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Research Article
Conditioned Medium of Mesenchymal Stromal Cells Inhibits Cell Proliferation and Promotes Sensitivity of Paclitaxel in MDA-MB-231 Cells

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Abstract

Background and Objective: Triple Negative Breast Cancer (TNBC) is an aggressive malignancy with a generally poor prognosis. The conditioned medium (CM) obtained from the mesenchymal stromal cells (MSCs) under different activation conditions could inhibit the cancer progression, however, its roles in TNBC are still unclear. This study aims to investigate the effects of conditioned medium (CM) of mesenchymal stromal cells (MSCs) on the cellular proliferation and sensitivity of cancer cells to paclitaxel (PTX). **Materials and Methods:** The MDA-MB-231 cells were divided into four groups including control group, MSCs-CM group, IT-CM group and BC-CM group. The CCK-8 assay was performed to evaluate the cellular viability in each group. Nuclear count was utilized to measure the sensitivity of MDA-MB-231 to the paclitaxel (PTX). Immunofluorescence analysis was conducted to measure the survival of cancer stem cells after PTX treatment in each group. Western blot analysis was performed to analyze the roles of apoptotic pathways in this process. **Results:** The IT-CM group showed significant toxicities to MDA-MB-231 cells. Besides, IT-CM could enhance the sensitivity of MDA-MB-231 cells to PTX. The survival rates of cancer stem cells expressing $CD44^+$ showed a significant increase in the BC-CM group and IT-CM group. The MSCs-CM and IT-CM led to no significant changes in the survival rates of cancer stem cells compared with the control group. Significant down-regulation of Bcl-2 and up-regulation of cleaved caspase-3 was observed in the IT-CM group. **Conclusion:** The IT-CM rather than BC-CM could inhibit cellular proliferation and promote sensitivity of PTX in MDA-MB-231 cells.

Key words: Interferon-γ, tumor necrosis factor-α, mesenchymal stem cells, MDA-MB-231 cell, conditioned medium

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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INTRODUCTION

The therapeutic effects of mesenchymal stem cells (MSCs) have been attributed to their homing, proliferation and differentiation capabilities[1]. However, the post-implantation survival of MSCs is usually too short[2]. Less than 1% of MSCs could survive *in vivo* for more than 1 week after systemic administration, suggesting that the exogenous MSCs are mediated by a variety of biologically active factors produced by paracrine mechanisms[3].

Conditioned medium (CM) is the most convenient source of biologically active factors[4]. Besides, the obtained CMs represent the complete components of the secretome derived from MSCs under different activation conditions[5]. The CM isolated from MSCs avoids the collection procedure of invasive cells, which is more economical and practical. Besides, it can also be mass-produced through pre-activated cell lines under controlled laboratory conditions[6]. There are some reports on the tumor suppressor or tumor-promoting effects of MSCs co-cultured with tumor cells[7]. The pro-inflammatory cytokines such as Interferon-γ (IFN-γ) and Tumor Necrosis Factor-α (TNF-α) can synergistically activate MSCs[8] and the preactivated MSCs can enhance the inhibitory effects of immune cells on hematological tumors *in vitro*[9]. However, some studies have raised the concerns as the combination of IFN-γ and TNF-α preactivated MSCs can promote the proliferation and metastasis of intestinal tumor cells[10,11]. In this study, the effects of CM prepared based on the pre-treatment of Human Umbilical MSCs (HUMSCs) were investigated using IFN-γ and TNF-α on breast cancer cells and analyzed the synergistic effects of the CM on the response of cancer cells to paclitaxel (PTX).

MATERIALS AND METHODS

Study area: The study was performed in the Department of Breast and Thyroid Surgery, Central Hospital Affiliated Shandong First Medical University from April, 2020 to January 2023.

Cell culture: The HUMSCs (Cat. No.: HUXU-01001; Cyagen, Taicang, China) were cultured on a complete medium (Cat. No.: HuXU-90011; Cyagen, Taicang, China) in an incubator (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 37°C with 5% CO_2. The cells were passaged upon reaching a confluence of 70-80%. Cells of passage 3 were used for the subsequent experiments.

Construction of GFP expression HUMSCs: The GFP-expressing lentivirus (i.e., PHBLV-CMV-MCS-EF1-GFP-T2A-Puro) was purchased from HanBiotech (Seoul, Korea). The HUMSCs (1×10^5) were seeded on a 24-wells cell culture plate in 5% CO_2 at 37°C. When the cell density reached 50%, the original culture medium was aspirated, followed by adding polybrene (6 μg mL^{-1}). The transfection process was performed using LipoFiter reagent (HB-LF-1000, HanBiotech, Seoul, Korea), according to the manufacturer's instructions. About 48 hrs after transfection, the lentivirus-containing medium was replaced with a fresh medium. The expression of GFP was detected by fluorescence microscopy (Olympus, Tokyo, Japan) as described by Soboleski *et al.*[12].

