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Decreased Inhibition of Proliferation and Induction of Apoptosis in Breast Cancer Cell Lines (T47D and MCF7) from Treatment with Conditioned Medium Derived from Hypoxia-Treated Wharton's Jelly MSCs Compared with Normoxia-Treated MSCs

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) are an appealing source of adult stem cells for cell therapy due to the high rate of proliferation, self-renewal capability, and applicable therapy. Wharton's jelly (WJ), the main component of the umbilical cord extracellular matrix, comprises multipotent stem cells with a high proliferation rate and self-renewal capability and has anti-cancer properties. MSCs have been reported to secrete a variety of cytokines that have a cytotoxic effect in various cancers. Oxygen tension affects MSCs proliferation, cytokines level but no in surface markers expression**,** MSCs' differentiation**.**
We explored the cytotoxic effect and inducing apoptosis of Wharton's jelly derived mesenchymal stem cells (WJMSCs) secretions from normoxic WJMSCs (WJMSCs-norCM) (CM: conditioned medium) and hypoxic WJMSCs (WJMSCs-hypoCM) in breast cancer cell lines (T47D and MCF7).
Materials and Methods: Cytotoxic activity was determined using the MTS assay. RT-PCR was performed to measure the expression of apoptosis-inducing genes, specifically P53, BAX, and CASP9, and the antiapoptotic gene BCL-2.
Results: WJMSCs-norCM and WJMSCs-hypoCM were potent inhibitors of the proliferation in both cell lines. WJMSCs-norCM had more anticancer activity in T47D and MCF7. The IC_{50} value of WJMSCs-norCM on MCF7 was 42.34%, and on T47D was 42.36%. WJMSCs-norCM significantly induced the gene expression of apoptotic P53, BAX, and CASP9 and insignificantly decreased the antiapoptotic gene BCL-2 in both MCF7 and T47D cells. WJMSCs-CM has anticancer activity by inducing P53, BAX, and CASP9 apoptotic genes.
Conclusion: WJMSCs-norCM has more anticancer activity than WJMSCs-hypoCM.

Keywords: Apoptosis; Breast cancer; Conditioned medium; Hypoxia; Wharton's jelly-derived mesenchymal stem cells

INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of total cancer cases and 14% of cancer deaths[1] Mesenchymal stem cells (MSCs) emerge as a potential cell source for clinical therapy because of their bioactive potential in secreting bioactive molecules, immunomodulatory function[2], self-renewal, and differentiation into various cell lineages[3]. Human bone marrow-derived mesenchymal stem cells (hBMMSCs) are widely used, but cell isolation involves invasive procedures and a lower growth rate[4] Wharton's jelly (WJ) un-invasive collection procedure and have rapidly available donor pool, and ethically noncontroversial, WJ from the umbilical cord can be used as an alternative source of MSCs. Wharton's jelly derived mesenchymal stem cells (WJMSCs) have multipotency, do not induce teratoma, and extensive *in vitro* expansion capabilities[5], have a higher proliferation rate and self-renewal capacity than adult tissue-derived MSCs, and have short doubling time[6,7,8,9]. WJMSCs have anticancer activity in rat mammary adenocarcinoma; the rats showed an improvement after WJMSCs treatment[10]. Human Wharton's jelly-derived mesenchymal stem cells (hWJMSCs) significantly reduced BC cell line growth. An amount of 100.000 hWJMSCs can induce apoptosis on BC cell line MCF7[11]. Umbilical cord-derived mesenchymal stem cells (UCMSCs) and adipose tissue-derived mesenchymal stem cells (ADMSCs) can efficiently induce both apoptosis and differentiation in the U251 human glioma cell line[12].

Oxygen concentration is important for stem cell growth, and most human embryonic stem cells (hESCs) are exposed to the hypoxia condition *in vivo*. The hypoxia condition can minimize spontaneous differentiation and maintain hESCs pluripotency[13,14]. A hypoxic microenvironment can increase the proliferation rate and decrease the population doubling time significantly of WJMSCs[9]. Tumor cell responses to hypoxia are important for tumor progression as well as tumor therapy[15].

MSCs or MSCs conditioned medium (CM) containing microparticles mediates therapeutic effects[16]. CM from normoxia-treated WJMSCs (WJMSCs-norCM) and CM from hypoxia-treated WJMSCs (WJMSCs-hypoCM) have anticancer activity toward various cancer cell lines (HeLa, SKOV3, PC3, HSC3, and HepG2) and have no cytotoxic effect toward mouse fibroblast (NIH3T3L1), human fibroblast, and MSCs[17]. CM from human umbilical cord blood-derived MSCs (hUCBMSCs-CM) significantly inhibited melanogenesis by suppressing melanin synthesis by regulating microphthalmia-associated transcription factor expression. hUCBMSCs-CM induced extracellular signal-regulated kinase (ERK1/2) activation in melanocytes[18].

MSCs have an intrinsic property for homing toward tumor sites and can be used as tumor therapy, but very few studies investigated the antitumor properties of MSCs secretion incubated in normoxic and hypoxic conditions. We investigated WJMSCs-hypoCM and WJMSCs-norCM in inhibiting proliferation and inducing apoptosis in MCF7 and T47D cell lines.

MATERIALS AND METHODS

Isolation and Cultivation of WJMSCs

WJMSCs were isolated as previously reported [9,17,19]. An approval after the donor's written informed consent as guidelines Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia. Fresh UC (n = 3) were collected from women donors (aged 25–40 ± 7.63 years) after full-term births (normal vaginal delivery). The UC was washed with phosphate-buffered saline (PBS; 0.9% w/v sodium chloride) and cut into small explants (1–2 mm) before placing them in tissue culture plates. The explants were cultured in minimum essential medium-α (MEM-α) with 2 mM GlutaMAX (Gibco, 35050061), supplemented with 20% fetal bovine serum (FBS, Gibco, 10270106) and 1% penicillin-streptomycin (Biowest, L0018). Cultures were incubated in a humidified atmosphere with 5% CO_2 at 37 ℃, with replacing medium every 5 days until 21 days. When cells reached 80%–90% confluence, they were harvested and replated at a density of 8×10^3 cells/cm^2. WJMSCs were cultured in 95%

air (21% O_2), 5% CO_2 for normoxic and hypoxic (2.5%, 5% O_2 and 5% CO_2) conditions. Hypoxia was conducted using a tri-gas incubator (CO_2 incubators with additional process controls; BINDER GmbH, Tuttlingen) with an internal O_2 and N_2 tank changer for connecting to separate gas tanks[9,19-21]

Detection of MSCs Markers and Multipotent Differentiation

WJMSCs were incubated in hypoxia 2.5%, 5% and normoxia until P4 and P8 were measured using surface marker detection for MSCs characterization, especially CD105, CD73, CD90, CD34, CD45, CD14, CD19, and HLA-II using a flow cytometer[9,17,19,20]. Multipotent differentiation includes osteogenic, chondrogenic, and adipogenic differentiation. WJMSCs (P4 and P8) were seeded at a density of 1×10^4 cells/cm^2 in culture dishes using the StemPro Osteogenesis Differentiation Kit (Gibco, A10072-01) for 3 weeks, using the StemPro Chondrogenesis Differentiation Kit (Gibco, A10071-01) for 2 weeks, and using the StemPro Adipogenesis Differentiation Kit (Gibco, A10070-01) for 2 weeks. Calcium deposits were visualized using Alizarin red S (Amresco, 9436), chondrocytes were visualized using Alcian blue (Amresco, 0298), and adipocytes were visualized using Oil Red O (Sigma-Aldrich, U0625) [12,17,19,21,22].

WJMSCs of P4 and P8 were used for this research. The WJMSCs were seeded at a density of 8×10^3 cells/cm^2. CM was prepared by incubating the cells under normoxia (21% O_2, 5% CO_2), hypoxia 2.5% (2.5% O_2, 5% CO_2), and hypoxia 5% (5% O_2, 5% CO_2) for 72 h. When cells reached 80%–90% confluence, they were harvested. The medium was collected and centrifuged at 2000 rpm for 4 min at room temperature, and the supernatant was filtered with a 0.22 µm syringe filter (Corning, 431219) and used as WJMSCs-hypo2.5%CM, WJMSCs-hypo5%CM, and WJMSCs-norCM conserved at −80 ℃ until use[12,17,23,24].

Cell Viability Assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) cell titer 96 ® AQueous One Solution (Promega, G3581) assay was used to determine the cell viability of MCF7 (ATCC® HTB22™) and T47D (ATCC® HB133™) cell lines, which were obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. This assay used an optimized reagent containing tetrazolium converted to fluorescent formazan by viable cells that absorb light at 490 nm. The medium consists of Dulbecco's modified Eagle's medium (DMEM, Biowest, L0104) for MCF7 and Roswell Park Memorial Institute medium (RPMI 1640, Biowest, L0495) for T47D. FBS 10% (Biowest, S181H) and 1% penicillin-streptomycin (Biowest, L0018) were then incubated (21% O_2, 5% CO_2, 37 ℃). After cells reached 80% confluence, they were seeded at a density of 5×10^3 in a 96-well plate for 24 h incubation[17,25,26]. Cells were supplemented with WJMSCs-hypo2.5%CM, WJMSCs-hypo5%CM, and WJMSCs-norCM in various concentrations (0%, 20%, 40%, 60%, and 80%) and then incubated for 72 h. MTS was added at 10 µL to each well, and the plate was incubated at 21% O_2, 5% CO_2, 37 ℃ for 4 h. The absorbance of the cells was measured at 490 nm using a microplate reader (Multiskan Go, Thermo Scientific Inc.). The data were presented as the number of viable cells, the percentage of viable cell, the percentage of cells inhibition, and the data of growth rate inhibition were calculated the median inhibitory concentration (IC_{50}) [9,17,25,26].

