

Assessment the Effect of Human Umbilical Cord Wharton's Jelly Stem Cells on the Expression of Homing Genes; CXCR4 and VLA-4 in Cell Line of Breast Cancer

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ABSTRACT

Background: Breast cancer is the most common cancer in women. The prevalence of breast cancer in Western women is one in eight. Although the prevalence of breast cancer in Iran is lower than in Western countries (one in every 10-12 women), the incidence of breast cancer in it is 5-10 years earlier than in Western countries. Breast cancer is the second leading cause of cancer death among women after lung cancer. Therefore, finding new therapeutic methods could potentially help to reduce breast cancer mortality and increase the survival rate. Wharton jelly stem cells with mesenchymal morphology play an important role in inhibiting the progression of ovarian, osteosarcoma, and breast cancer by inducing apoptosis and reducing metastasis. Several environmental and genetic factors are involved in the occurrence of breast cancer. CXCR4 and VLA-4 genes are important genetic factors in breast cancer that play a role in cell survival, migration, proliferation, and metastasis of several types of cancer, especially breast cancer. Therefore, inhibition of these two genes by Wharton's Jelly Stem Cells could be a novel and effective therapeutic target in breast cancer. The aim of this study was to investigate the effect of Wharton jelly stem cells secretion on the expression of CXCR4 and VLA-4 genes in cancer cells.

Materials and Methods: These cells were exposed to Wharton's Jelly Stem Cells after culturing breast cancer cells. RNA was extracted from treated cells. The expression of CXCR4 and VLA-4 genes was evaluated by real-time PCR.

Results: The results of the MTT and Scratching tests showed a significant difference compared to the control group. Also, the results of Real-time PCR showed a significant decrease in the expression of CXCR4 and VLA-4 genes compared to the control group.

Conclusion: The results of this study showed that different concentrations of Wharton Jelly Stem Cells reduce cancer cell growth and expression of CXCR4 and VLA-4 homing genes in MDA-MB-231 breast cancer cells. Therefore, Wharton Jelly Stem Cells can be considered as an effective treatment for breast cancer.

Keywords: Wharton jelly; CXCR4; Genes, VLA-4; Breast cancer

INTRODUCTION

Human Wharton jelly Stem Cells (hWJSCs) are derived from the umbilical cord that contains stromal cells such as myofibroblasts¹. These stromal

cells originate from the ectopic mesoderm²⁻⁴. hWJSCs are mainly organized from mucopolysaccharides (hyaluronic acid and chondroitin sulfate). This gelatinous substance is

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provided to support and cushioning for the umbilical cord artery. The Wharton jelly primary mesenchymal cells are trapped at very early fetal ages and retain the primary stem cells feature and have high telomerase activity^{5,6}. Various research groups have found that hWJSCs can inhibit various human cancers⁷⁻¹⁰. In addition, hWJSCs do not cause tumor formation in mice with immunodeficiency unlike MSCs obtained from other sources¹¹. Recent studies have shown the anti-cancer activity of mesenchymal stem cells (MSC) in vivo and in vitro. Human bone marrow mesenchymal stem cells (hBMMSCs), which are infused by intravenous injection, have the inherent property of tumor suppressor in Capsicum sarcoma mice [12]. According to studies by Isova and Ganta, human and mouse umbilical cord stem cells can inhibit breast cancer^{9,13}.

Breast cancer is the most common malignant neoplasm among women, affecting one-eighth of the population worldwide. It is the second leading cause of cancer mortality. BC has increased dramatically in recent years¹⁴. Metastasis is the main cause of cancer death¹⁵. Early tumors located adjacent to the healthy tissue can be removed by surgery or radiation therapy, but when the tumor crosses physiological boundaries, these procedures are either ineffective or help to accelerate death. After cancer metastasis, the breast cancer five-year survival rate is reduced from 100% to less than 25%¹⁶. Hence, the most important achievement will be metastatic inhibition in cancer treatment. Among the various cancer types, breast cancer has the highest metastasis, with an estimated more than 90 percent of all breast cancer deaths due to metastasis¹⁷. Cancer cell metastasis to distant parts of the body involves several continuous stages, including the tumor cells' entry from primary tissue into blood vessels, survival in the bloodstream, migration to secondary organs, and proliferation of cancer cells in the target tissue¹⁸. The available treatments are mostly temporary and in most cases, the long-term survival is rare when metastases are clinically identified. Effective strategies for inhibiting and controlling metastatic progression require understanding the molecular mechanism, host-tumor interaction, and molecules that regulate the metastasis process at various stages¹⁹.

