was examined via wound healing assay. Cell-cycle distribution and apoptosis were determined using flow cytometry.

**Results:** Based on MTT assay hAECs-CM was cytotoxic against cancerous cell lines in a dose-time-dependent manner. After 48 h of treatment with hAECs-CM pure, the cell viability of breast cancer cells includes MCF-7 and MDA-MB-231 reached to 73.2% and 65.5%, respectively. In the same situation, HeLa cervical cancer cell line revealed the lowest viability by 47.3%. The wound-healing assay displayed an incomplete wound closure of scratched MDA-MB-231 cells and significant inhibition of cell migration after hAECs-CM treatment. The results also revealed that hAECs-CM exerted anti-proliferation activity by prompting cell cycle arrest and apoptosis of cancer cells.

Conclusions: hAECs-CM is a potent candidate for inducing apoptosis and simultaneously inhibition of the proliferation and migration of cancer cells via inhibiting cell cycle blockade.

## Keywords

Human amniotic epithelial stem cell, cancer, conditioned medium, proliferation, cell cycle arrest, apoptosis

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## Introduction

Breast cancer and cervical cancer are the foremost common leading causes of cancer death in women.[1] The side effects and the ineffectiveness of traditional therapies have drawn the attention of researchers to new cancer therapeutic strategies.[2,3] Among them, stem cell therapy has been very promising in recent decades.[4,5] In this regard, the human amniotic membrane (hAM) has been considered an available, attractive, and novel potential stem cell source for cellular therapy.[5,6] The amnion is the innermost layer of the placenta that covers and forms the amniotic cavity around the developing fetus. This tissue is split into five layers, including a) epithelium, b) basement membrane, c) compact layer, d) fibroblast layer, and e) spongy layer.[7] Two types of cells with differing plasticity levels were isolated from the amnion, including human amnion epithelial cells (hAECs) and human amnion mesenchymal stromal cells (hAMSCs).[5,8] hAECs are considered to have stem-cell-like plasticity, anti-inflammatory, immune-privilege, angiomodulatory, and anticancer properties, as well as no ethical issues. These unique features, along with other additional benefits such as easy access and a non-invasive application process, make hAECs a remarkable candidate for use in medical applications.[3,6,7] In recent years, the cytotoxic effect of amniotic cells has been reported.[6,9,10] The hAECs secretary factors have been exposed to trigger apoptosis of activated T-cells.[11] Furthermore, hAECs prevent the proliferation of peripheral blood mononuclear cells (PBMCs) earlier triggered by mitogens.[12] In addition to the AM and its stem cells, recent research has been performed on the conditioned medium extract from amnion-derived cells.[13] Some studies have verified that B and T cells proliferation as well as the chemotactic activities of neutrophils and macrophages can be repressed by the supernatant from hAECs cultures,[14] while others have revealed that direct cell-to-cell contact is essential for the immunomodulatory effects of AECs.[12,15,16] Since a limited number of studies have been performed to evaluate the effect of hAEC-derived conditioned media on cancer cells, this work was designed to investigate whether hAECs-CM possess antitumor properties. Our hypothesis was that the supernatant of amniotic epithelial cells is the source of antitumor properties of the amnion. In this study, we examined the cytotoxic effect of hAECs-CM on human breast cancer MDA-MB-231 and MCF-7 cells and Hela human cervical cancer cells. Moreover, the effect of hAECs-CM on the migration rate, cell cycle distribution, and apoptosis rate of the MDA-MB-231 cell was assessed.

## Methods

### Cell line and reagent

In this case-control study, all cell lines were acquired from the Pasteur Institute of Iran. MDA-MB-231 and MCF-7 human breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA), while Hela was cultured in RPMI-1640 medium (Gibco, CA), both supplemented with 10% of heat-inactivated fetal bovine serum (FBS) and 1% antibiotic (penicillin 100 U $ml^{-1}$, 0.1 g/L streptomycin). Cells were grown in a 95% humidified atmosphere at 37°C with 5% CO2. The culture media was changed every 2–3 days, with the passage carried out with a confluence of 85%–90%.