Evaluation of biological function of the GFP-expressing HUMSCs: Flow cytometry was conducted to evaluate the HUMSCs labelling after lentivirus transfection. Briefly, GFP-MSCs was resuspended with DPBS containing 1% BAS until a concentration of 3×10^6 cells. Then 100 μL cell suspension was added to each centrifuge tube, followed by 2 μL of corresponding primary antibody against CD105, CD29 and CD73. Cells were resuspended with 100 μL DPBS containing 1% BAS, followed by incubating with 2 μL fluorescent secondary antibody. The samples were incubated at 4°C for 30 min in dark and were washed twice with 1 mL DPBS. Upon centrifugation at 1,000 rpm for 5 min, the supernatant was removed and the cells were resuspended with 400 μL of DPBS containing 1% BAS. Finally, the results were analyzed using FlowJo software (v10.8.1).

Preparation of preactivated CM: Three types of CM were prepared including BC-CM, IT-CM and MSCs-CM. To prepare the BC-CM, the GFP-HUMSCs (10^6 cells) were inoculated on 2.5 mL complete medium and 2.5 mL MDA-MB-231 culture supernatant (logarithmic growth phase), followed by culture for 48 hrs. For the preparation of IT-CM, the GFP-HUMSCs (10^6 cells) were incubated with 10 μL IFN-γ and TNF-α with a concentration of 100 μg mL^{-1}. For the MSCs-CM, GFP-HUMSCs (10^6 cells) were incubated with 5 mL complete medium. Finally, the medium was removed and then the FBS-free basal medium was added to each group and cultured for 12 hrs, followed by centrifugation at 1,000 rpm for 10 min under a temperature of 4°C. The supernatants were the collected preactivated CM.

Study design: To evaluate the effects of different CM on the cellular viability and differentiation capacity of cancer cells, human breast cancer MDA-MB-231 cells were divided into four groups, including, (i) Control group, cells (1×10^6 cells) cultured on complete medium, (ii) MSCs-CM group, cells (1×10^6) subjected to pretreatment using MSCs-CM, (iii) BC-CM group, cells (1×10^6) subjected to pretreatment using BC-CM

and (iv) IT-CM group, cells (1×10^6) subjected to pretreatment using IT-CM. To investigate whether the CM could synergize with the PTX against cancer cells, cells in each group were incubated with PTX for 24 hrs. Then the synergistic effects were determined.

CCK-8 assay: The CCK-8 assay was performed to determine the cellular viability after treating with different CM. In brief, cells (1×10^5) were seeded in 96-well plates and were preincubated at 37°C in 5% CO_2 in an incubator. About 24 hrs later, 10 µL of CCK-8 solution was added, followed by incubation for 2 hrs. The cellular viability was measured using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA) under a wavelength of 450 nm.

Nuclear staining with Hoechst 33342: The cell death was evaluated using nucelar staining with Hoechst 33342, according to the description by Crowley *et al.*[13]. Briefly, Hoechst 33342 was added to the cultured cells with a final concentration of 3 µg mL^{-1} and then incubated for 5 min at 37°C. The images were observed at a wavelength of 350 nm under a laser scanning confocal microscope (Olympus, Tokyo, Japan). ImageJ software (NIH, 1.49 version) was utilized for the calculation of staining cells.

Immunofluorescence analysis: This analysis was performed to evaluate the rate of cancer stem cell in each group. Cells were planted in confocal glass bottom Petri dishes and washed with PBS. The cells were incubated with 0.25% Triton X-100 in PBS for 30 min at room temperature. Then goat serum was added to the mixture, followed by incubating with normal goat serum (ZLI-9021, Sant Cruz) for 1 hr at room temperature. Blocked goat serum was diluted with primary anti-CD44 antibody (1: 300, ab254530, Abcam) and anti-CD24 antibody (1: 300, ab202073, Abcam) overnight. Subsequently, the cells were rinsed three times with PBS and incubated with the goat anti-rabbit IgG H&L (Alexa Fluor® 647) pre-adsorbed secondary antibodies for 1 hr at room temperature. The nuclei were stained with DAPI. Finally, the images were observed under a confocal microscope (Platinum Elmer Ultraview VOX) and the positive rate was calculated using IMARIS.

Western blot analysis: Total protein was extracted from MDA-MB-231 cells with commercial kit (Sigma-Aldrich, California, USA) and the protein concentration was measured with BCA method. Protein samples were subject to SDS-PAGE and were transferred to the PDVF membrane (Milipore, Sigma-Aldrich, California, USA). Then the membranes were blocked in 5% skim milk and were incubated with the primary antibodies including Bcl-2 (ab141523, Abcam) and cleaved caspase-3 (ab32042, Abcam) overnight. Subsequently, the secondary antibodies were added and incubated at 37°C for 2 hrs. The same membrane probed with beta-actin served as internal standard. All the bands were visualized using the ImageJ software, for the semi-quantitative analysis.

Statistical analysis: GraphPad Prism 7.0 statistics software was used for statistical analysis. Results are presented as Mean±Standard Deviation. Differences between groups were compared by One-way Analysis of Variance (ANOVA). The $p<0.05$ was considered statistically significant.

RESULTS

GFP-expressing HUMSCs did not change the cellular viability: The GFP-expressing HUMSCs were identified by the green fluorescence, together with the flow cytometry for the detection of cells labelling on the surface of GFP-HUMSCs after transfection (Fig. S1 and S2). Compared with HUMSCs, the cellular viability showed no statistical differences in the GFP-HUMSCs group ($p>0.05$).