Recent studies have shown that chemokine receptors play an essential role in the metastasis process, which their abnormal expression can lead to cancer²⁰. CXCR4 expression is higher in breast cancer cells compared with normal breast tissue. Previous studies have shown that CXCR4 has an important role in cell survival, migration, proliferation, and metastasis in several types of cancer, including BC. The interaction between CXCR4 and its ligand (SDF-1) causes cancer cells to leave the bloodstream and enter other tissues, including bone, liver, and lungs, resulting in invasive tumors²¹.

The integrin $\alpha 4$ and $\beta 1$ are essential components of cell migration to the inflammation site²². The VLA4 and VLA families of integrin are located in leukocytes and not only contribute to extracellular matrix adhesion but also act as receptors for fibronectin as well as cell-to-cell adhesion receptors²³.

In the present study, we studied whether hWJMSC may reduce the MDA-MB-231 breast tumor cells growth in vitro. We treated MDA-MB-231 cells with a different concentration of hWJMSC. We also measured the CXCR4 and VLA4 genes expression involved in the metastasis process.

MATERIALS AND METHODS

Cell culture

The MDA-MB-231 cell lines. The MDA-MB-231 cell lines were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The MDA-MB-231 cell line was mixed in DMEM High glucose culture medium (GIBCO) with 100 μ l penicillin, 100 mg/ml Streptomycin (GIBCO), and 10% cow embryo serum (FBS) (GIBCO). This compound was incubated at 37. C with 5% Co₂.

Human Wharton's jelly stem cells. The umbilical cord of the fetus born by cesarean was prepared under sterile conditions and inside the physiological serum from Tabriz International Hospital. The umbilical cord was washed with 70% alcohol. Then it was crushed into 2cm small pieces under the hood and washed by PBS and HBSS buffers. Finally, the pieces were cut in half and the Wharton jelly was removed and transferred to the culture medium. Measurement of cell viability the inhibitory effect of growth inhibition and toxicity of HWJSCs using the

MTT method (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) according to the manufacturer's instructions (Sigma-Aldrich, USA).

Purification of HWJSCs from fetal umbilical cord

The fetal umbilical cord born by cesarean section was prepared from Tabriz International Hospital under sterile conditions inside a physiological serum. The umbilical cord was washed by 70% alcohol and crushed into 2cm small pieces under the hood. It was then washed by PBS and HBSS buffer and cut in half. Finally, after extracting the HWJSCs, they were transferred to the culture medium.

Exposure of MDA-MB231 cancer cells to hWJSC

To study the cell apoptosis, MDA-MB-231 cancer cell culture medium was exposed to 20% and 40% concentrations of hWJSC.

Cell morphology

Trypan blue staining assay was used to detect cells morphology treated with hWJSC compared with control. At first, 100µl uniform cell suspension was poured into a 0.2ml microtube under sterile conditions. Then, 100µl Trypan Blue was added and completely piped. Then the cells were counted by using the Neubauer Haemocytometry. (Trypan Blue is one of the staining that does not enter the living cell due to the membrane pumps and only penetrates the dead cells, so the dead cells turn blue. The living cells are seen in white).

Cell viability

Cell viability, growth inhibition, and the toxicity of HWJSCs were evaluated using the MTT assay.

Cell migration assay

The scratching test was used to evaluate the MDA-MB231 cancer cell migration rate. To perform this test, cells were cultured in DMEM high glucose medium containing 20%FBS. After sufficient growth, they were added to the medium containing 1%FBS. After 24 hours, the migration rate of the cells was photographed and measured at different hours (6, 12, 24, and 48 hours) by scraping and treating the cultured cells at 20% and 40% concentrations of HWJSCs.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The Real-time PCR method was used to evaluate the CXCR4 and VLA-4 genes expression after exposure of the MDA-MB231 cell class with 20% and 40% concentrations of HWJSCs. The B2M gene was used as an internal control gene. CXCR4 and VLA-4 genes primers are shown in Table 1.