### Isolation and culture of hAECs

The study was approved by the Human Ethics Committee of Shahid Beheshti University of Medical Sciences. Full-term human placentas were obtained from healthy women undergoing uncomplicated elective Caesarean section. All deliveries which were positive for infectious agents including hepatitis B virus, hepatitis C virus, infectious syphilis, and HIV and those with pre-diagnosed genetic abnormalities were excluded from this study. In each case, proper written informed consent was acquired from placenta donors. The placenta was rapidly transferred to the laboratory under sterile conditions. The amniotic membrane (AM) was separated from the chorion by peeling, washed three times with cold PBS to remove blood, and cut into 3 × 3 $cm^2$ pieces with scissors. The epithelial cells were isolated enzymatically. Briefly, the amnion fragments were firstly incubated for 10 minutes at 37°C in trypsin/EDTA solution (0.15% w/v). After removing debris, the pieces were incubated in a new trypsin/EDTA solution for 40 minutes in a 37°C water bath, while the tissue was gently agitated. To terminate digestion, the trypsin inactivated by adding two to three volumes of DMEM/F12 medium supplemented with 10% FBS. The cell suspensions from the second digest were filtered through a 100 µm cell strainer, centrifuged at 1200 rpm for 10 min, and supernatant discarded. Characterization of freshly isolated hAECs was performed at the protein expression level by immunocytochemistry according to a previously described protocol.[17] Trypan blue (Sigma-Aldrich, USA) dye exclusion was used for determining the viability of the isolated cells. Isolated hAECs were re-suspended in DMEM/F12 supplemented with 10% FBS, 1% Pen/Strep, and 1% epidermal growth factor (EGF, 10 ng/ml) and incubated at standard culture conditions.

### Preparation of CM from hAECs

As explained above, isolated hAECs were cultured in T25 flask in complete DMEM/F12 contain EGF until 85% confluent. Then, the non-adherent cells were removed by changing the medium, and the attached cells at the primary passage (passage 0) were washed with PBS and fed with a fresh complete DMEM/F12 media (without EGF). After

overnight, the supernatant was collected from, filtered through a 0.22 μm filter to remove debris and avoid contamination before using for cell culture, and stored at −80°C for use in subsequent experiments as CM. The steps of hAECs isolation and its conditioned media preparation process are displayed in Figure 1.

## Cell viability assay

The cell viability assay was performed with 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, USA). Mentioned cell lines were seeded at a density of $10^4$ cells/well in a 96-well plate and incubated at 37°C to a confluence of 85%. Then cells treated in triplicate with 0 (in control group), 25%, 50%, 75%, and 100% hAECs-CM diluted with complete DMEM/F12 media. MTT assay was carried out 24, 48, and 72 hours after cell seeding. To this end, 20 μl MTT solutions (5 mg/ml in PBS) was added to each well and the plate incubated in 37°C for 4 h. Then, the medium was gently removed and 100 μl dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The plate was shaken for 15 minutes, and then the absorbance (OD) of each well was read at 570 nm, with the background subtraction of 630 nm (OD blank) using a microplate reader (ELx 800, BioTek, USA).

The percentage of cell viability was determined based on the subsequent formula:

Cell viability rate = (OD treatment-OD blank)/(OD control-OD blank) × 100

## Migration assay

Wound healing assay was used to assess cell migration of cancer cells upon hAECs-CM treatment. MDA-MB-231 cells ($1 \times 10^5$ cells/well) were seeded into 24-well plates incubated under standard conditions to reach complete confluence. Thereafter, cells were scratched with a sterile 200-μl pipette tip and washed by PBS to remove detached cells. The cells were then incubated with hAECs-CM pure and hAECs-CM ½ diluted with a complete DMEM/F12 medium. In the control group, the cells were treated with a complete medium. The images of cells were captured at 0 hours. After 24 h of continuous culture, the medium was aspirated from the cells. Then the cells were washed with PBS, fixed using 4% formaldehyde, and stained with crystal violet. After that, the images of migrating cells were taken at 24 h. ImageJ software was used to draw the cell-free region bounds in each case.[18] All experiments were carried out three times.

## Cell cycle analysis

MDA-MB-231 cells ($1 \times 10^6$ cells) were treated with hAECs-CM or standard culture medium (in control group) for 48 h. Then, treated and control cells collected and fixed with 70% cold ethanol at −20°C overnight. After that, the DNA of cells was incubated with propidium iodide (PI, 100 μg/ml) in the presence of 1% RNAase A for 30 minutes at 37°C in the dark and analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA). CELLQuest Version 3.3 software was used to analyze the percentage of cells in different phases of cell cycle (G0/G1, S, and G2/M).

## Apoptosis analysis

Early and late apoptosis stages have been widely distinguished by initially staining the cells with the combination of fluorescein isothiocyanate-labeled Annexin V (Annexin V-FITC) and propidium iodide (PI) solution followed by flow cytometry detection (Kay and Grinstein 2011). The MDA-MB-231 cells were cultured in standard DMEM/F12. After cells reach 80% confluence, the medium was changed with hAECs-CM. The MDA-MB-231 cells cultured in complete DMEM/F12 medium (without hAECs-CM) were considered as control. After 48 h incubation, cells were harvested and stained by Annexin V-FITC kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The FACS Calibur Flow Cytometer has been used for analyzing the percentage of apoptotic cells. The data were analyzed using version 3.3 of Cell Quest software.