Effects of different CM on the viability of MDA-MB-231 cells: In this section, we determined the cellular viability in each group at 0, 24, 48 and 72 hrs, respectively. The cellular viability in the MDA-MB-231 in MSCs-CM group showed significant decrease compared with that of control at 24, 48 and 72 hrs, respectively (Fig. 1a-b). The MDA-MB-231 cells in the IT-CM group showed significant decrease in the cellular viability at 24, 48 and 72 hrs, respectively (all $p<0.05$, Fig. 1a-b). Cells in the BC-CM group showed significant decrease in viability at 24 hrs ($p<0.05$) rather than 48 and 72 hrs ($p>0.05$) compared with the control group (Fig. 1a-b). This implied that IT-CM showed anti-cancer effects on the BC cancer cells.

IT-CM could enhance the sensitivity of MDA-MB-231 cells to PTX: Figure 2a showed the relative nuclear count of the BC cells with or without PTX treatment in each group. Before PTX treatment, there were no statistical differences in the relative nuclear count in the cells of MSCs-CM group, BC-CM group and IT-CM group compared with that of control group ($p>0.05$). In the presence of PTX, compared with the control group, there were statistical decrease in the relative nuclear count in the MSCs-CM group and BC-CM group (all $p<0.05$). In addition, significant decrease was seen in the relative nuclear count in the cells of IT-CM group after PTX treatment compared with that of the control group, MSCs-CM group and BC-MSCs-CM group (all $p<0.05$, Fig. 2b). This strongly implied that IT-CM could exert synergic effects on the killing of cancer cells by PTX.

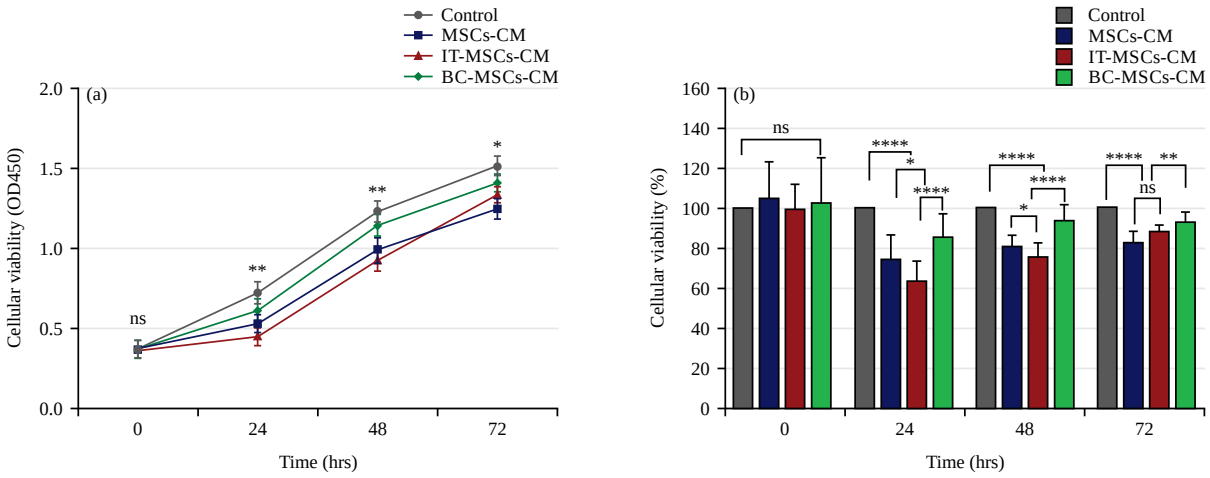

Fig. 1(a-b): Cellular viability after preatreating with MSCs-CM, IT-CM and BC-CM, (a-b) Cellular viability in the MSCs-CM group

IT-CM group and BC-CM group showed significant decrease compared with that of control at 24, 48 and 72 hrs, ^{ns}Non-significant, *p<0.05, **p<0.01 and ****p<0.01