Real-Time PCR Assay for Apoptotic Induction of T47D and MCF7

Total RNA was isolated from MCF7 and T47D cells using Aurum total RNA mini kit reagent (Bio-Rad #732-6820) based on the manufacturer's instructions. The total RNA yield was estimated using a spectrophotometer at 260 and 280 nm. RNA quality was confirmed from the results of electrophoresis, RNA purity was measured and then RNA was reverse-transcribed into

cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad #170-8841). The mixture was incubated at 25 ℃ for 5 min, at 42 °C for 30 min, and at 85 °C for 5 min. PCR amplification was performed using real-time PikoReal (Thermo Scientific Inc.). The qPCR conditions were as follows: pre-denaturing at 95 °C for 30 s, and the cycle of qPCR is 40 cycles with conditions denaturized at 95 °C for 5 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 30 s. βActin as the housekeeping gene was used as an internal control[27-29]. The primers used for real-time PCR are summarized in Table 1.

Table 1. Primers for real-time PCR

Gene	Primer sequences	
	Forward	Reverse
βActin	5′-TCT GGC ACC ACA CCT TCT ACA ATG-3′	5′-AGC ACA GCC TGG ATA GCA ACG-3′
P53	5′-AGA GTC TAT AGG CCC ACC CC-3′	5′-GCT CGA CGC TAG GAT CTG AC-3′
CASP9	5′-CAT GCT CAG GAT GTA AGC CA-3′	5′-AGG TTC TCA GAC CGG AAA CA-3′
BAX	5′-TGC TTC AGG GTT TCA TCC AG-3′	5′-GGC GGC AAT CAT CCT CTG-3′
BCL2	5′-GGT CAT GTG TGT GGA GAG CG-3′	5′-GGT GCC GGT TCA GGT ACT CA-3′

Statistical Analysis

Statistical tests were performed using the SPSS 20.0 program**.** Data were presented as means and standard deviation (M ± SD). The result was analyzed using ANOVA and continued by Tukey's honest significant difference (HSD) *post hoc* test, 95% confidence interval; a p-value < 0.05 was considered to be statistically significant. The IC_{50} value of the cytotoxic effect was analyzed using probit analysis[17].

RESULTS

WJMSCs Markers and Multipotent Differentiation

WJMSCs markers were detected using flow cytometry analysis and showed cultured cells under normoxia and hypoxia (5% O_2, 2.5% O_2) for P4 and P8. WJMSCs were positive (more than 95%) for MSCs markers CD105, CD73, and CD90 and negative (less than 2%) for CD34, CD45, CD14, CD19, and HLA-II. The surface marker expressions of WJMSCs (P4 and P8) on hypoxia and normoxia were not significantly different (data not presented). WJMSCs differentiation into osteocytes, chondrocytes, and adipocytes has been assessed (data not presented). Therefore, hWJMSCs were confirmed based on the surface marker and differentiation capability[17].

Effect of WJMSCs-hypoCM and WJMSCs-norCM toward the Growth Inhibition of BC Cells

To determine the anticancer activity of WJMSCs-norCM, WJMSCs-hypo2.5%CM, and WJMSCs-hypo5%CM from P4 and P8 toward T47D and MCF7 cell lines, we counted the inhibition of cells and IC_{50} on T47D and MCF7 cell lines by the MTS assay. The effect of WJMSCs-norCM and WJMSCs-hypoCM on the inhibition of cancer cells can be seen in Table 2. The IC_{50} value of WJMSCs-norCM and WJMSCs-hypoCM (concentration of the anticancer candidate that could inhibit 50% cell proliferation) was found to be 42.34%–62.84% (Table 3).

Table 2 shows that WJMSCs-CM was able to inhibit the proliferation of BC cells. WJMSCs-norCM and WJMSCs-hypoCM could inhibit the proliferation of BC cells significantly compared with control.

Tables 2 and 3 and Figure 1 show that WJMSCs-norCM-P4 possessed the highest anticancer activity in MCF7 and T47D cell lines with IC_{50} = 42.34% and 42.36%, respectively, and WJMSCs-hypo2.5%CM-P8 possessed the lowest anticancer activity in MCF7 and T47D cell lines with IC_{50} = 62.84% and 58.96%, respectively. WJMSCs-CM which incubation in normoxia and hypoxia both early and late passage could inhibit

BC cell proliferation with IC_{50} range 42.34%–62.84%. Figure 1 shows that control or untreated BC cells (T47D and MCF7) exhibited a high density of living cells; this figure was confirmed with the highest cell number, viability, and lowest inhibition (Table 2). BC cells (T47D and MCF7) treated with WJMSCs-norCM and WJMSCs-hypoCM exhibited lower density and abundant cell debris formation. The WJMSCs-norCM treatment had the most cytotoxic activity; it exhibited the lowest density of living cells and most cell debris formation.

Table 2. Effect of WJMSCs-norCM and WJMSCs-hypoCM toward proliferation inhibition of T47D and MCF7 cancer cells (data are expressed as means, standard deviation, Tukey's HSD *post hoc* test)

Cell lines	Oxygen tension Passage	0%	20%	Concentrations 40%	60%	80%
MCF7	WJMSCs-norCM-P4	0.00 ± 0.85 [a]	30.55 ± 1.98 [b]	44.74 ± 5.89 [c]	54.29 ± 5.85 [c]	69.96 ± 3.27 [d]
	WJMSCs-norCM-P8	0.00 ± 0.85 [a]	34.33 ± 5.37 [b]	44.22 ± 6.27 [b]	57.18 ± 5.45 [c]	69.20 ± 2.83 [c]
	WJMSCs-hypo5%CM-P4	0.00 ± 0.85 [a]	27.02 ± 4.03 [b]	35.61 ± 4.02 [c]	49.37 ± 3.02 [d]	70.46 ± 0.11 [e]
	WJMSCs-hypo5%CM-P8	0.00 ± 0.85 [a]	26.42 ± 6.92 [b]	34.53 ± 4.55 [bc]	46.53 ± 5.13 [c]	64.07 ± 3.24 [d]
	WJMSCs-hypo2.5%CM-P4	0.00 ± 0.85 [a]	27.57 ± 3.13 [b]	39.59 ± 4.54 [c]	47.56 ± 2.66 [c]	63.64 ± 2.83 [d]
	WJMSCs-hypo2.5%CM-P8	0.00 ± 0.85 [a]	27.65 ± 8.91 [b]	41.47 ± 1.47 [c]	56.33 ± 4.73 [d]	65.12 ± 2.76 [d]
T47D	WJMSCs-norCM-P4	0.00 ± 5.51 [a]	34.47 ± 2.05 [b]	40.81 ± 5.52 [b]	60.99 ± 2.19 [c]	70.00 ± 8.86 [c]
	WJMSCs-norCM-P8	0.00 ± 5.51 [a]	28.73 ± 1.54 [b]	37.65 ± 7.10 [b]	56.39 ± 3.99 [c]	64.28 ± 9.55 [c]
	WJMSCs-hypo5%CM-P4	0.00 ± 5.51 [a]	25.57 ± 6.96 [b]	35.00 ± 1.37 [b]	46.66 ± 2.61 [c]	59.64 ± 2.25 [d]
	WJMSCs-hypo5%CM-P8	0.00 ± 5.51 [a]	28.46 ± 6.19 [b]	39.05 ± 1.83 [bc]	48.05 ± 9.40 [cd]	56.27 ± 2.00 [d]
	WJMSCs-hypo2.5%CM-P4	0.00 ± 5.51 [a]	26.86 ± 7.27 [b]	34.66 ± 5.08 [b]	55.06 ± 8.89 [c]	60.47 ± 5.23 [c]
	WJMSCs-hypo2.5%CM-P8	0.00 ± 5.51 [a]	28.72 ± 6.86 [b]	38.99 ± 8.28 [bc]	52.20 ± 0.30 [cd]	61.11 ± 4.99 [d]

Data are expressed as mean ± standard deviation; different superscript small letters (a, b, bc, c, cd, d) in the same row (among concentrations of CM in T47D and MCF7 cells) are significant differences at $p < 0.05$ (Tukey's HSD *post hoc* test).

Table 3. IC_{50} of WJMSCs-norCM and WJMSCs-hypoCM in BC cell lines for 72 h incubation

Cell lines	IC_{50} (%)					
	WJMSCs-norCM		WJMSCs-hypo5%CM		WJMSCs-hypo2.5%CM	
	Passage 4	Passage 8	Passage 4	Passage 8	Passage 4	Passage 8
MCF7	42.34 ± 4.94	49.51 ± 2.13	52.10 ± 10.16	54.1 ± 3.32	57.93 ± 15.49	62.84 ± 7.95
T47D	44.87 ± 3.50	42.36 ± 2.49	51.98 ± 2.16	48.06 ± 4.08	56.27 ± 5.42	58.96 ± 7.22

Data are expressed as IC_{50} values (%); the anticancer activity of the WJMSCs-CM effect toward BC cell lines (MCF7 and T47D). The experiment was conducted in triplicate for each treatment.