## Caspase 3 activity assay

Caspase 3 activity was determined using a colorimetric technique based on the hydrolysis of the peptide substrate acetylAsp-Glu-Val-Asp p-nitroanilide (Ac-DEVDpNa) by caspases-3. This process results in the release of the p-nitroaniline (pNA) moiety by measuring its concentration at 405 nm from the absorbance values. The comparison between the absorption of pNA from an apoptotic sample and the untreated control enables the assessment of the fold increase in the activity of caspase-3. In the present study, the caspase 3 activity assay was performed according to the manufacturer's instructions. MDA-MB-231 cells were cultured in a T25 flask until 90% confluent. Cells were then treated with hAECs-CM for 24 h under standard culture conditions. Following the treatment period, the cells were trypsinized and centrifuged (1000 rpm × 5 min). The supernatant is gently removed and discarded, and the cell pellet is incubated in lysis buffer on ice for 10 min. Cell lysates were again centrifuged (15 min at 15,000 rpm at 4°C). Then, the supernatant was transferred to a new tube on ice and the proteins were extracted. After that, 2X reaction buffer/DTT and Caspase-3 colorimetric substrate (DEVD-pNA) were added and incubation was done at 37°C for 2 h. Finally, optical density (OD) was measured at 405 nm wavelengths. Activity of caspase-3 is presented as the fold change compared to the control group.

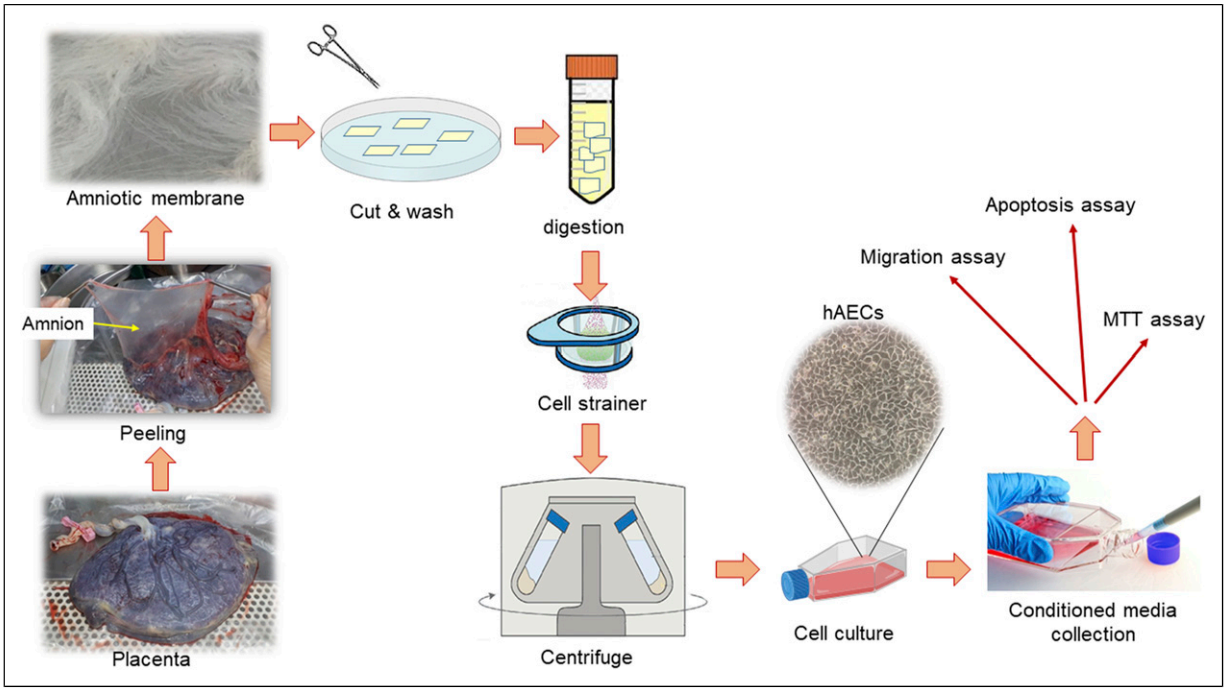


**Figure 1.** Schematic illustration showing the process of hAECs isolation and their conditioned media.

### *Statistical analyses*

All statistical analyses were performed using Graph Pad Prism software 8.0 (GraphPad Software, Inc.). Quantitative data were summarized as mean ± SD. For testing between two groups, the unpaired two-tailed Student's t-test was used. When more than two groups were considered, ANOVA analysis was used. Any $p$-value <0.05 was considered as statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