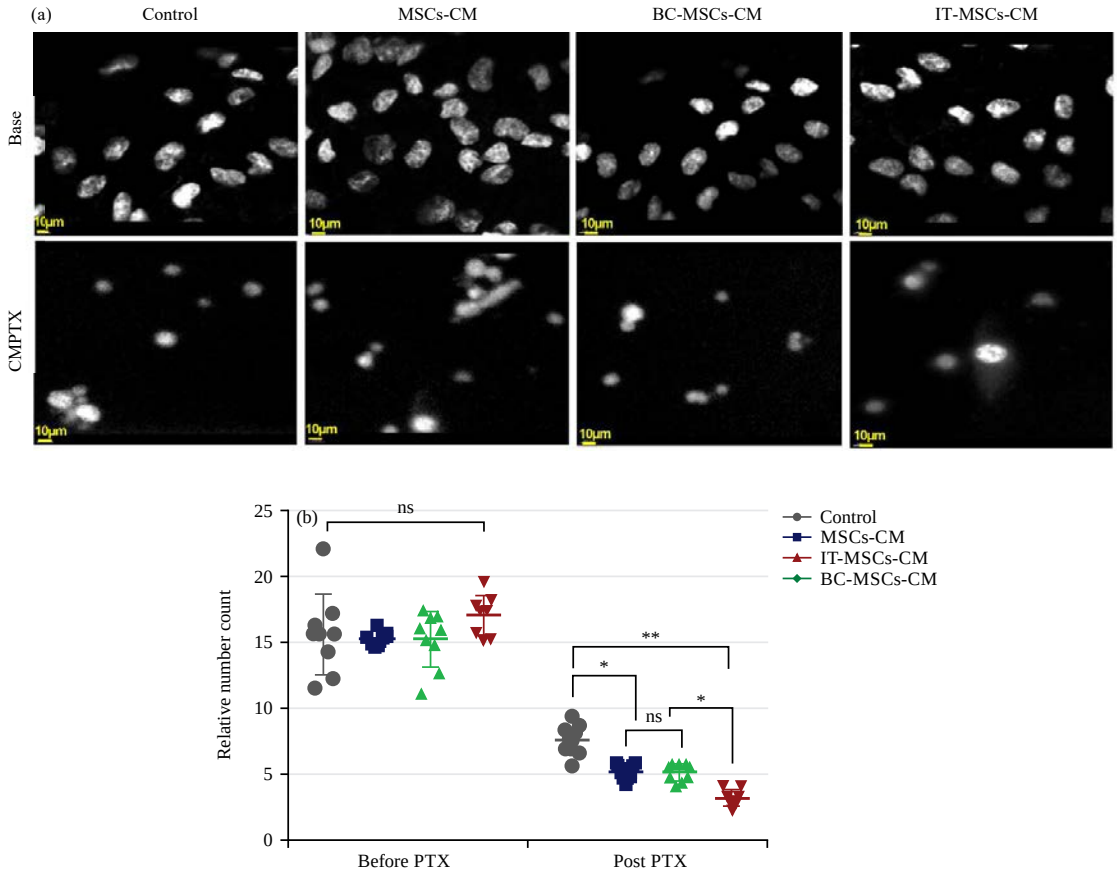

Fig. 2(a-b): Nuclear count of MDA-MB-231 cells before and after PTX treatment in each group, (a) Nuclear count among the control group, MSCs-CM group, IT-CM group and BC-CM group and (b) Compared with the control group

There were no statistical differences in the relative nuclear count in the MSCs-CM group and BC-CM group after PTX treatment (all p>0.05). In contrast, significant decrease was seen in the relative nuclear count in the cells of IT-CM group after PTX treatment compared with that of the other groups, ns: Non-significant, *p<0.05 and **p<0.01