Table 1. CXCR4 and VLA-4 genes primers

gene	sequence	size
CXCR4	F 5'CGCCACCAACAGTCAGAG 3' R 5'AACACAACCCACAAGTC 3'	177
VLA-4	F 5'CAAGAATCCAAACTACGGAC 3' R 5'TTGCATTCAAGTGTGTGGGA 3'	145

Statistical analysis

Statistical data were evaluated using Student's t-Test and Bivariate Correlate tests by SPSS V.22.0 software. All charts were plotted using GraphPad PRISM Version 6.01 software. P <0.05 was considered as the significant difference between cancer and control cells.

RESULTS

Cell morphology

Microscopic images of MDA-MB231 cells that had been apoptosis by HWJSCs, as well as images of Jelly Wharton cells and MDA-MB231 uninfected cells (Figure 1).

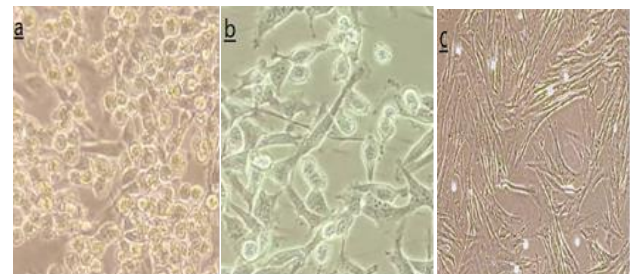


Figure1: Cell morphology. a: Cells induced by Wharton jelly that have been apoptotic. b: MDA-MB231 cancer cells without induction. Image c: Wharton jelly Stem cells (* 1000).

Cell viability

Cancer cells were treated with different doses of HWJSCs for 24 and 48 hours to investigate the toxicity effect of HWJSCs and the inhibitory concentration of IC50 on the breast cancer cells line. The MTT assay for the MDA-MB-231 cell class showed that the HWJSCs IC50 inhibitory concentration was 40µM (p <0.0001) and 20µM (p <0.0001) at 24 and 48 hours, respectively. According to the results, the difference in IC50 concentration was significant for this cell line at 24 and 48 hours (p <0.001), so it is clear that HWJSCs have an inhibitory effect on the MDA-MB-231 cell line growth (Figure 2).

Cell migration assay

To study the effect of HWJSCs on breast cancer cells migration, these cells were treated with different concentrations of HWJSCs and evaluated by a scratching test. The images were analyzed by the Cell Science software. The distance between the two sides of the scratch layer from all concentration migration images at the specified times was calculated by the Cell Science software program (Figure 3).

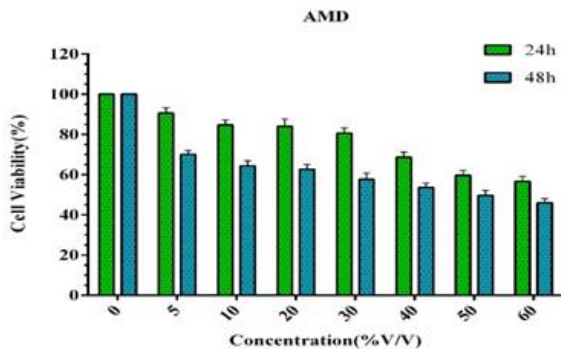


Figure 2: MDA-MB-231 cell biomass percentage diagram in treatment with different doses of HWJSCs for 24 and 48 hours

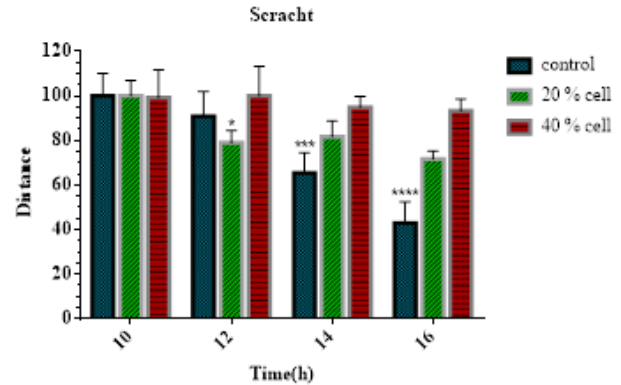


Figure3: HWJSCs effect on the cancer cells migration with Scratching test (sign * means P <0.05 and sign *** and **** means P <0.0001).

The results showed that 20% and 40% concentrations were significant compared to the control group. Images taken from concentrations at four times, 10-12-14, and 16 hours are shown in Figure 4.