## Results

### *The hAECs-CM inhibited proliferation of cancerous cell lines*

To evaluate the effects of hAECs-CM on cell viability, MTT assays were done. Data on mitochondrial activity collected from OD of cell culture plates of the experimental groups were converted in percentages about the control group, considered 100%. To avoid any false-positive effect caused due to deficient nutrition in CM, we tested the influence of hAEC-derived CM following a dose-dependent manner. AEC-derived CM was mixed with fresh culture medium in proportion for the experiments. As expected, in comparison to the control group, hAECs-CM decreased viability of cancer cell lines in a dose-dependent manner. In order to better compare the effect of CM concentration on cell viability, MTT results after 48-h treatment of cell lines with different concentrations of hAECs-CM are shown in Figure 2(a). A trend of decrease in the number of viable cancer cells was seen with CM at concentrations of 25%–100%. After 48 h of exposure to hAECs-CM 25%, the viability of cancer cell lines decreased, but difference between the treated cells and their control groups was not statistically significant in any of three cell lines ($p > 0.05$). The hAECs-CM 50% caused meaningfully reduction in the viability of Hela cells to 68% compared with the control group ($p < 0.05$). Treatment with hAECs-CM 75% caused a significant decrease in the number of viable MDA-MB-231 and Hela cells (70% and 65%, respectively; $p < 0.05$). Following exposure with hAECs-CM 100%, a noteworthy decrease in the percentage of viable cells was observed in three cancer cells lines as compared to controls, reaching 73% and 65% reduction in MCF-7 and MDA-MB-231 cells, respectively ($p < 0.05$), and 47% in Hela cells ($p < 0.01$).

As displayed in Figure 2(b), hAECs-CM also attenuated the viability of cancer cell lines in a time-dependent manner. All cancer cell lines exhibited a decrease in viability after 24 h incubation with CM, however, this decrease was only significant for HeLa cells. The results following 48 h CM-treatment are described above. After 72 h incubation with hAECs-CM, a remarkable diminish was observed in the viability of MCF-7, MDA-MB-231, and Hela cells, including 68%, 60% ($p < 0.05$), and 42%,

respectively ($p < 0.01$). However, no statistically significant difference was found for cell viability between 48 h and 72 h incubation.

### The hAECs-CM suppresses the migration of MDA-MB-231 cell line

In the wound healing assay, hAECs-CM significantly decreased the migration of MDA-MB-231 cells compared with the untreated control, in a dose-dependent manner (Figure 3). In the control group, cell migration was dynamic, achieving a value of 80.6% after 24 h. Using hAECs-CM, the motility of the MDA-MB-231 cells was inhibited. Of note, increasing the dose of CM from 50% to 100% showed the difference more clearly, from 47.7% ($p < 0.01$) to 29.8% ($p < 0.001$) (Figure 3).

### The hAECs-CM induces cell cycle arrest at the G2/M phase

The cell cycle distribution of MDA-MB-231 cells was assessed after hAECs-CM treatment. Our results indicate that 23% of cells were arrested in the G2/M phase of the cell cycle after treatment with hAECs-CM (Figure 4(a)). These results displayed that hAECs-CM effectually induced cell cycle arrest and cell apoptosis in MDA-MB-231 cells ($p < 0.05$).

### The hAECs-CM promotes cell apoptosis in breast cancer cells

To examine whether treatment with hAECs-CM inhibits the viability of cancer cells by inducing apoptosis, the