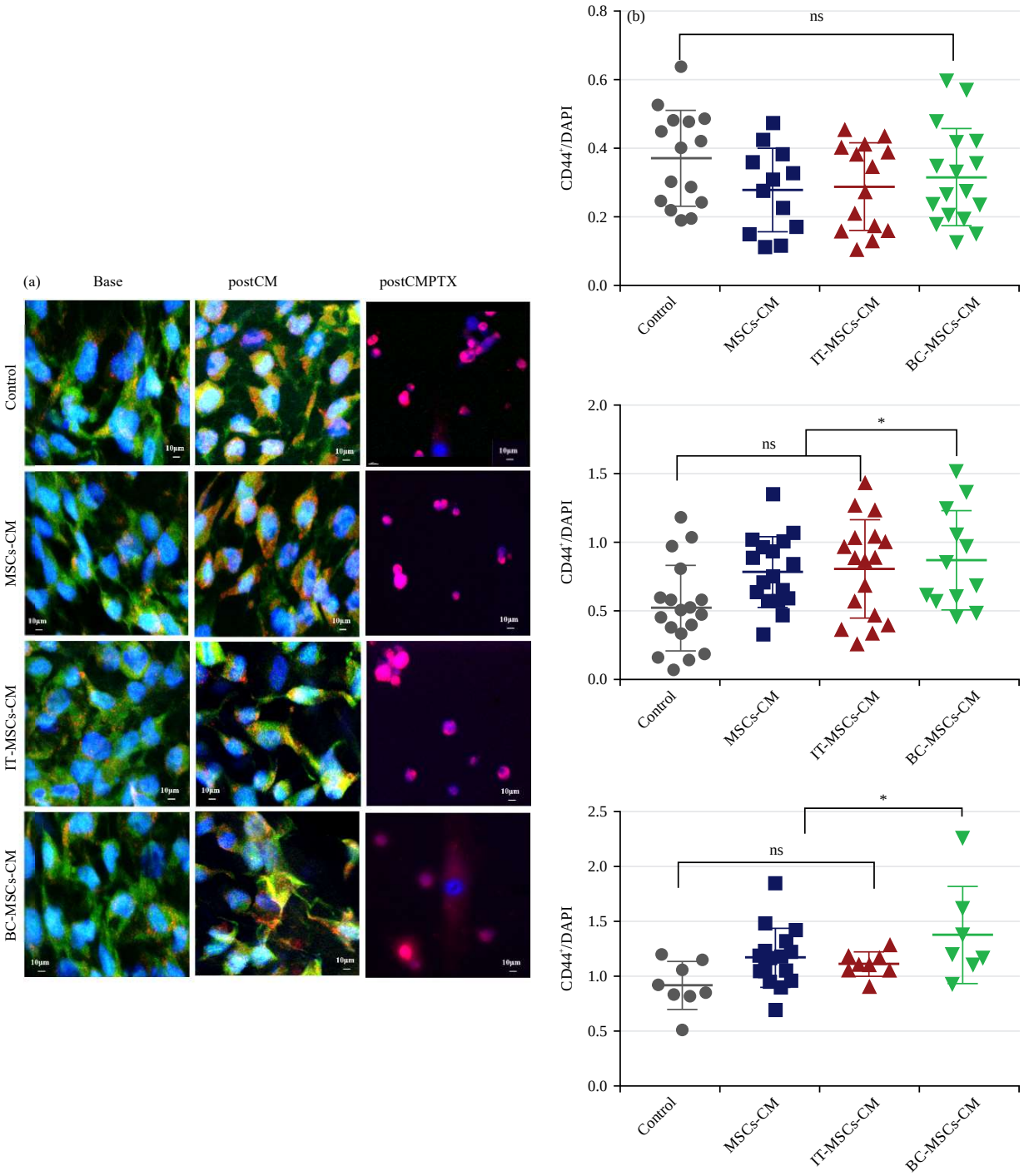

Fig. 3(a-b): Survival of cancer stem cells in each group, (a) Fluorescence staining of the cancer stem cells expressing $CD44^+$ and (b) Semi-quantitative staining of the cancer stem cells expressing $CD44^+$ in each group

The groups were Base, postCM and postCMPTX from top to bottom. Base: Group with no CM or PTX treatment, postCM: After CM treatment, postCMPTX: After PTX treatment, ns: Non-significant and *p<0.05

Survival of cancer stem cells in each group after PTX treatment: Before CM or PTX treatment, the survival of cancer stem cells in each group showed no statistical differences (p>0.05). After treating with the CM, the survival rates of cancer stem cells expressing $CD44^+$ showed significant increase in the BC-CM group compared with control group (p<0.05). After PTX treatment, the survival rate of cancer stem cells expressing $CD44^+$ in the BC-CM group showed

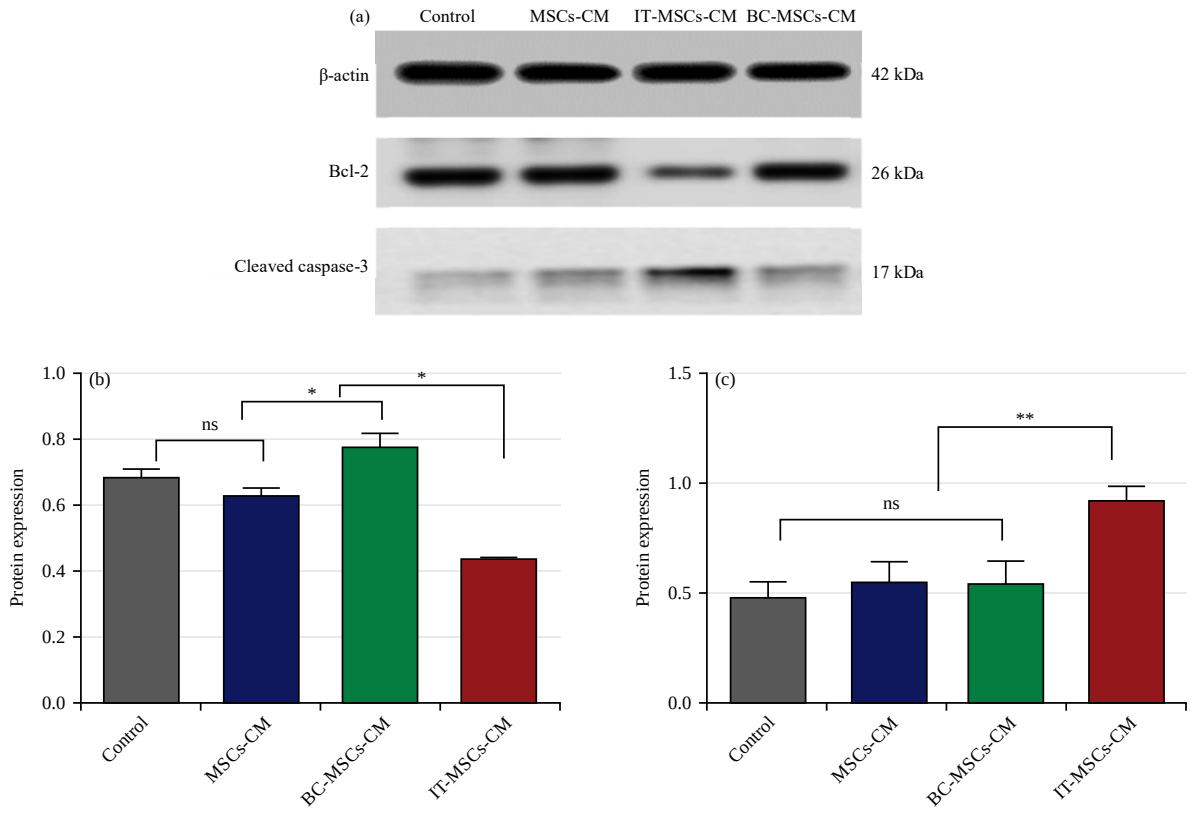

Fig. 4(a-c): Roles of apoptotic pathway in the MDA-MB-231 cells treated with different CMs, (a) Western blot analysis results and (b-c) Semi-quantitative analysis for the bands

Significant down-regulation of Bcl-2 and up-regulation of cleaved caspase-3 in the IT-CM group, **p<0.01, *p<0.05 and ns: Non-significant

significant increase compared with the other groups (all p<0.05, Fig. 3a-b). The MSCs-CM and IT-CM led to no significant changes in the survival rates of cancer stem cells compared with control group in the presence or absence of PTX (all p>0.05). All these indicated that IT-CM would not increase the residual cancer stem cells *in vitro*.