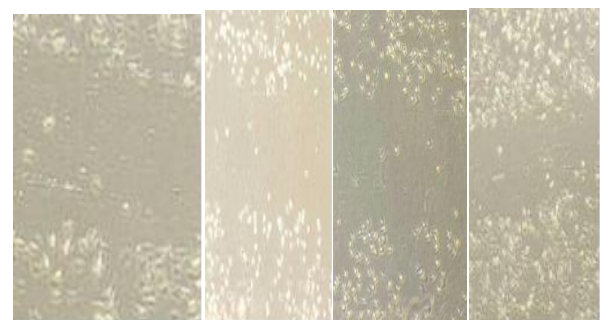
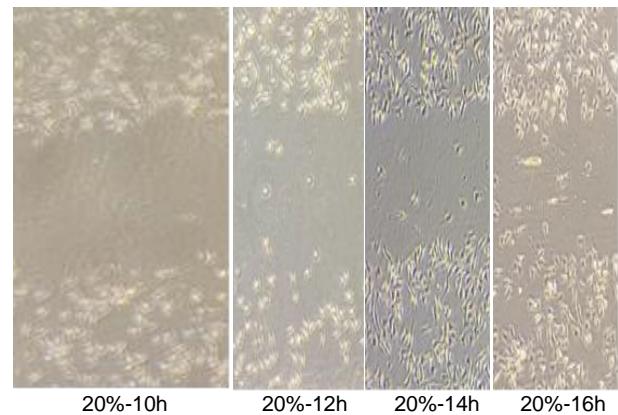


Figure4: HWJSCs effect on the cancer cells migration, with Scratching test at 20% and 40% concentration at 10 to 16 hours (* 400)

Quantitative real-time polymerase chain reaction (qRT-PCR)

The CXCR4 and VLA-4 expression in the MDA-MB-231 cell line decreased significantly after treatment with 20% and 40% concentrations of HWJSCs (P-value <0.001). The CXCR-4 expression decreased at different concentrations of HWJSCs. Therefore, CXCR4 expression at 40% concentration was higher than the 20% concentration. The VLA-4 expression that was dose-dependent in the 40% concentration of HWJSCs had a significant effect on this gene (Figure 5).

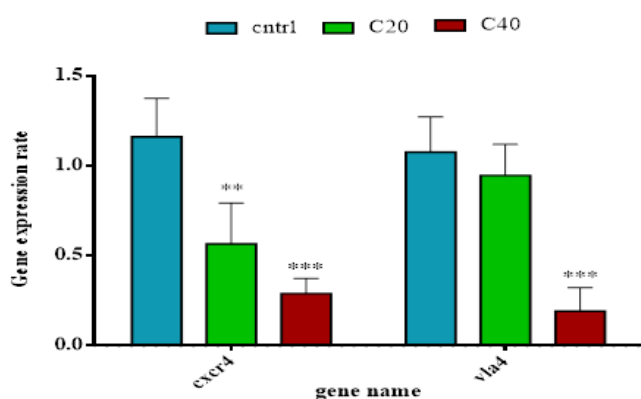


Figure 5: CXCR-4 and VLA-4 expression in the MDA-MB-231 cell lines after treatment with HWJSCs.

DISCUSSION

In our study, HWJSCs decreased the cancer cell growth and CXCR4 and VLA-4 expression involved in cancer metastasis. The MTT assay results showed that the HWJSCs decreased the biological percentage of breast cancer cell line in a dose and time-dependent manner. In the Scratching test, which was performed at 20 and 40% concentrations at 10, 12, 14, and 16 hours, the results showed that cell migration decreased with increasing time and HWJSCs concentration. This showed the anti-cancer effect of HWJSCs on cancer cells, which prevents the growth of cells at different concentrations and times. Our results were consistent with findings of Kalamegam et al and by Ganta et al⁹. Kalamegam et al. found that CL had a greater inhibitory effect on tumor cells than CM. They also reported that HWJSCs also have anti-cancer effects on a variety of cancers, including ovarian and osteosarcoma. An interesting

point in their studies was that the anti-cancer effect was different between the cell lines, so its effect was more severe on osteosarcoma and less on ovarian cancer²⁴. Various invitro and invivo studies have shown that hBMMSC and other types of MSCs inhibit tumor growth. In studies by Khakoo et al., Injection of hBMMSC derived from HWJSCs into the Kaposi sarcoma mouse model inhibited tumor growth by the dose-dependent procedure¹². Secchiero et al. showed that hBMMSC can reduce tumor growth in SCIP mice. In a 2009 study, Ayuzawa also confirmed that HWJSCs functioned in immunocompromised mice, which completely destroyed the tumor by intravenous or intramuscular injection^{13, 25}. According to previous studies on HWJSCs as well as our study, it can be said that HWJSCs have anti-cancer effects. While various studies have reported that HWJSCs play roles in different messaging mechanisms, including the initiation and transcription activity, the differential expression of functional genes, and the reprogramming of specific cells²⁴.