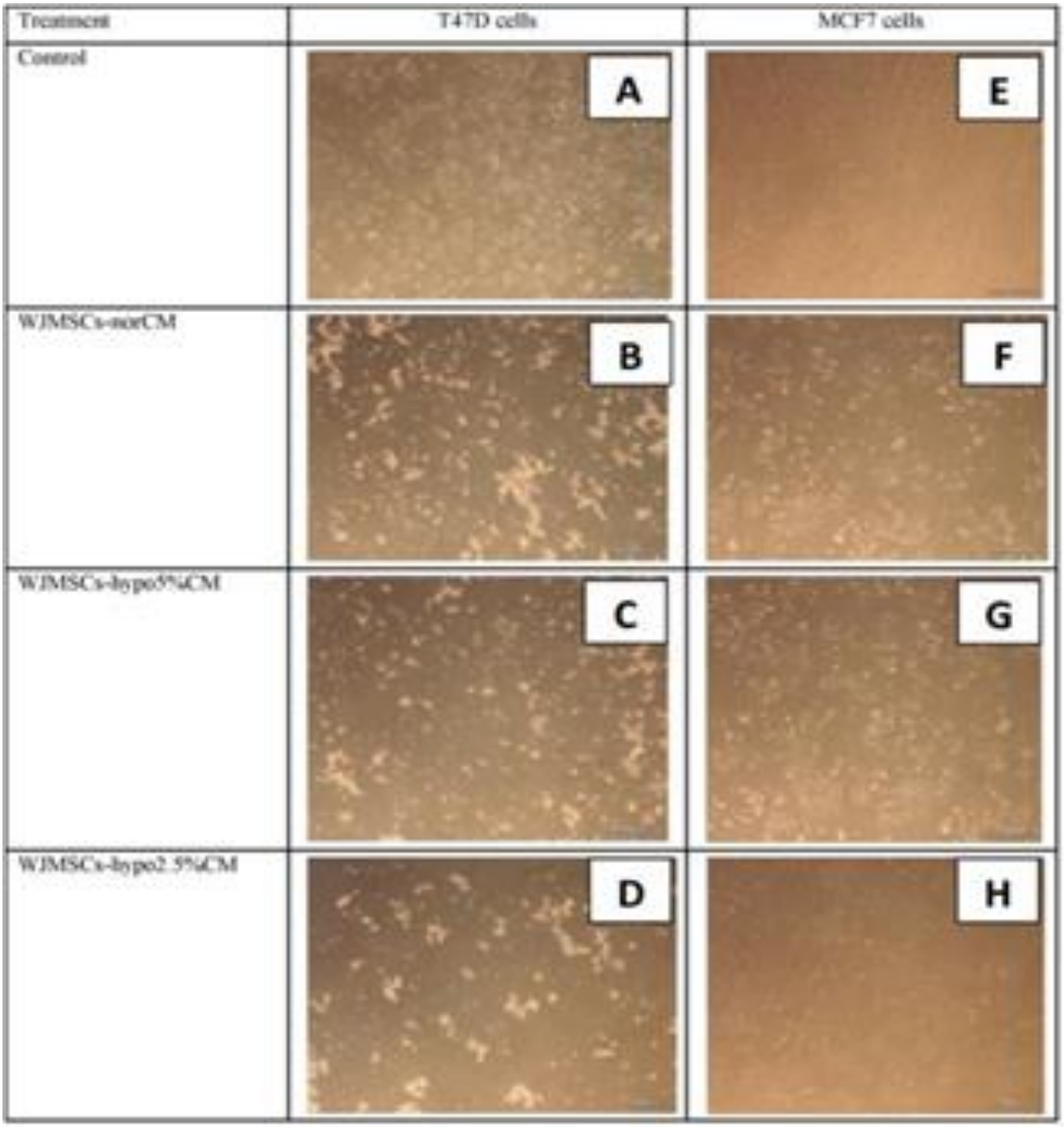

Figure 1. Morphological appearance of T47D (A–D) and MCF7 (E–H) cells treated with WJMSCs-CM using an inverted microscope. A and F: breast cancer untreated; B and F: breast cancer treated with 50% concentration of WJMSCs-norCM; C and G: breast cancer treated with 50% concentration of WJMSCs-hypo5%CM; D and H: breast cancer treated with 50% concentration of WJMSCs-hypo2.5%CM. Scale bar: 100 µm. Control or untreated cells (both T47D and MCF7) showed high density and the highest cell number compared with treated cells. Treatment WJMSCs-norCM in T47D and MCF7 showed the lowest cell number and cell debris formation compared with control, WJMSCs-hypo5%CM, and WJMSCs-hypo2.5%CM. Both T47D and MCF7 cells treated with WJMSCs-hypo2.5%CM showed the lowest anticancer activity with less cell debris formation and less cell density.

Effect of WJMSCs-hypoCM and WJMSCs-norCM toward the Apoptosis Gene Expression of BC Cells

To determine the apoptotic inducer of WJMSCs in T47D and MCF7, gene expressions were determined by RT-PCR. We continued the research for gene expression based on the anticancer activity that the average anticancer of WJMSCs-hypoCM and WJMSCs-norCM with an IC_{50} range of 42.34%–62.84%. Furthermore, we continued to measure the expression of proapoptotic genes, especially P53, BCL2-Associated X Protein (BAX), cysteine-aspartic proteases 9 (CASP9), and B-Cell CLL/Lymphoma 2 (BCL-2). Apoptotic genes were detected in both T47D and MCF7 cells using concentrations of 50% of WJMSCs-CM. The most detected P53 and BAX gene expressions in MCF7 and T47D cell lines were found at WJMSCs-norCM. WJMSCs-norCM and WJMSCs-hypoCM were insignificantly different to decrease the antiapoptotic BCL2 gene expression (Table 4). Table 4 shows that WJMSCs-CM at 50% concentration possessed the highest P53, BAX, and CASP9 expression, but there was an insignificant difference toward BCL2 gene expression in both MCF7 and T47D.

DISCUSSION

The development of specific methods for the isolation and identification of MSCs is highly important, to study the molecular mechanisms behind the multipotentiality and self-renewable capability of stem cells and also for the establishment of stem cell-based regenerative therapeutics. The environmental factor such as O_2 concentration is a key that might play a vital role in stem cell fate and function[30]. Therefore, the *in vitro* manipulation of MSCs cultured by using different O_2 concentrations in hypoxia or normoxia cultured conditions before the clinical trial is necessary to yield homogeneous cell populations with multilineage differentiation potential without causing side effects in the treatments.

The normoxia or hypoxia condition could lead to cell expansion with MSCs characteristics, and the cell's functional ability to differentiate into osteocytes, chondrocytes, and adipocytes has been assessed. The data of surface markers and multipotent differentiation of WJMSCs under normoxia and hypoxia (2.5% O_2, 5% O_2) for P4 and P8 are not presented; they have been presented previously[9,17,19]. The data of MSCs differentiated to three main mesenchymal lineages (osteocytes, chondrocytes, and adipocytes) in normoxia and hypoxia (2.5% O_2, 5% O_2) conditions for P4 and P8 are not presented; they have been presented previously[17,19] The differentiation of MSCs into osteocytes, chondrocytes, and adipocytes (trilineage) is a unique biological property of MSCs[21]. MSCs differentiated into osteoblasts most rapidly in the normoxia condition with 21% O_2, while treatment with 5% O_2 showed a reduced differentiation potential. Therefore, there was no significant difference in osteogenic markers when the O_2 concentration was 5% and 21%[31] and hypoxic incubation of WJMSCs did not change the osteogenic, chondrogenic, and adipogenic differentiation capacity [9,17].

The result of this study suggested that WJMSCs-norCM and WJMSCs-hypoCM could inhibit the proliferation of BC cell lines (both T47D and MCF7) with an IC_{50} range of 42.34%–62.84% (Table 2). This result was validated with our previous study in which WJMSCs-norCM and WJMSCs-hypo5%CM could inhibit cancer cell proliferation, including HeLa, SKOV3, PC3, HSC3, and HepG2, with an IC_{50} range of 51%–74%[17]. In the hypoxic condition, hypoxia-inducible factor-1α (HIF-1α) on metabolic regulation by suppressing mitochondrial respiration. The reduction of mitochondrial O_2 consumption was associated with decreasing reactive oxygen species production[32]. MSCs-CM inhibited the growth of several cancers, including breast adenocarcinoma, ovarian carcinoma, osteosarcoma, benign neoplastic keloid cells, bladder tumor, and lymphoma cells *in vitro*. In an

in vivo study, MSCs-CM inhibited mammary carcinoma, osteosarcoma, and pancreatic and lung tumor growth[26]. CM contains a broad variety of cytokines, growth factors, and putatively also microvesicles containing (micro) RNA, which are responsible for the beneficial effects in anticancer therapy[33,34]. WJMSCs-CM contained soluble factors such as interleukins, cell adhesion molecules, hyaluronic acid, growth factors, and glycosaminoglycans that probably possess tumoricidal activity[34-36]. The soluble factors from MSCs could also inhibit tumor angiogenesis by downregulating vascular endothelial growth factor (VEGF) expression in tumor cells[34,37]. Table 4 shows that WJMSCs-CM upregulated P53, BAX, and CASP9 gene expression in BC cell lines. These data were validated with a previous study in which hUCMSCs can induce apoptosis in the BC MDA-MB 231 cell line[38]. The BC cells (MCF7) were treated using 30% and 60% CM of co-culture WJMSCs, and IL-2-induced NK (WJMSC-CM, (WJMSCs + NK)-CM, and (WJMSCs + IL12-NK)-CM) for 24 h showed that WJMSC-CM, WJMSC-CM + NK, and (WJMSCs + IL12-NK)-CM inhibited BC cell proliferation[28]. CM from WJMSCs, WJMSCs + NK, and WJMSCs + IL12-NK induced apoptosis BC cells (respectively 33.11%, 34.70%, and 36.50%)[28].