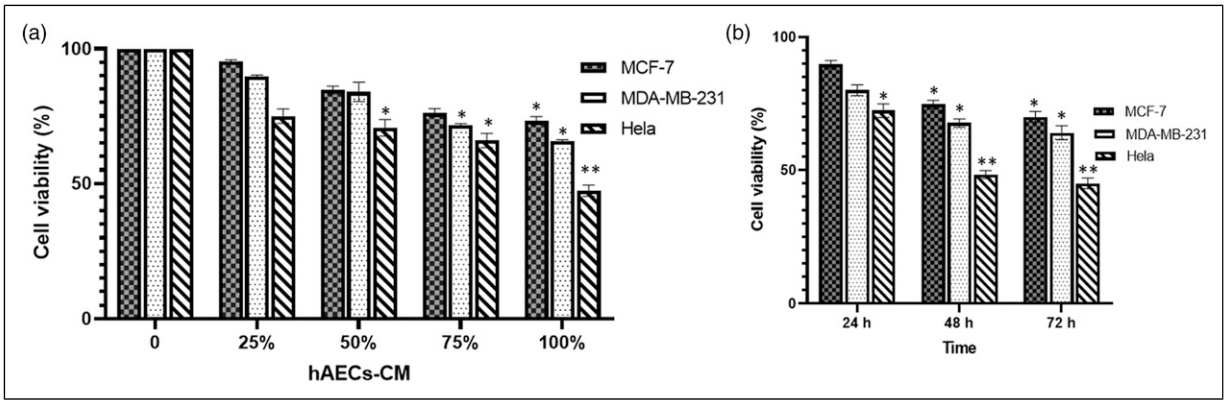


**Figure 2.** The effect of hAECs-CM on proliferation of cancer cells. (a) hAECs-CM suppresses the viability of cancer cells in a dose-dependent manner. Cancer cell lines, including MCF-7, MDA-MB-231, and Hela, were treated with hAECs-CM (25%, 50%, 75%, and 100%) for 48 h, and then subjected to an MTT assay. (b) hAECs-CM suppresses the viability of cancer cells in a time-dependent manner. The mentioned cell lines were treated with hAECs-CM 100% for 24, 48, and 72 h, and then subjected to an MTT assay. Data are represented as mean ± SD ($n = 3$), *$p < 0.05$, **$p < 0.01$, compared with the control (untreated group).

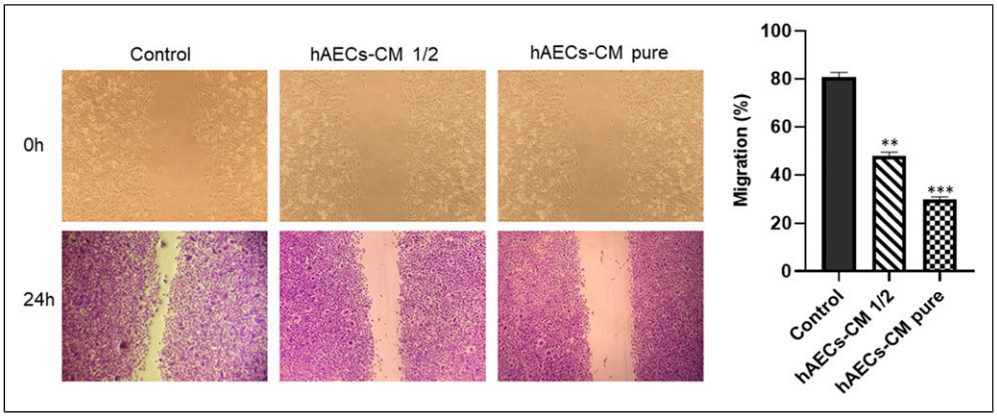


**Figure 3.** Effect of hAECs-CM on migration of MDA-MB-231 cells. Representative images from the wound healing assay of MDA-MB-231 cells treated with hAECs-CM 50% and hAECs-CM 100%. Data values are presented as a percentage of migration variation between starting (0 h) and ending (24 h) time points. Wound healing assay showing significant inhibition of cell migration after 24 h of exposure to hAECs-CM (**$p < 0.01$, ***$p < 0.001$).