Apoptosis is a regulated mechanism that leads to the programmed death of damaged cells. The apoptosis in malignant cells without damaging normal cells is an effective but challenging anticancer approach²⁶. In our study, the apoptosis in the breast cancer cell lines treated with HWJSCs was morphologically demonstrated in these cells. According to the MTT assay results, MDA-MB-231 cell lines can induce apoptosis with different concentrations of Wharton jelly stem cells. In a study by Hideshima et al., SDF-1 α stimulated proliferation, migration, and resistance to apoptosis-induced dexamethasone through mitogen activation in myeloma cells²⁷. Other studies have shown that the SDF-1 / CXCR4 pathway is activated in most cancer cells²⁸. Therefore, according to our study, it can be concluded that HWJSCs can lead to apoptosis in cancer cells by affecting and preventing the SDF-1 α pathway.

The anticancer mechanism of HWJSCs has not been precisely understood. The chemokine receptors, such as CXCR-4, play an essential role in metastasis and carcinogenesis. CXCR4 expression, which plays an important role in cell survival, migration, proliferation, and metastasis is greatly increased in

breast cancer^{29, 30}. In our study, the evaluation of CXCR-4 gene expression after treatment of breast cancer cell lines with HWJSCs showed CXCR-4 decreased expression at 20 and 40 μ l concentrations. The CXCR4 expression decreased significantly at 20%, while its expression was lower at higher concentrations (40%). Our results showed that the CXCR-4 expression depends on the HWJSCs concentration which gene expression decreases further at higher concentrations. Given that the chemokine receptor CXCR4 is involved in cellular metastasis, it can be said that CXCR4 low expression reduces the cancer cell metastasis. Zhang et al. by study the effects of umbilical cord stem cells on the MDA-MB-231 cell lines Showed that umbilical cord cells were involved in the apoptosis of pulmonary metastases³¹. CXCR4 antibacterial antagonists may inhibit cancer growth. Evidence suggests that the CXCR4 receptor is involved in proliferation, invasion, and metastasis processes in breast cancer; therefore, it is considered as a diagnostic marker and a suitable therapeutic target; however, clinical information is deficient about the CXCR4 expression in breast cancer³².

In our study, the evaluation of VLA-4 gene expression after treatment of MDA-MB-231 cell lines with Wharton Jelly stem cell showed decreased expression of VLA-4 in breast cancer. There was no significant difference in gene expression at the 20% concentration, but the difference in VLA-4 expression was significant at the 40% concentration of HWJSCs, indicating that the VLA-4 gene expression depends on the HWJSCs concentration. The VLA-4 is involved in extracellular matrix adhesion as well as a receptor for fibronectin and a cell-to-cell adhesion, and has an important role in the homing process³³. Low expression of this gene leads to decrease cancer cell metastasis. Honn Tang showed that the VLA-4 expression and function change extensively in tumor cells compared to the normal ones³⁴. Similar results were found in a study conducted by Albelda et al. in 1993³⁵.

CONCLUSION

Based on our results, HWJSCs had cell proliferation inhibitory and apoptotic effect in a dose-dependent manner in MDA-MB-231 cell lines. However,

anticancer effects and regulatory mechanisms appear to be different due to different molecular features of cell lines. Several messaging mechanisms and pathways may be involved in the interaction between different cancer cells and HWJSCs. In our study, which the expression of homogenous genes in the MDA-MB-231 cell lines studied, the anti-cancer effects of Jelly Wharton have a new perspective on the cancer biology and treatment, which will be followed by other studies to confirm laboratory results as well as animal models. In previous studies, HWJSCs were converted to tumor-associated fibroblasts (TAFs) that are involved in tumor metastasis. So, the anti-tumor feature of HWJSCs, along with its resistance to TAF phenotype, makes it to become an important and safe stem cell for use in future clinical applications²⁴.

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