MSCs-CM significantly inhibited proliferation, induced apoptosis, significantly upregulated the apoptotic genes of both CASP3 and CASP9, significantly downregulated the antiapoptotic genes such as SURVIVIN and X-linked inhibitor of apoptosis (XIAP), and induced and completed differentiation in the human U251 cell line[12]. The anticancer effect of WJMSCs-CM was via an apoptotic mechanism[39]. WJMSCs-CM (50%) showed increases in the sub-G1 phase; G2/M cell population and annexin V-FITC positive culture[39]. WJMSCs-CM increased the pro-apoptotic BAX gene and decreased the antiapoptotic BCL2 and SURVIVIN genes in three cancer cell lines, including human ovarian cancer cells (TOV-112D), osteosarcoma cells (MG-63), and breast adenocarcinoma cells (MDA-MB-23)[39]. MSCs-CM has the potential for autoimmune, inflammatory, and malignant diseases and tissue regeneration. The most important factors present in MSCs-CM that can be considered protagonists of MSCs physiological effects include hepatocyte growth factor (HGF), transforming growth factor-beta (TGFβ), VEGF, tumor suppressor gene (TSG6), prostaglandin E 2 (PGE2), and galectins 1 (GAL1) and GAL9[34,40]. Interferon-beta (IFNβ) in CM induces the extrinsic and intrinsic apoptosis pathways in lung cancer, lowers the CASP8 and CASP9 expression, and increases cleaved CASP3[41]. WJMSCs-CM significantly decreased cell viability and cell proliferation by inducing apoptosis and cell cycle arrest in human Burkitt's lymphoma cell line (Rams and CRL1596). The mechanism of WJMSCs-CM was activated by the secretory product-mediated induction of cell cycle arrest at sub-G1 phase and reduction in entry to G2/M, increase in oxidative stress, or decrease in glutathione peroxidase (GPX)[42]. WJMSCs-CM induced apoptosis and autophagy in human foreskin fibroblast (CCD-112sk) and significantly downregulated the expression of TBP-associated factor (TAF) in keloid cells[43]. Microvesicles of WJMSCs induced apoptosis in T24 bladder tumor cells with altered morphology, including membrane damage, cell shrinkage, blebbing, and cell debris formation[44].

WJMSCs-norCM had more anticancer activity than WJMSCs-hypo5%CM and WJMSCs-hypo2.5%CM in both MCF7 and T47D cell lines. This result was not consistent with our previous study where WJMSCs-hypoCM was more active in the cervical (HeLa), prostate (PC3), human squamous carcinoma (HSC3), and liver (HepG2) cancer cells but less active in ovarium (SKOV3) cells[17]. WJMSCs-hypoCM was less active in inhibiting BC cell proliferation and lowering apoptosis induction genes (P53, BAX, and CASP3) than WJMSCs-norCM. WJMSCs-hypo2.5%CM had the lowest anticancer activity compared with WJMSCs-norCM based on proliferation cells and apoptosis induction genes. This research was in line with previous research in which WJMSCs incubated in the hypoxic condition released higher levels of growth factor (VEGF) compared with normoxic incubation[35]. VEGFα is a member of the VEGF family and induces expression of the antiapoptotic proteins BCL2 and A1 in endothelial cells mediated by the PI3K/Akt

pathway[45]. VEGFα influences the susceptibility and aggressiveness of BC cells[46]. Several solid tumors with high levels of VEGF expression have been associated with poor clinical outcomes[47]. hBMMCs secreted VEGF 230 pg/mL in normoxia and 450 pg/mL in hypoxia conditions[48]. VEGF plays a role in angiogenesis and is important in regeneration; higher VEGF resulted in lower anticancer activity[35,49].

MSCs have been readily engineered to express antiproliferative, proapoptotic, and antiangiogenic agents that specifically target different cancer types[50]. This result was validated with previous findings in which the hypoxic preincubation of amniotic fluid MSCs (AFMSCs) induced the secretome, namely the upregulation of various secretable factors such as VEGF and TGFβ1[51-53]. Hypoxia (2% O_2) significantly upregulated the mRNA expression level of βFGF, IGF1, VEGFα, HGF, indoleamine 2,3-dioxygenase (IDO)[39,53], TGFβ2, TGFβ3, interleukin (IL1β), IL6, and IL8 of BMMSCs compared with normoxia incubation[54]. This result was in line with our previous research in which WJMSCs-hypo2.5%CM secreted the highest VEGF compared with WJMSCs-hypo5%CM and WJMSCs-norCM[34], in agreement with RT-PCR data in which hypoxia significantly increased the levels of βFGF, VEGFα, IL6, and IL8 using ELISA[34,54]. Our previous research indicated that hypoxic incubation upregulated the concentration level of VEGF, IL1, IL6, and IL8 compared with normoxia. Lower oxygen tension (2.5% O_2) resulted in higher VEGF, IL1, IL6, and IL8 than hypoxic 5% O_2 [34,55].

The IL1 network of cytokines and receptors controls the tumor cell subpopulation expression of other protumorigenic cytokines such as the angiogenic/growth factor, IL8 [56]. The IL1 family of cytokines may be important in regulating protumorigenic activities within the human BC tumor microenvironment[57]. IL1 promotes tumor growth, induces prometastatic genes such as matrix metalloproteinase (MMP), and stimulates adjacent cells to produce angiogenic proteins and growth factors such as VEGF, IL8, IL6, tumor necrosis factor (TNFα), and TGFβ[58]. IL1 contributes to tumor progression and metastasis[59]. IL6 and IL8 can directly or indirectly promote tumor growth via induction of VEGF expression[59]. Our previous research was in line with these data in which WJMSCs-norCM secreted the lowest IL1 level[34] and had higher anticancer potency.

IL8 signaling increases the proliferation and survival of endothelial and cancer cells and potentiates the migration of cancer cells, endothelial cells, and infiltrating neutrophils at the tumor site. IL8 expression correlates with the angiogenesis, tumorigenicity, and metastasis of tumors in numerous xenograft and orthotropic *in vivo* models[60]. IL8 promotes BC progression by increasing cell invasion, angiogenesis, and metastases and is upregulated in human epidermal growth factor receptor 2-positive (HER2-positive) cancers. IL8 via its cognate receptors, CXC chemokine receptor type 1 (CXCR1) and CXCR2, is also involved in regulating cancer stem cell (CSC) activity[60]. IL8 can promote CSC invasion, metastases, and treatment resistance[61]. IL8 upregulated the antiapoptotic gene BCL2, downregulated the proapoptotic gene SASP3 via the PI3K/AKT signal pathway, and significantly inhibited the apoptosis of MCF7 cells[62]. IL8 binds CXCR2 receptors, on the surface of nearby cancer cells. The binding of IL8 to CXCR2 produces signals within tumor cells that activate molecule transcription factors, including Nuclear factor kappa B (NFkB) and Activator protein-1 (AP1), via the activated protein kinase (AKT) and mitogen-activated protein kinase (MAPK) signaling pathways, ultimately causing the growth, and survival of colon cancer cells[64]. Our previous research was in line with this result in which WJMSCs-norCM secreted the lowest IL8 level[34] and had higher anticancer potency.

TGFβ contributes to the development of colorectal cancer[64]. TGFβ plays a role in regulating cancer formation and progression while acting as a cancer inhibitor in normal cells and early carcinomas. The use of TGFβ antagonists is critical for the development of novel anticancer therapies[65]. Therapeutic approaches for colorectal cancer are based on

the inhibition of TGFβ-dependent IL6 trans-signaling[64]. IL6 enhances human skin carcinoma cell invasiveness by inducing the overexpression of MMP1[66]. IL6 contributes to mediating epithelial-mesenchymal transition (EMT) and metastasis neck squamous cell carcinoma (HNSCC)[67]. Cytokines are involved in inflammation-related carcinogenesis, such as hypoxia-inducible factor (HIF1α), MMP2 and MMP9, BCLX, BCL2, VEGF, and IL6[68,69]. IL6 promotes prostate cancer cell proliferation and inhibits apoptosis via multiple signal pathways: Janus tyrosine family kinase (JAK), signal transducer and activator of transcription pathway, extracellular signal-regulated kinase 1 and 2 (ERK1/2), MAPK pathway, and phosphoinositide 3-kinase (PI3-K) pathway[69].

Table 4: Gene expression of 50% concentration of WJMSCs-hypo5%CM-P4 and WJMSCs-hypo2.5%CM-P4 in T47D and MCF7 cells for 72 h incubation

Cell line	Treatment	Gene				
		RNA purity	P53	BAX	CASP9	BCL2
MCF7	Control (untreatment)	2.4828 ± 0.3640	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00
	WJMSCs-norCM-P4	2.5885 ± 0.4776	24.67 ± 7.04 [b]	14.12 ± 2.82 [b]	8.62 ± 1.93 [b]	0.37 ± 0.20
	WJMSCs-hypo5%CM-P4	2.1204 ± 0.0627	6.08 ± 2.71 [a]	2.39 ± 0.56 [a]	5.98 ± 4.18 [a]	0.52 ± 0.24
	WJMSCs-hypo2.5%CM-P4	2.5239 ± 0.4063	5.01 ± 1.02 [a]	3.27 ± 0.81 [a]	6.15 ± 0.36 [a]	0.90 ± 0.58
T47D	Control (untreatment)	2.6439 ± 0.4787	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00
	WJMSCs-norCM-P4	2.6908 ± 0.3683	10.45 ± 0.46 [b]	3.94 ± 1.25 [b]	13.86 ± 2.57 [b]	0.11 ± 0.05
	WJMSCs-hypo5%CM-P4	2.7166 ± 0.1896	1.74 ± 0.30 [a]	1.61 ± 0.56 [ab]	14.43 ± 3.84 [b]	0.81 ± 0.10
	WJMSCs-hypo2.5%CM-P4	2.3367 ± 0.4628	1.79 ± 0.20 [a]	2.01 ± 0.05 [ab]	10.18 ± 3.00 [ab]	0.80 ± 0.09

Data are expressed as mean ± standard deviation; different letters (a, ab, b) in the same column (among WJMSCs-norCM, WJMSCs-hypo5%, and WJMSCs-hypo2.5% in T47D and MCF7 cell lines) are significant differences at $p < 0.05$ (Tukey's HSD *post hoc* test)

CONCLUSION

In conclusion, WJMSCs-hypoCM was less active in inducing apoptosis because CM contained higher cytokines, chemokines, and growth factors that increase BC proliferation. Our previous research was in line with this result : that WJMSCs-norCM secreted the lowest VEGF level[35] and had higher anticancer potency. However, WJMSCs-norCM has more anticancer activity by inducing apoptotic gene expression in BC cells (T47D and MCF7) compared with WJMSCs-hypoCM.

CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

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Decreased Inhibition of Proliferation and Induction of Apoptosis in Breast Cancer Cell Lines (T47D and MCF7) from Treatment with Conditioned

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Decreased Inhibition of Proliferation and Induction of Apoptosis in Breast Cancer Cell Lines (T47D and MCF7) from Treatment with Conditioned Medium Derived from Hypoxia-Treated Wharton's Jelly MSCs Compared with Normoxia-Treated MSCs

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) are an appealing source of adult stem cells for cell therapy due to the high rate of proliferation, self-renewal capability, and applicable therapy. Wharton's jelly (WJ), the main component of the umbilical cord extracellular matrix, comprises multipotent stem cells with a high proliferation rate and self-renewal capability and has anti-cancer properties. MSCs have been reported to secrete a variety of cytokines that have a cytotoxic effect in various cancers. Oxygen tension affects MSCs proliferation, cytokines level but no in surface markers expression, MSCs' differentiation.

We explored the cytotoxic effect and inducing apoptosis of Wharton's jelly derived mesenchymal stem cells (WJMSCs) secretions from normoxic WJMSCs (WJMSCs-norCM) (CM: conditioned medium) and hypoxic WJMSCs (WJMSCs-hypoCM) in breast cancer cell lines (T47D and MCF7).

Materials and Methods: Cytotoxic activity was determined using the MTS assay. RT-PCR was performed to measure the expression of apoptosis-inducing genes, specifically P53, BAX, and CASP9, and the antiapoptotic gene BCL-2.

Results: WJMSCs-norCM and WJMSCs-hypoCM were potent inhibitors of the proliferation in both cell lines. WJMSCs-norCM had more anticancer activity in T47D and MCF7. The IC_{50} value of WJMSCs-norCM on MCF7 was 42.34%, and on T47D was 42.36%. WJMSCs-norCM significantly induced the gene expression of apoptotic P53, BAX, and CASP9 and insignificantly decreased the antiapoptotic gene BCL-2 in both MCF7 and T47D cells. WJMSCs-CM has anticancer activity by inducing P53, BAX, and CASP9 apoptotic genes.

Conclusion: WJMSCs-norCM has more anticancer activity than WJMSCs-hypoCM.

Keywords: Apoptosis; Breast cancer; Conditioned medium; Hypoxia; Wharton's jelly-derived mesenchymal stem cells

INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of total cancer cases and 14% of cancer deaths[1] Mesenchymal stem cells (MSCs) emerge as a potential cell source for clinical therapy because of their bioactive potential in secreting bioactive molecules, immunomodulatory function[2], self-renewal, and differentiation into various cell lineages[3]. Human bone marrow-derived mesenchymal stem cells (hBMMSCs) are widely used, but cell isolation involves invasive procedures and a lower growth rate[4] Wharton's jelly (WJ) un-invasive collection procedure and have rapidly available donor pool, and ethically noncontroversial, WJ from the umbilical cord can be used as an alternative source of MSCs. Wharton's jelly derived mesenchymal stem cells (WJMSCs) have multipotency, do not induce teratoma, and extensive *in vitro* expansion capabilities[5], have a higher proliferation rate and self-renewal capacity than adult tissue-derived MSCs, and have short doubling time[6,7,8,9]. WJMSCs have anticancer activity in rat mammary adenocarcinoma; the rats showed an improvement after WJMSCs treatment[10]. Human Wharton's jelly-derived mesenchymal stem cells (hWJMSCs) significantly reduced BC cell line growth. An amount of 100.000 hWJMSCs can induce apoptosis on BC cell line MCF7[11]. Umbilical cord-derived mesenchymal stem cells (UCMSCs) and adipose tissue-derived mesenchymal stem cells (ADMSCs) can efficiently induce both apoptosis and differentiation in the U251 human glioma cell line[12].

Oxygen concentration is important for stem cell growth, and most human embryonic stem cells (hESCs) are exposed to the hypoxia condition *in vivo*. The hypoxia condition can minimize spontaneous differentiation and maintain hESCs pluripotency[13,14]. A hypoxic microenvironment can increase the proliferation rate and decrease the population doubling time significantly of WJMSCs[9]. Tumor cell responses to hypoxia are important for tumor progression as well as tumor therapy[15].

MSCs or MSCs conditioned medium (CM) containing microparticles mediates therapeutic effects[16]. CM from normoxia-treated WJMSCs (WJMSCs-norCM) and CM from hypoxia-treated WJMSCs (WJMSCs-hypoCM) have anticancer activity toward various cancer cell lines (HeLa, SKOV3, PC3, HSC3, and HepG2) and have no cytotoxic effect toward mouse fibroblast (NIH3T3L1), human fibroblast, and MSCs[17]. CM from human umbilical cord blood-derived MSCs (hUCBMSCs-CM) significantly inhibited melanogenesis by suppressing melanin synthesis by regulating microphthalmia-associated transcription factor expression. hUCBMSCs-CM induced extracellular signal-regulated kinase (ERK1/2) activation in melanocytes[18].

MSCs have an intrinsic property for homing toward tumor sites and can be used as tumor therapy, but very few studies investigated the antitumor properties of MSCs secretion incubated in normoxic and hypoxic conditions. We investigated WJMSCs-hypoCM and WJMSCs-norCM in inhibiting proliferation and inducing apoptosis in MCF7 and T47D cell lines.

MATERIALS AND METHODS

Isolation and Cultivation of WJMSCs

WJMSCs were isolated as previously reported [9,17,19]. An approval after the donor's written informed consent as guidelines Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia. Fresh UC (n = 3) were collected from women donors (aged 25–40 ± 7.63 years) after full-term births (normal vaginal delivery). The UC was washed with phosphate-buffered saline (PBS; 0.9% w/v sodium chloride) and cut into small explants (1–2 mm) before placing them in tissue culture plates. The explants were cultured in minimum essential medium-α (MEM-α) with 2 mM GlutaMAX (Gibco, 35050061), supplemented with 20% fetal bovine serum (FBS, Gibco, 10270106) and 1% penicillin-streptomycin (Biowest, L0018). Cultures were incubated in a humidified atmosphere with 5% CO_2 at 37 °C, with replacing medium every 5 days until 21 days. When cells reached 80%–90% confluence, they were harvested and replated at a density of 8×10^3 cells/cm^2. WJMSCs were cultured in 95%

air (21% O_2), 5% CO_2 for normoxic and hypoxic (2.5%, 5% O_2 and 5% CO_2) conditions. Hypoxia was conducted using a tri-gas incubator (CO_2 incubators with additional process controls; BINDER GmbH, Tuttlingen) with an internal O_2 and N_2 tank changer for connecting to separate gas tanks[9,19-21]

Detection of MSCs Markers and Multipotent Differentiation

WJMSCs were incubated in hypoxia 2.5%, 5% and normoxia until P4 and P8 were measured using surface marker detection for MSCs characterization, especially CD105, CD73, CD90, CD34, CD45, CD14, CD19, and HLA-II using a flow cytometer[9,17,19,20]. Multipotent differentiation includes osteogenic, chondrogenic, and adipogenic differentiation. WJMSCs (P4 and P8) were seeded at a density of 1×10^4 cells/cm^2 in culture dishes using the StemPro Osteogenesis Differentiation Kit (Gibco, A10072-01) for 3 weeks, using the StemPro Chondrogenesis Differentiation Kit (Gibco, A10071-01) for 2 weeks, and using the StemPro Adipogenesis Differentiation Kit (Gibco, A10070-01) for 2 weeks. Calcium deposits were visualized using Alizarin red S (Amresco, 9436), chondrocytes were visualized using Alcian blue (Amresco, 0298), and adipocytes were visualized using Oil Red O (Sigma-Aldrich, U0625) [12,17,19,21,22].

WJMSCs of P4 and P8 were used for this research. The WJMSCs were seeded at a density of 8×10^3 cells/cm^2. CM was prepared by incubating the cells under normoxia (21% O_2, 5% CO_2), hypoxia 2.5% (2.5% O_2, 5% CO_2), and hypoxia 5% (5% O_2, 5% CO_2) for 72 h. When cells reached 80%–90% confluence, they were harvested. The medium was collected and centrifuged at 2000 rpm for 4 min at room temperature, and the supernatant was filtered with a 0.22 µm syringe filter (Corning, 431219) and used as WJMSCs-hypo2.5%CM, WJMSCs-hypo5%CM, and WJMSCs-norCM conserved at –80 °C until use[12,17,23,24].

Cell Viability Assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) cell titer 96 ® AQueous One Solution (Promega, G3581) assay was used to determine the cell viability of MCF7 (ATCC® HTB22™) and T47D (ATCC® HB133™) cell lines, which were obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. This assay used an optimized reagent containing tetrazolium converted to fluorescent formazan by viable cells that absorb light at 490 nm. The medium consists of Dulbecco's modified Eagle's medium (DMEM, Biowest, L0104) for MCF7 and Roswell Park Memorial Institute medium (RPMI 1640, Biowest, L0495) for T47D. FBS 10% (Biowest, S181H) and 1% penicillin-streptomycin (Biowest, L0018) were then incubated (21% O_2, 5% CO_2, 37 °C). After cells reached 80% confluence, they were seeded at a density of 5×10^3 in a 96-well plate for 24 h incubation[17,25,26]. Cells were supplemented with WJMSCs-hypo2.5%CM, WJMSCs-hypo5%CM, and WJMSCs-norCM in various concentrations (0%, 20%, 40%, 60%, and 80%) and then incubated for 72 h. MTS was added at 10 µL to each well, and the plate was incubated at 21% O_2, 5% CO_2, 37 °C for 4 h. The absorbance of the cells was measured at 490 nm using a microplate reader (Multiskan Go, Thermo Scientific Inc.). The data were presented as the number of viable cells, the percentage of viable cell, the percentage of cells inhibition, and the data of growth rate inhibition were calculated the median inhibitory concentration (IC_{50}) [17,25,26].