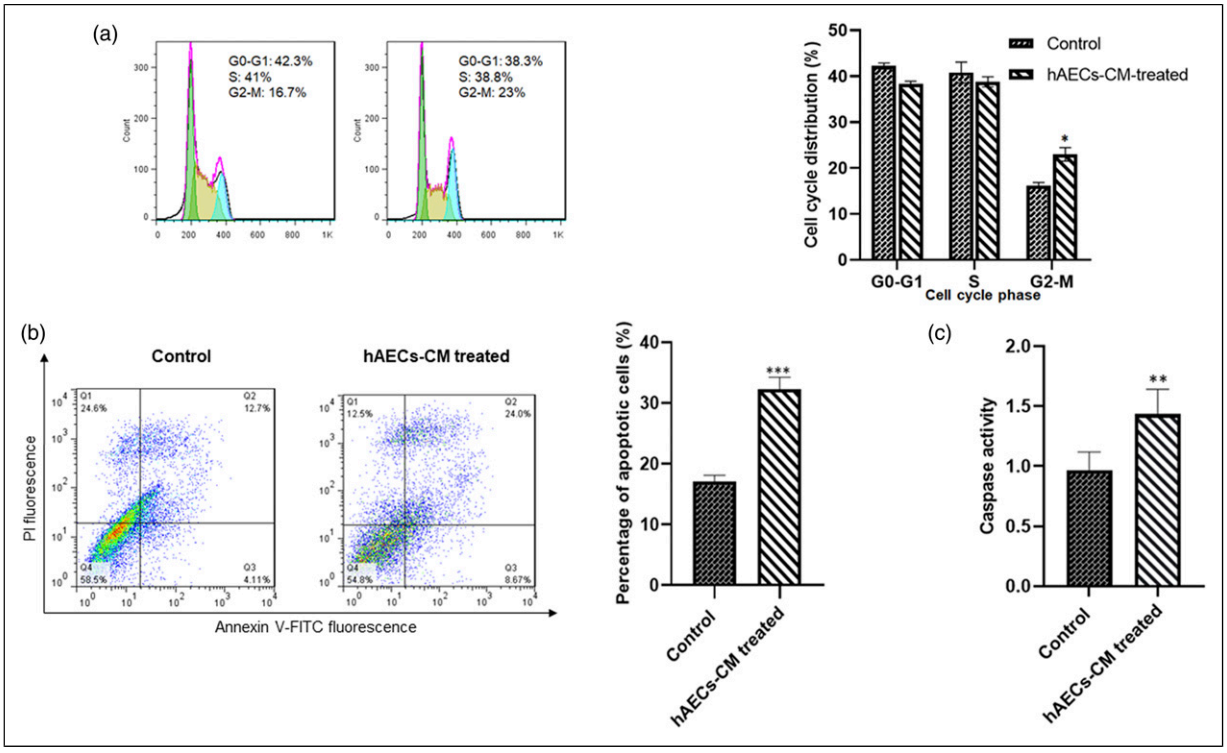


**Figure 4.** Cell cycle distribution, apoptosis, and caspase 3 activity analysis of MDA-MB-231 cells after treated with hAECs-CM for 48 h. (a) The percentage of cell increased after treatment with hAECs-CM in the G2 phase cells, leading to G2/M cell cycle arrest. (b) The percentage of apoptotic cells (Q2 + Q3) in treated group was significantly higher than control group (32.67% vs 16.81%). The percentage of cells was measured using flow cytometry. (c) hAECs-CM increases the caspase 3 activity of MDA-MB-231 cells (**$p < 0.01$, ***$p < 0.001$). The experiments were carried out in triplicate.

percentage of apoptotic cells was measured using the Annexin V/PI assay. Flow cytometry analysis revealed that percentages of apoptotic cells in the treated group were significantly greater than those in the control group. After treatment of MDA-MB-231 cells with hAECs-CM, the percentage of apoptotic cells increased from 17.01% to 32.26% ($p < 0.001$; Figure 4(b)). These results show that hAECs-CM diminishes cancer cell viability by prompting apoptosis.

### *The hAECs-CM increases the activity of caspase 3*

Caspase 3 activity assay was analyzed using a caspase 3 colorimetric kit. According to Figure 4(c), the caspase 3 activity was significantly increased in the hAECs-CM-treated group compared to the control group (by 1.5-fold $p < 0.01$). This result suggests that caspase-3 activation is an underlying mechanism of hAECs-CM-induced apoptosis.

## Discussion

Stem cells derived from hAM have several advantages in cancer therapy, including that hAM is not normally used after birth, small membrane pieces contain large numbers of single-expanded stem cells, and hAM is less immunogenic.[19] Over the past decade, several research groups investigated the anticancer activity of hAM using hAM-derived cells, hAM homogenate, and the conditioned medium prepared using hAM-derived cells.[13,20–22] Growing evidence demonstrated anti-angiogenic, pro-apoptotic, and anti-proliferation effects of hAECs and hAECs-CM on cancer cells.[3,13]

Many researches have shown that increasing the passage number of hAECs result in the acquisition of mesenchymal markers as well as epithelial-mesenchymal transition.[23,24] Considering these key findings, we used hAECs at passage 0 in our experiments to avoid such worsening phenotypic change. In the current study, the cytotoxic effect of hAECs-CM on cervical cancer Hela cells and breast cancer cells (MDA-MB-231 and MCF-7) was initially investigated. The results suggest that hAECs-CM, especially in high concentrations, significantly inhibits the viability of these cancer cells in a dose-time dependent manner. The inhibitory impact observed in this study was not caused by toxic metabolic waste products or a lack of nutrients in the CM, since 25%, 50%, or 75% hAECs-CM also inhibited cell viability on the stated cancer cell lines.

In the study herein, MDA-MB-231 cells are more sensitive than MCF-7 cells, and Hela cell line is the most sensitive to the cytotoxic effects of hAECs-CM. These data indicated that the inhibitory effect of hAECs-CM on cancer cell proliferation is a cell-type-specific effect. In other words, various types of cancer cells contain specific factors and different targets for hAECs-CM that may modulate their sensitivity, and thus lead to different responses.