Expression of apoptotic factors in each group: To determine the potential mechanisms involved in the apoptosis mediated by the preconditioned medium, western blot analysis was performed to measure the expression of apoptotic proteins (i.e., Bcl-2 and cleavage caspase-3). The expression of Bcl-2 showed no difference in MSCs-CM group compared with the control group, while it was up-regulated in the BC-CM group compared with that of control group (p<0.05). Compared with the control group, the expression of Bcl-2 in the IT-CM group was significantly down-regulated (p<0.05). In the BC-CM group, the expression of Bcl-2 was significantly up-regulated compared with the IT-CM group and MSCs-CM group (all p<0.05, Fig. 4a-c). There were no statistical differences in the expression of cleavage caspase-3 in MSCs-CM group and BC-CM group compared with that of the control group. Whereas, significant up-regulation was seen in the expression of cleavage caspase-3 in the IT-CM group compared with the other groups (all p<0.05).

DISCUSSION

In this study, the MDA-MB-231 cells were pretreated with three CMs including MSCs-CM, BC-CM and IT-CM, respectively. Current study data showed that these CMs could inhibit the proliferation of MDA-MB-231 cells *in vitro*, especially the IT-CM. Besides, the IT-CM showed synergistic effects on the killing of MDA-MB-231 cells by PTX.

The PTX has been currently considered the standard treatment regimen for TNBC, but many patients present a high frequency of recurrence and distant metastasis. Yeh *et al.*[14] used the CM of adipose-derived MSCs from breast cancer patients to treat the MDA-MB-231 breast cancer cell line, which demonstrated that CXCL1 secreted by adipose-derived MSCs elicited doxorubicin resistance through miR-106a-mediated ABCG2 upregulation. Current study data

showed that the BC-CM and MSCs-CM showed no significant synergistic effects on PTX in MDA-MB-231 cells. It has been reported that promoting the secretion of TNF-α by immune cells and the expression of TNF-related apoptosis-inducing ligands can enhance the apoptotic effect of chemotherapeutic agents, such as gemcitabine and PTX on bladder cancer cells[15]. In this study, IFN-γ and TNF-α combined with MSCs-CM can improve the sensitivity of PTX to treat MDA-MB-231 cells, which may be related to these two factors. In the future, more studies are required to investigate the potential mechanisms in it.

The $CD44^+/CD24^-$ is the commonly used surface marker of breast cancer stem cells. As a cell surface glycoprotein and a ligand of P-selectin, CD24 mediated the interaction with endothelial cells and platelets in the process of breast cancer cell metastasis, thereby playing a role in promoting tumor progression and metastasis[16,17]. The CD44 is another cell surface glycoprotein that acts as a specific receptor for hyaluronic acid and plays a key role in the adhesion, motility, migration and invasion of breast cancer cells[18]. The CD44 is involved in efficient epithelial-mesenchymal and mesenchymal-epithelial transitions and the functional properties of CD44 explained the recurrence and high metastasis of MSCs-enriched tumors[19]. The $CD44^+/CD24^-$ cells often have a mesenchymal stem-like phenotype or mesenchymal type and are located at tumor margins. Thus, the $CD44^+/CD24^-$ phenotype in breast cancer cells has been identified as having greater tumorigenicity. It plays a major role in the invasion and metastasis of breast cancer[20]. Baccelli and Trumpp[21] reported that for TNBC, regardless of the effects of chemotherapy, breast cancer stem cells will metastasize to distant tissues or organs. In this study, various CMs were used to pretreat MDA-MB-231 cells and then PTX was used to intervene MDA-MB-231 cells. The results showed that $CD24^+$ cells were almost invisible in each group and the residual cells were all $CD44^+$ cells. This implied that tumor stem cells with a CD44-positive/CD24-negative phenotype would survive. The BC-CM could increase the percentage of CD44 positive cells in MDA-MB-231 cells, the effects of IT-CM was the same as that of MSCs-CM, which were all lower than that of BC-CM.

The PTX, a naturally occurring anti-microtubule agent, has been shown to exert important cell-killing activity in a variety of tumor cells by inducing apoptosis[22]. At present, there are three main apoptotic pathways, which are mitochondrial apoptotic pathway, death receptor pathway and endoplasmic reticulum pathway. Among them, the mitochondrial apoptotic pathway is the most classic apoptotic pathway, which is also one of the main signaling pathways for anticancer drugs to induce apoptosis[23]. The expression of apoptosis-related factors, such as Bcl-2 and cleaved caspase-3 in MDA-MB-231 cells treated with various CMs was determined. The results suggested that IT-CM could reduce the expression of Bcl-2 protein and increase the protein expression of cleaved caspase-3 after PTX intervention. The BC-CM inhibited the expression of cleaved caspase-3 and promoted the protein expression of Bcl-2. Current study found that different CMs had different effects on apoptosis and anti-apoptosis. Although the BC-CM failed to affect the expression level of cleaved caspase-3 protein in residual cancer stem cells, it promoted the expression of Bcl-2 protein, which was significantly higher than that of the control group treated with PTX alone. It was also higher than the MSCs-CM and the IT-CM. This suggested that the BC-CM may play a role in promoting cell survival by up-regulating the expression of Bcl2 protein. Meanwhile, IT-CM could down-regulate the Bcl2 expression, thereby promoting the PTX-induced apoptosis.