Real-Time PCR Assay for Apoptotic Induction of T47D and MCF7

Total RNA was isolated from MCF7 and T47D cells using Aurum total RNA mini kit reagent (Bio-Rad #732-6820) based on the manufacturer's instructions. The total RNA yield was estimated using a spectrophotometer at 260 and 280 nm. RNA quality was confirmed from the results of electrophoresis, RNA purity was measured and then RNA was reverse-transcribed into

cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad #170-8841). The mixture was incubated at 25 °C for 5 min, at 42 °C for 30 min, and at 85 °C for 5 min. PCR amplification was performed using real-time PikoReal (Thermo Scientific Inc.). The qPCR conditions were as follows: pre-denaturing at 95 °C for 30 s, and the cycle of qPCR is 40 cycles with conditions denaturized at 95 °C for 5 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 30 s. βActin as the housekeeping gene was used as an internal control[27-29]. The primers used for real-time PCR are summarized in Table 1.

Table 1. Primers for real-time PCR

Gene	Primer sequences	
	Forward	Reverse
βActin	5′-TGT GGC ACC ACA CCT TCT ACA ATG-3′	5′-AGC ACA GCC TGG ATA GCA ACG-3′
P53	5′-AGA GTC TAT AGG CCC ACC CC-3′	5′-GCT CGA CGC TAG GAT CTG AC-3′
CASP9	5′-CAT GCT CAG GAT GTA AGC CA-3′	5′-GG TTC TCA GAC CGG AAA CA-3′
BAX	5′-TGC TTC AGG GTT TCA TCC AG-3′	5′-GGC GGC AAT CAT CCT CTG-3′
BCL2	5′-GGT CAT GTG TGT GGA GAG CG-3′	5′-GGT GCC GGT TCA GGT ACT CA-3′

Statistical Analysis

Statistical tests were performed using the SPSS 20.0 program. Data were presented as means and standard deviation (M ± SD). The result was analyzed using ANOVA and continued by Tukey's honest significant difference (HSD) *post hoc* test, 95% confidence interval; a p-value < 0.05 was considered to be statistically significant. The IC_{50} value of the cytotoxic effect was analyzed using probit analysis[17].

RESULTS

WJMSCs Markers and Multipotent Differentiation

WJMSCs markers were detected using flow cytometry analysis and showed cultured cells under normoxia and hypoxia (5% O_2, 2.5% O_2) for P4 and P8. WJMSCs were positive (more than 95%) for MSCs markers CD105, CD73, and CD90 and negative (less than 2%) for CD34, CD45, CD14, CD19, and HLA-II. The surface marker expressions of WJMSCs (P4 and P8) on hypoxia and normoxia were not significantly different (data not presented). WJMSCs differentiation into osteocytes, chondrocytes, and adipocytes has been assessed (data not presented). Therefore, hWJMSCs were confirmed based on the surface marker and differentiation capability[17].

Effect of WJMSCs-hypoCM and WJMSCs-norCM toward the Growth Inhibition of BC Cells

To determine the anticancer activity of WJMSCs-norCM, WJMSCs-hypo2.5%CM, and WJMSCs-hypo5%CM from P4 and P8 toward T47D and MCF7 cell lines, we counted the inhibition of cells and IC_{50} on T47D and MCF7 cell lines by the MTS assay. The effect of WJMSCs-norCM and WJMSCs-hypoCM on the inhibition of cancer cells can be seen in Table 2. The IC_{50} value of WJMSCs-norCM and WJMSCs-hypoCM (concentration of the anticancer candidate that could inhibit 50% cell proliferation) was found to be 42.34%–62.84% (Table 3).

Table 2 shows that WJMSCs-CM was able to inhibit the proliferation of BC cells. WJMSCs-norCM and WJMSCs-hypoCM could inhibit the proliferation of BC cells significantly compared with control.

Tables 2 and 3 and Figure 1 show that WJMSCs-norCM-P4 possessed the highest anticancer activity in MCF7 and T47D cell lines with IC_{50} = 42.34% and 42.36%, respectively, and WJMSCs-hypo2.5%CM-P8 possessed the lowest anticancer activity in MCF7 and T47D cell lines with IC_{50} = 62.84% and 58.96%, respectively. WJMSCs-CM which incubation in normoxia and hypoxia both early and late passage could inhibit

BC cell proliferation with IC_{50} range 42.34%–62.84%. Figure 1 shows that control or untreated BC cells (T47D and MCF7) exhibited a high density of living cells; this figure was confirmed with the highest cell number, viability, and lowest inhibition (Table 2). BC cells (T47D and MCF7) treated with WJMSCs-norCM and WJMSCs-hypoCM exhibited lower density and abundant cell debris formation. The WJMSCs-norCM treatment had the most cytotoxic activity; it exhibited the lowest density of living cells and most cell debris formation.

Table 2. Effect of WJMSCs-norCM and WJMSCs-hypoCM toward proliferation inhibition of T47D and MCF7 cancer cells
(data are expressed as means, standard deviation, Tukey's HSD *post hoc* test)

Cell lines	Oxygen tension Passage	Concentrations 0%	20%	40%	60%	80%
MCF7	WJMSCs-norCM-P4	0.00 ± 0.85 ^{a}	30.55 ± 1.98 ^{b}	44.74 ± 5.89 ^{c}	54.29 ± 5.85 ^{c}	69.96 ± 3.27 ^{d}
	WJMSCs-norCM-P8	0.00 ± 0.85 ^{a}	34.33 ± 5.37 ^{b}	44.22 ± 6.27 ^{b}	57.18 ± 5.45 ^{c}	69.20 ± 2.83 ^{c}
	WJMSCs-hypo5%CM-P4	0.00 ± 0.85 ^{a}	27.02 ± 4.03 ^{b}	35.61 ± 4.02 ^{c}	49.37 ± 3.02 ^{d}	70.46 ± 0.11 ^{e}
	WJMSCs-hypo5%CM-P8	0.00 ± 0.85 ^{a}	26.42 ± 6.92 ^{b}	34.53 ± 4.55 ^{bc}	46.53 ± 5.13 ^{c}	64.07 ± 3.24 ^{d}
	WJMSCs-hypo2.5%CM-P4	0.00 ± 0.85 ^{a}	27.57 ± 3.13 ^{b}	39.59 ± 4.54 ^{c}	47.56 ± 2.66 ^{c}	63.64 ± 2.83 ^{d}
	WJMSCs-hypo2.5%CM-P8	0.00 ± 0.85 ^{a}	27.65 ± 8.91 ^{b}	41.47 ± 1.47 ^{c}	56.33 ± 4.73 ^{d}	65.12 ± 2.76 ^{d}
T47D	WJMSCs-norCM-P4	0.00 ± 5.51 ^{a}	34.47 ± 2.05 ^{b}	40.81 ± 5.52 ^{b}	60.99 ± 2.19 ^{c}	70.00 ± 8.86 ^{c}
	WJMSCs-norCM-P8	0.00 ± 5.51 ^{a}	28.73 ± 1.54 ^{b}	37.65 ± 7.10 ^{b}	56.39 ± 3.99 ^{c}	64.28 ± 9.55 ^{c}
	WJMSCs-hypo5%CM-P4	0.00 ± 5.51 ^{a}	25.57 ± 6.96 ^{b}	35.00 ± 1.37 ^{b}	46.66 ± 2.61 ^{c}	59.64 ± 2.25 ^{d}
	WJMSCs-hypo5%CM-P8	0.00 ± 5.51 ^{a}	28.46 ± 6.19 ^{b}	39.05 ± 1.83 ^{bc}	48.05 ± 9.40 ^{cd}	56.27 ± 2.00 ^{d}
	WJMSCs-hypo2.5%CM-P4	0.00 ± 5.51 ^{a}	26.86 ± 7.27 ^{b}	34.66 ± 5.08 ^{b}	55.06 ± 8.89 ^{c}	60.47 ± 5.23 ^{c}
	WJMSCs-hypo2.5%CM-P8	0.00 ± 5.51 ^{a}	28.72 ± 6.86 ^{b}	38.99 ± 8.28 ^{bc}	52.20 ± 0.30 ^{cd}	61.11 ± 4.99 ^{d}

Data are expressed as mean ± standard deviation; different superscript small letters (a, b, bc, c, cd, d) in the same row (among concentrations of CM in T47D and MCF7 cells) are significant differences at $p < 0.05$ (Tukey's HSD *post hoc* test).

Table 3. IC_{50} of WJMSCs-norCM and WJMSCs-hypoCM in BC cell lines for 72 h incubation

Cell lines	IC_{50} (%)					
	WJMSCs-norCM		WJMSCs-hypo5%CM		WJMSCs-hypo2.5%CM	
	Passage 4	Passage 8	Passage 4	Passage 8	Passage 4	Passage 8
MCF7	42.34 ± 4.94	49.51 ± 2.13	52.10 ± 10.16	54.1 ± 3.32	57.93 ± 15.49	62.84 ± 7.95
T47D	44.87 ± 3.50	42.36 ± 2.49	51.98 ± 2.16	48.06 ± 4.08	56.27 ± 5.42	58.96 ± 7.22

Data are expressed as IC_{50} values (%); the anticancer activity of the WJMSCs-CM effect toward BC cell lines (MCF7 and T47D). The experiment was conducted in triplicate for each treatment.