One of the limitations of the present study is the lack of investigating the effect of hAECs-CM on non-cancerous cells. However, based on the findings of our and others' previous studies, we refrained from repeating the effect of the CM on normal cells. We have previously explored the effect of a conditioned media derived from the intact amniotic membrane (hAM-CM) on the survival of non-cancerous cells in addition to cancer cells.[25] We observed a significant reduction in the viability of hAM-CM-treated breast cancer cells. Interestingly, no cell death was observed in the non–cancerous MCF10A breast cell lines when exposed to identical conditions. Similarly, Riedel et al. to analyze the antitumor properties of hAM-CM on hepatocellular carcinoma cells treated these cells with pure or diluted hAM-CM. They used the same cancer cells cultured with complete DMEM/F12 (untreated without hAM-CM) as a control group. They proved that the viability of hepatocarcinoma cells was significantly reduced after treatment with CM, while the viability of control cells was increased.[13] A recent study from Janev et al. demonstrated that hAM homogenate significantly decreased the adhesion, growth, and proliferation of human bladder invasive and papillary cancer urothelial cells, but did not affect normal urothelial cells.[26] Besides, Protein extracts obtained from processed hAM have been shown to suppress metabolic activity in hepatocarcinoma cell lines, but had no effect on metabolic activity inhibition or protein and DNA content in non-tumorigenic cell lines.

Although the precise factors and mechanisms responsible for the anti-cancer roles of amniotic membrane remain unknown, previous studies have hypothesized biological mechanisms, including down-regulation of cancer cells' gene expression associated with cell cycle progression, inhibition of heat shock protein 90, and secretion of some mediators, such as interleukin (IL)-2, IL-4, IL-8, IL-10, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TNF-β), along with indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), and HLA-G.[6,9,26,27] Kang and colleagues established that hAECs via expression of cytotoxic factors, such as TNF-α, TGF-ß, or ILs, reduced the amount of growth and proliferation of MDA-MB-231 cancer cells.[9] A plenty of studies shown the link between inflammation and development of cancer. Hence, anti-inflammatory agents are believed to have promise for cancer therapy. In recent study, Wu et al. demonstrated that AECs-CM decreased the infiltration of inflammatory cells, including B cells, Th2 cells, eosinophils, and mast cell, and it blocked the release of inflammatory factors. Notably, treatment with CM secreted by AECs increased the levels of anti-inflammatory factors TGF-β and IL-10 as well as the expression of Foxp3, a protein involved in immune system responses.[28] A study showed that soluble factors contained in AM-CM significantly reduced the inflammatory response on human limbal myofibroblast by inhibiting NF-κB activity and subsequently abrogating the synthesis and secretion of proinflammatory chemokines CCL2, CCL5, and CXCL10.[29] Another report has shown that the addition of rat AECs-CM to lipopolysaccharide-activated macrophages evoked anti-inflammatory effects via a decrease in TNF-α expression and inhibited tumor cell proliferation in vitro and in vivo.[30] Other investigations have also confirmed the role of the factors in apoptosis induction.[9,31]

We also evaluate the effect of hAECs-CM on the migration rate of cancer cells. Metastasis may be a multi-step process that includes migration and invasion of cancerous cells.[32,33] Developing anti-tumor agents to reduce cancer cell migration is very critical. A highly malignant cell line with well-known metastatic characteristics, MDA-MB-231, was selected to elucidate the effects of hAECs-CM on cell migration. The hAECs-CM effectively inhibits breast cancer cell migration in vitro, in a dose-dependent manner. After 24 h incubation, the cell migration rate in the control group was 80.3%. This rate dramatically reduced by 47.7% and 29.8% after exposure to 50% and 100% hAECs-CM, respectively. These findings open new viewpoints for the use of hAECs-CM to inhibit breast cancer metastasis. Our findings contradict the report of Zhao et al., which states that the hAECs-CM contributes to keratinocyte proliferation and migration via activation of extracellular signal-regulated kinases 1/2 (ERK1/2), JNK, and Akt signaling pathways.[34] It is also reported that hAECs-CM protects against chemotherapy-induced ovarian damage by decreasing apoptosis, increasing angiogenesis, and regulating follicular development. The discrepancy between these studies may be due to the different responses of normal and cancer cells to the conditioned media.

Several studies revealed inhibition of DNA synthesis and induction of cell cycle arrest of cancer cells by protein extracts from hAM or diluted hAM-CM.[13,35] Activation of the DNA damage response pathway by increasing the expression of p53 and p21 tumor suppressors and subsequent inhibition of cyclin-dependent cyclins and kinases (CDKs) leads to cell cycle arrest.[36] In the current study, we demonstrated that G2/M phase cell cycle arrest and G2/M phase accumulation peaked following treatment with hAECs-CM in the MDA-MB-231 breast cell line, indicating the presence of factors in this condition that interfere with cell cycle controls in tumor cells. Once cell cycle

arrest occurs at the G2/M phase, it is verified that the intracellular DNA damage and errors are difficult for repairing during cell division.[37] In their work, Bu et al. indicated that hAECs endow potential anti-cancer properties on epithelial ovarian cancer, which was believed partially mediated by hAEC-secreted TGF-β1-induced cell cycle arrest,[38] suggesting that hAEC-secreted cytokines might promise for cancer treatment as an interesting agent.