There are some limitations in this study. The exact mechanism of how the CMs could synergize with the PTX in MDA-MB-231 cells could not find out. In the future, the expression of certain genes may knockdown, in order to identify which gene or pathway may involve in this process.

CONCLUSION

This study was designed to investigate the effects of the different CM of HUMSCs on the cellular proliferation and PTX sensitivity of MDA-MB-231 cells. The IT-CM could inhibit the proliferation of MDA-MB-231 cells *in vitro* and present synergistic effects on PTX by increasing the sensitivity of cancer cells to PTX. This may act through targeting the apoptosis related signaling pathway. It may promote the establishment of regimens for treating breast cancer.

SIGNIFICANCE STATEMENT

Patients with TNBC usually show a poor response. The MSCs can secrete a variety of bioactive molecules according to different microenvironmental conditions. Many studies support the beneficial effects of MSCs were related to these bioactive molecules. The generated CM based on pre-activation of MSCs using culture medium of the tumor cells or the pro-inflammatory cytokines (e.g., IFN-γ and TNF-α) showed different effects on tumor cells. However, few studies have been performed in the TNBC. This study was designed to investigate the effects of the different CM of HUMSCs on the cellular proliferation and PTX sensitivity of MDA-MB-231 cells. The IT-CM could inhibit the proliferation of MDA-MB-231 cells *in vitro* and present synergistic effects on PTX by increasing the sensitivity of cancer cells.

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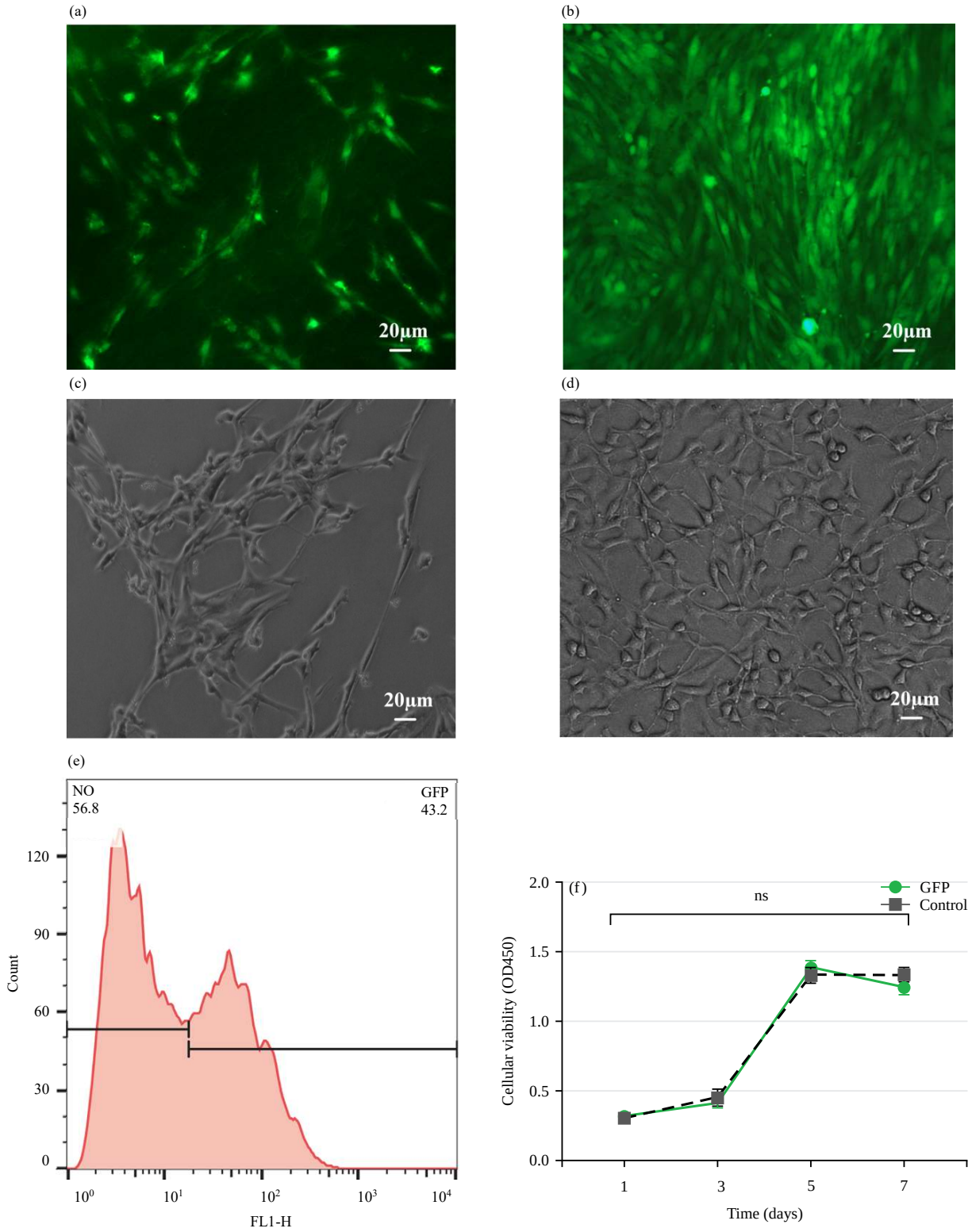