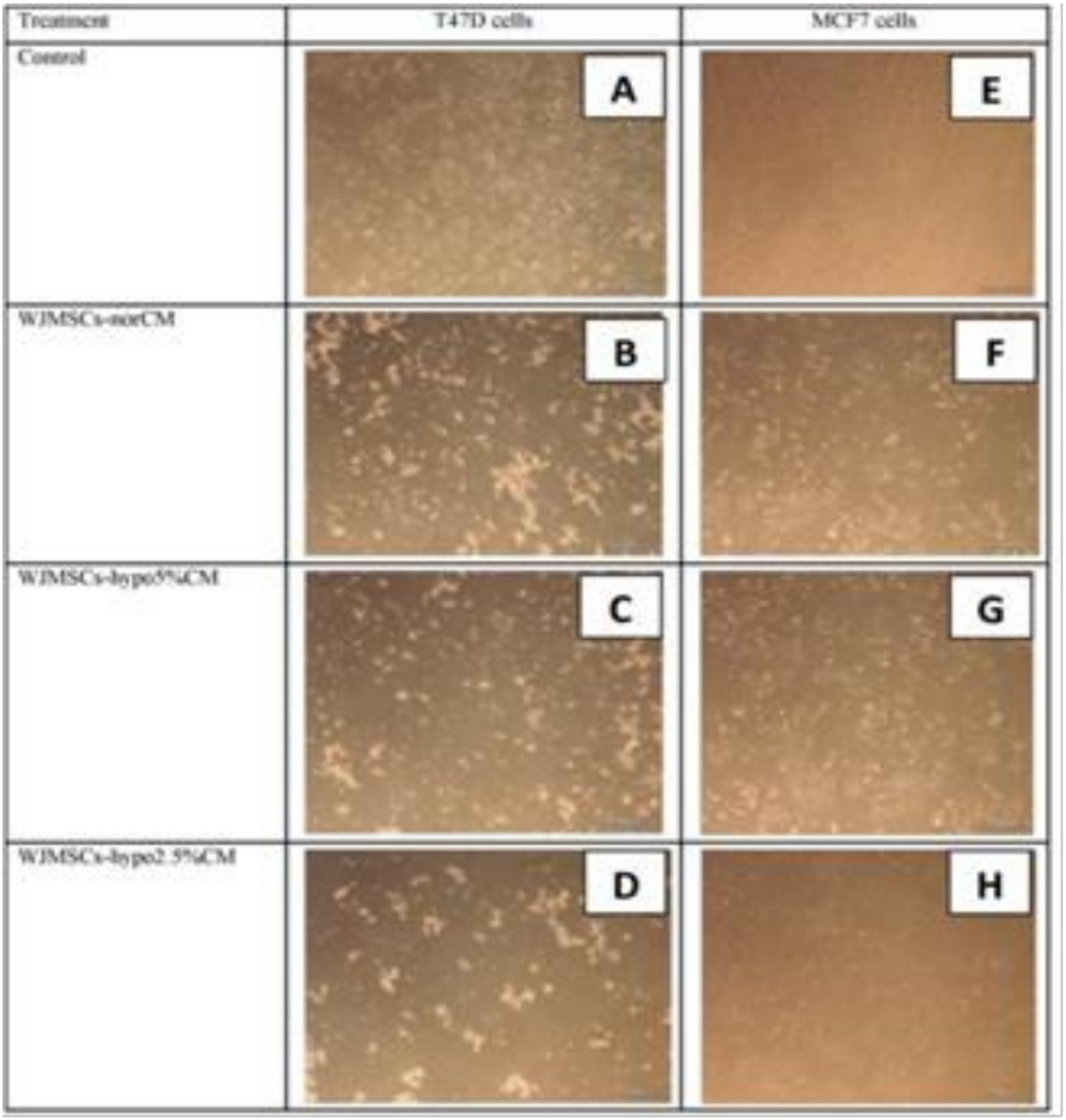

Figure 1. Morphological appearance of T47D (A–D) and MCF7 (E–H) cells treated with WJMSCs-CM using an inverted microscope. A and F: breast cancer untreated; B and F: breast cancer treated with 50% concentration of WJMSCs-norCM; C and G: breast cancer treated with 50% concentration of WJMSCs-hypo5%CM; D and H: breast cancer treated with 50% concentration of WJMSCs-hypo2.5%CM. Scale bar: 100 μm. Control or untreated cells (both T47D and MCF7) showed high density and the highest cell number compared with treated cells. Treatment WJMSCs-norCM in T47D and MCF7 showed the lowest cell number and cell debris formation compared with control, WJMSCs-hypo5%CM, and WJMSCs-hypo2.5%CM. Both T47D and MCF7 cells treated with WJMSCs-hypo2.5%CM showed the lowest anticancer activity with less cell debris formation and less cell density.

Effect of WJMSCs-hypoCM and WJMSCs-norCM toward the Apoptosis Gene Expression of BC Cells

To determine the apoptotic inducer of WJMSCs in T47D and MCF7, gene expressions were determined by RT-PCR. We continued the research for gene expression based on the anticancer activity that the average anticancer of WJMSCs-hypoCM and WJMSCs-norCM with an IC_{50} range of 42.34%–62.84%. Furthermore, we continued to measure the expression of proapoptotic genes, especially P53, BCL2-Associated X Protein (BAX), cysteine-aspartic proteases 9 (CASP9), and B-Cell CLL/Lymphoma 2 (BCL-2). Apoptotic genes were detected in both T47D and MCF7 cells using concentrations of 50% of WJMSCs-CM. The most detected P53 and BAX gene expressions in MCF7 and T47D cell lines were found at WJMSCs-norCM. WJMSCs-norCM and WJMSCs-hypoCM were insignificantly different to decrease the antiapoptotic BCL2 gene expression (Table 4). Table 4 shows that WJMSCs-CM at 50% concentration possessed the highest P53, BAX, and CASP9 expression, but there was an insignificant difference toward BCL2 gene expression in both MCF7 and T47D.

DISCUSSION

The development of specific methods for the isolation and identification of MSCs is highly important, to study the molecular mechanisms behind the multipotentiality and self-renewable capability of stem cells and also for the establishment of stem cell-based regenerative therapeutics. The environmental factor such as O_2 concentration is a key that might play a vital role in stem cell fate and function[30]. Therefore, the *in vitro* manipulation of MSCs cultured by using different O_2 concentrations in hypoxia or normoxia cultured conditions before the clinical trial is necessary to yield homogeneous cell populations with multilineage differentiation potential without causing side effects in the treatments.

The normoxia or hypoxia condition could lead to cell expansion with MSCs characteristics, and the cell's functional ability to differentiate into osteocytes, chondrocytes, and adipocytes has been assessed. The data of surface markers and multipotent differentiation of WJMSCs under normoxia and hypoxia (2.5% O_2, 5% O_2) for P4 and P8 are not presented; they have been presented previously[9,17,19]. The data of MSCs differentiated to three main mesenchymal lineages (osteocytes, chondrocytes, and adipocytes) in normoxia and hypoxia (2.5% O_2, 5% O_2) conditions for P4 and P8 are not presented; they have been presented previously[17,19] The differentiation of MSCs into osteocytes, chondrocytes, and adipocytes (trilineage) is a unique biological property of MSCs[21]. MSCs differentiated into osteoblasts most rapidly in the normoxia condition with 21% O_2, while treatment with 5% O_2 showed a reduced differentiation potential. Therefore, there was no significant difference in osteogenic markers when the O_2 concentration was 5% and 21%[31] and hypoxic incubation of WJMSCs did not change the osteogenic, chondrogenic, and adipogenic differentiation capacity [9,17].

The result of this study suggested that WJMSCs-norCM and WJMSCs-hypoCM could inhibit the proliferation of BC cell lines (both T47D and MCF7) with an IC_{50} range of 42.34%–62.84% (Table 2). This result was validated with our previous study in which WJMSCs-norCM and WJMSCs-hypo5%CM could inhibit cancer cell proliferation, including HeLa, SKOV3, PC3, HSC3, and HepG2, with an IC_{50} range of 51%–74%[17]. In the hypoxic condition, hypoxia-inducible factor-1α (HIF-1α) on metabolic regulation by suppressing mitochondrial respiration. The reduction of mitochondrial O_2 consumption was associated with decreasing reactive oxygen species production[32]. MSCs-CM inhibited the growth of several cancers, including breast adenocarcinoma, ovarian carcinoma, osteosarcoma, benign neoplastic keloid cells, bladder tumor, and lymphoma cells *in vitro*. In an

in vivo study, MSCs-CM inhibited mammary carcinoma, osteosarcoma, and pancreatic and lung tumor growth[26]. CM contains a broad variety of cytokines, growth factors, and putatively also microvesicles containing (micro) RNA, which are responsible for the beneficial effects in anticancer therapy[33,34]. WJMSCs-CM contained soluble factors such as interleukins, cell adhesion molecules, hyaluronic acid, growth factors, and glycosaminoglycans that probably possess tumoricidal activity[34-36]. The soluble factors from MSCs could also inhibit tumor angiogenesis by downregulating vascular endothelial growth factor (VEGF) expression in tumor cells[34,37]. Table 4 shows that WJMSCs-CM upregulated P53, BAX, and CASP9 gene expression in BC cell lines. These data were validated with a previous study in which hUCMSCs can induce apoptosis in the BC MDA-MB 231 cell line[38]. The BC cells (MCF7) were treated using 30% and 60% CM of co-culture WJMSCs, and IL-2-induced NK (WJMSC-CM, (WJMSCs + NK)-CM, and (WJMSCs + IL12-NK)-CM) for 24 h showed that WJMSC-CM, WJMSC-CM + NK, and (WJMSCs + IL12-NK)-CM inhibited BC cell proliferation[28]. CM from WJMSCs, WJMSCs + NK, and WJMSCs + IL12-NK induced apoptosis BC cells (respectively 33.11%, 34.70%, and 36.50%)[28].