Our study suggested the induction of apoptosis as another mechanism for the anti-cancer properties of AEC-derived CM that may be resulted from the paracrine effect of AECs. It seems that hAEC-CM can trigger cell apoptosis by bioactive molecules and DNA damaging agents. The balance between cell division and cell death is lost in cancer.[39–41] This problem may occur at any stage along the path of apoptosis, which plays a key role in tumor development and progression.[42]

In the abovementioned experiments, we observed that hAECs-CM effectively induced the apoptosis of MDA-MB-231 cells. According to the literature, hAECs express different Toll-like receptors (TRLs) on their surface, including TLR3, TLR4, and TLR5, which are crucial regulators of the innate immune system.[31] Ligand engagement and activation of these TLRs trigger cellular apoptosis through the nuclear factor-κB (NF-κB) signaling pathway, PI3K/AKT pathway, and induction of TGF-β1 expression, thereby inhibiting tumor cell growth, survival, proliferation, and migration.[43] Consistent with the results obtained from flow cytometry, the expression of cleaved-caspase 3, which is activated during cell apoptosis was also evaluated. Caspase family members play central roles in the regulation of apoptosis.[44] Both caspase-8 and-9, upstream of caspase-3, are the initiators of death receptor and mitochondrial pathways.[44] Caspase-3 is the key executioner caspase within the apoptosis pathway, and therefore caspase-3 activity assay can be used as a marker of apoptotic cells.[45] In this light, we show that hAECs-CM significantly induces caspase-3 activation in MDA-MB-231 cells. The exclusive activation of caspase-3 by hAECs-CM corresponded to the induction of apoptosis. In a prior study by immunocytochemistry and TUNEL assay, we confirmed a robust cytotoxic effect of the supernatant of the hAM on cancer cells by increasing the apoptosis signaling pathway and caspase-3 and caspase-8 expression.[6] Jiao et al. concluded that the decreased ratio of Bcl-2/Bax thanks to overexpression of apoptotic markers (Bax, caspase-3, and caspase 8) and low expression of anti-apoptotic marker Bcl-2 is responsible for apoptosis induction of the glioma cells after treatment with amniotic stem cells.[46] As a result, these cells promote anti-cancer effects by stimulating the caspase cascade and apoptosis-stimulating factors via the stimulation of internal and external apoptosis pathways.

Our results show the capability of conditioned media derived from hAECs to target several hallmarks of cancer cells and its potential anti-cancer effects by inducing apoptosis and stopping the cell cycle while simultaneously inhibiting proliferation and migration of cancer cells, without having a toxic effect on normal cells. Our investigations, however, cannot rule out the possibility that some of the effects are caused by exosomes rather than soluble substances. More research is needed to better analyze this point. Exosomes and microvesicles can be released by hAECs and play a role in anti-proliferative and anti-inflammatory responses and induction of cancer cells apoptosis.[47] Our further study has confirmed the existence of exosomes in hAEC-derived-CM (data not shown). Therefore, these specific proteins and microRNAs in hAECs-CM-exosomes and their function in preventing cancer progression will be identified in future studies.

## Conclusion

The study concluded that CM derived from hAECs is a potent candidate for inducing apoptosis and simultaneously inhibition of the proliferation and migration of cancer cells via inhibiting cell cycle blockade. Based on our results, hAEC-CM can be considered as a non-invasive, safe, accessible, and cost-effective option for the treatment of breast and cervical cancer in the future. Further well-designed experimental and clinical trials are needed to prove this claim as well as to elucidate the mechanisms of paracrine effects of hAECs-CM and achieve its successful clinical applications.

### Author contributions

A.J., H.N., and M.R. contributed to study design. A.J. performed the experiments, analyzed the results, wrote the manuscript. A.J., S.G., and Z.J. contributed to data collection and analysis. A.J. and R.S.F. revised the manuscript. H.N. and M.R. contributed to supervision. All authors have read and approved the final version of this manuscript.

### Declaration of conflicting interests

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## Ethical approval

Ethical approval for the study was obtained from the Research Ethics Committee of Shahid Beheshti University of Medical Sciences (approval No.: REC 1399.927).

## Informed consent

Written informed consent was obtained from all subjects before the study.

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