Fig. S1(a-f): Growth status of GFP-MSCs after lentivirus transfection, (a-b) GFP-MSCs on day 2 and 5 after passage, (c-d) MSCs on day 2 and 5 after passage, (e) Positive rate of GFP expression of MSCs was about 43.22% by flow cytometry sorting after lentivirus transfection and (f) Growth curve of cells in the two groups

Black line is GFP-MSCs and the gray bolt line is MSCs in the control group

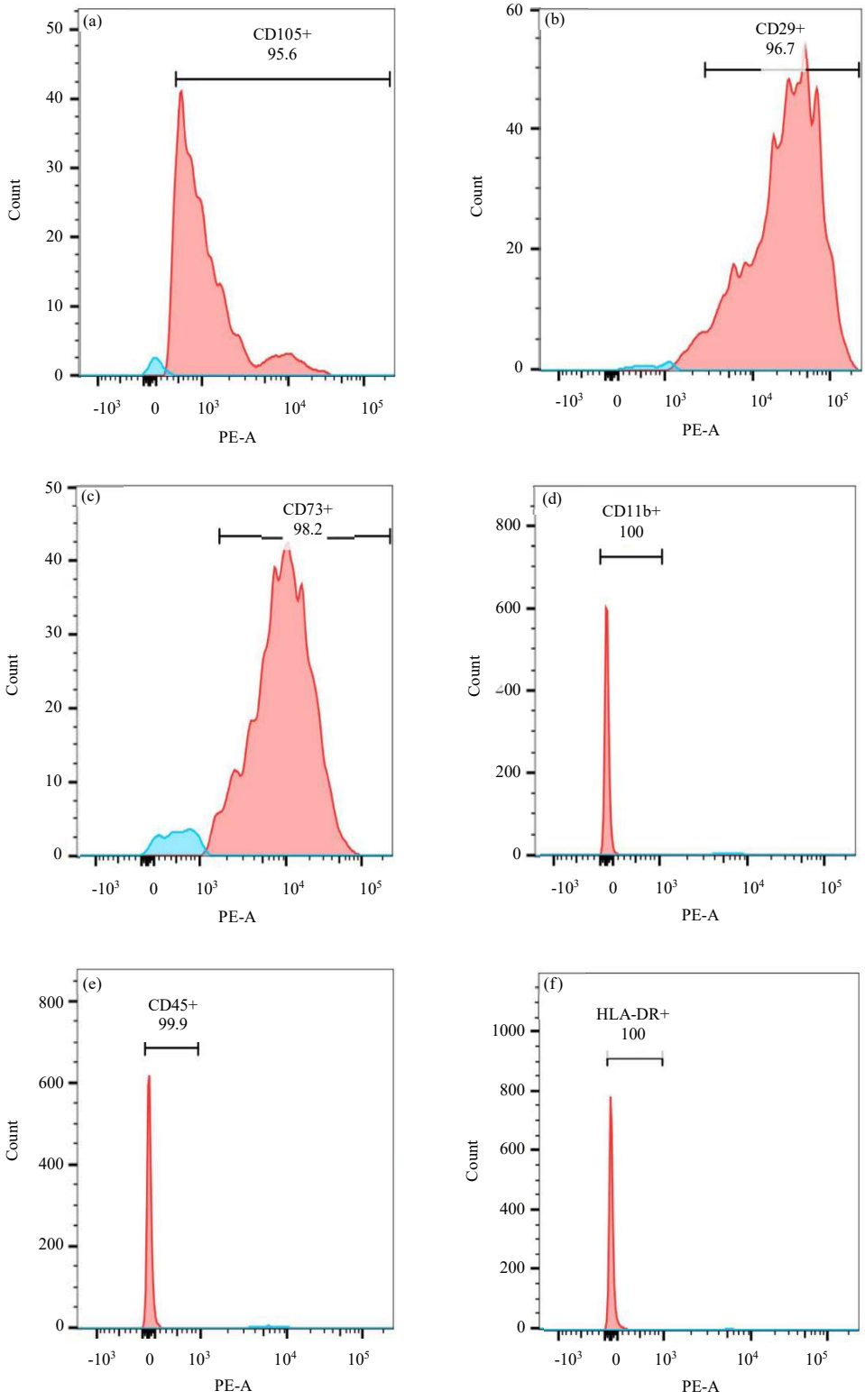

Fig. S2(a-f): Flow cytometry detection of stem cell labeling on the surface of GFP-MSCs after lentivirus transfection. The flow cytometry data were analyzed by FlowJo v10.8.1, (a) CD105 positive rates were 95.6%, (b) CD29 positive rates were 96.7%, (c) CD73 positive rates were 98.2%, (d) CD11b was negative, (e) CD45 was negative and (f) HLA-DR was negative