MSCs-CM significantly inhibited proliferation, induced apoptosis, significantly upregulated the apoptotic genes of both CASP3 and CASP9, significantly downregulated the antiapoptotic genes such as SURVIVIN and X-linked inhibitor of apoptosis (XIAP), and induced and completed differentiation in the human U251 cell line[12]. The anticancer effect of WJMSCs-CM was via an apoptotic mechanism[39]. WJMSCs-CM (50%) showed increases in the sub-G1 phase; G2/M cell population and annexin V-FITC positive culture[39]. WJMSCs-CM increased the pro-apoptotic BAX gene and decreased the antiapoptotic BCL2 and SURVIVIN genes in three cancer cell lines, including human ovarian cancer cells (TOV-112D), osteosarcoma cells (MG-63), and breast adenocarcinoma cells (MDA-MB-23)[39]. MSCs-CM has the potential for autoimmune, inflammatory, and malignant diseases and tissue regeneration. The most important factors present in MSCs-CM that can be considered protagonists of MSCs physiological effects include hepatocyte growth factor (HGF), transforming growth factor-beta (TGFβ), VEGF, tumor suppressor gene (TSG6), prostaglandin E 2 (PGE2), and galectins 1 (GAL1) and GAL9[34,40]. Interferon-beta (IFNβ) in CM induces the extrinsic and intrinsic apoptosis pathways in lung cancer, lowers the CASP8 and CASP9 expression, and increases cleaved CASP3[41]. WJMSCs-CM significantly decreased cell viability and cell proliferation by inducing apoptosis and cell cycle arrest in human Burkitt's lymphoma cell line (Rams and CRL1596). The mechanism of WJMSCs-CM was activated by the secretory product-mediated induction of cell cycle arrest at sub-G1 phase and reduction in entry to G2/M, increase in oxidative stress, or decrease in glutathione peroxidase (GPX)[42]. WJMSCs-CM induced apoptosis and autophagy in human foreskin fibroblast (CCD-112sk) and significantly downregulated the expression of TBP-associated factor (TAF) in keloid cells[43]. Microvesicles of WJMSCs induced apoptosis in T24 bladder tumor cells with altered morphology, including membrane damage, cell shrinkage, blebbing, and cell debris formation[44]. WJMSCs-norCM had more anticancer activity than WJMSCs-hypo5%CM and WJMSCs-hypo2.5%CM in both MCF7 and T47D cell lines. This result was not consistent with our previous study where WJMSCs-hypoCM was more active in the cervical (HeLa), prostate (PC3), human squamous carcinoma (HSC3), and liver (HepG2) cancer cells but less active in ovarium (SKOV3) cells[17]. WJMSCs-hypoCM was less active in inhibiting BC cell proliferation and lowering apoptosis induction genes (P53, BAX, and CASP3) than WJMSCs-norCM. WJMSCs-hypo2.5%CM had the lowest anticancer activity compared with WJMSCs-norCM based on proliferation cells and apoptosis induction genes. This research was in line with previous research in which WJMSCs incubated in the hypoxic condition released higher levels of growth factor (VEGF) compared with normoxic incubation[35]. VEGFα is a member of the VEGF family and induces expression of the antiapoptotic proteins BCL2 and A1 in endothelial cells mediated by the PI3K/Akt

pathway[45]. VEGFα influences the susceptibility and aggressiveness of BC cells[46]. Several solid tumors with high levels of VEGF expression have been associated with poor clinical outcomes[47]. hBMMCs secreted VEGF 230 pg/mL in normoxia and 450 pg/mL in hypoxia conditions[48]. VEGF plays a role in angiogenesis and is important in regeneration; higher VEGF resulted in lower anticancer activity[35,49].

MSCs have been readily engineered to express antiproliferative, proapoptotic, and antiangiogenic agents that specifically target different cancer types[50]. This result was validated with previous findings in which the hypoxic preincubation of amniotic fluid MSCs (AFMSCs) induced the secretome, namely the upregulation of various secretable factors such as VEGF and TGFβ1[51-53]. Hypoxia (2% O_2) significantly upregulated the mRNA expression level of βFGF, IGF1, VEGFα, HGF, indoleamine 2,3-dioxygenase (IDO)[39,53], TGFβ2, TGFβ3, interleukin (IL1β), IL6, and IL8 of BMMSCs compared with normoxia incubation[54]. This result was in line with our previous research in which WJMSCs-hypo2.5%CM secreted the highest VEGF compared with WJMSCs-hypo5%CM and WJMSCs-norCM[34], in agreement with RT-PCR data in which hypoxia significantly increased the levels of βFGF, VEGFα, IL6, and IL8 using ELISA[34,54]. Our previous research indicated that hypoxic incubation upregulated the concentration level of VEGF, IL1, IL6, and IL8 compared with normoxia. Lower oxygen tension (2.5% O_2) resulted in higher VEGF, IL1, IL6, and IL8 than hypoxic 5% O_2 [34,55].

The IL1 network of cytokines and receptors controls the tumor cell subpopulation expression of other protumorigenic cytokines such as the angiogenic/growth factor, IL8 [56]. The IL1 family of cytokines may be important in regulating protumorigenic activities within the human BC tumor microenvironment[57]. IL1 promotes tumor growth, induces prometastatic genes such as matrix metalloproteinase (MMP), and stimulates adjacent cells to produce angiogenic proteins and growth factors such as VEGF, IL8, IL6, tumor necrosis factor (TNFα), and TGFβ[58]. IL1 contributes to tumor progression and metastasis[59]. IL6 and IL8 can directly or indirectly promote tumor growth via induction of VEGF expression[59]. Our previous research was in line with these data in which WJMSCs-norCM secreted the lowest IL1 level[34] and had higher anticancer potency.

IL8 signaling increases the proliferation and survival of endothelial and cancer cells and potentiates the migration of cancer cells, endothelial cells, and infiltrating neutrophils at the tumor site. IL8 expression correlates with the angiogenesis, tumorigenicity, and metastasis of tumors in numerous xenograft and orthotropic *in vivo* models[60]. IL8 promotes BC progression by increasing cell invasion, angiogenesis, and metastases and is upregulated in human epidermal growth factor receptor 2-positive (HER2-positive) cancers. IL8 via its cognate receptors, CXC chemokine receptor type 1 (CXCR1) and CXCR2, is also involved in regulating cancer stem cell (CSC) activity[60]. IL8 can promote CSC invasion, metastases, and treatment resistance[61]. IL8 upregulated the antiapoptotic gene BCL2, downregulated the proapoptotic gene SASP3 via the PI3K/AKT signal pathway, and significantly inhibited the apoptosis of MCF7 cells[62]. IL8 binds CXCR2 receptors, on the surface of nearby cancer cells. The binding of IL8 to CXCR2 produces signals within tumor cells that activate molecule transcription factors, including Nuclear factor kappa B (NFkB) and Activator protein-1 (AP1), via the activated protein kinase (AKT) and mitogen-activated protein kinase (MAPK) signaling pathways, ultimately causing the growth, and survival of colon cancer cells[64]. Our previous research was in line with this result in which WJMSCs-norCM secreted the lowest IL8 level[34] and had higher anticancer potency.

TGFβ contributes to the development of colorectal cancer[64]. TGFβ plays a role in regulating cancer formation and progression while acting as a cancer inhibitor in normal cells and early carcinomas. The use of TGFβ antagonists is critical for the development of novel anticancer therapies[65]. Therapeutic approaches for colorectal cancer are based on

the inhibition of TGFβ-dependent IL6 trans-signaling[64]. IL6 enhances human skin carcinoma cell invasiveness by inducing the overexpression of MMP1[66]. IL6 contributes to mediating epithelial-mesenchymal transition (EMT) and metastasis neck squamous cell carcinoma (HNSCC)[67]. Cytokines are involved in inflammation-related carcinogenesis, such as hypoxia-inducible factor (HIF1α), MMP2 and MMP9, BCLX, BCL2, VEGF, and IL6[68,69]. IL6 promotes prostate cancer cell proliferation and inhibits apoptosis via multiple signal pathways: Janus tyrosine family kinase (JAK), signal transducer and activator of transcription pathway, extracellular signal-regulated kinase 1 and 2 (ERK1/2), MAPK pathway, and phosphoinositide 3-kinase (PI3-K) pathway[69].

Table 4: Gene expression of 50% concentration of WJMSCs-hypo5%CM-P4 and WJMSCs-hypo2.5%CM-P4 in T47D and MCF7 cells for 72 h incubation

Cell line	Treatment	Gene				
		RNA purity	P53	BAX	CASP9	BCL2
MCF7	Control (untreatment)	2.4828 ± 0.3640	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00
	WJMSCs-norCM-P4	2.5885 ± 0.4776	24.67 ± 7.04 [b]	14.12 ± 2.82 [b]	8.62 ± 1.93 [b]	0.37 ± 0.20
	WJMSCs-hypo5%CM-P4	2.1204 ± 0.0627	6.08 ± 2.71 [a]	2.39 ± 0.56 [a]	5.98 ± 4.18 [a]	0.52 ± 0.24
	WJMSCs-hypo2.5%CM-P4	2.5239 ± 0.4063	5.01 ± 1.02 [a]	3.27 ± 0.81 [a]	6.15 ± 0.36 [a]	0.90 ± 0.58
T47D	Control (untreatment)	2.6439 ± 0.4787	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00
	WJMSCs-norCM-P4	2.6908 ± 0.3683	10.45 ± 0.46 [b]	3.94 ± 1.25 [b]	13.86 ± 2.57 [b]	0.11 ± 0.05
	WJMSCs-hypo5%CM-P4	2.7166 ± 0.1896	1.74 ± 0.30 [a]	1.61 ± 0.56 [ab]	14.43 ± 3.84 [b]	0.81 ± 0.10
	WJMSCs-hypo2.5%CM-P4	2.3367 ± 0.4628	1.79 ± 0.20 [a]	2.01 ± 0.05 [ab]	10.18 ± 3.00 [ab]	0.80 ± 0.09

Data are expressed as mean ± standard deviation; different letters (a, ab, b) in the same column (among WJMSCs-norCM, WJMSCs-hypo5%, and WJMSCs-hypo2.5% in T47D and MCF7 cell lines) are significant differences at $p < 0.05$ (Tukey's HSD *post hoc* test)

CONCLUSION

In conclusion, WJMSCs-hypoCM was less active in inducing apoptosis because CM contained higher cytokines, chemokines, and growth factors that increase BC proliferation. Our previous research was in line with this result : that WJMSCs-norCM secreted the lowest VEGF level[35] and had higher anticancer potency. However, WJMSCs-norCM has more anticancer activity by inducing apoptotic gene expression in BC cells (T47D and MCF7) compared with WJMSCs-hypoCM.

CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

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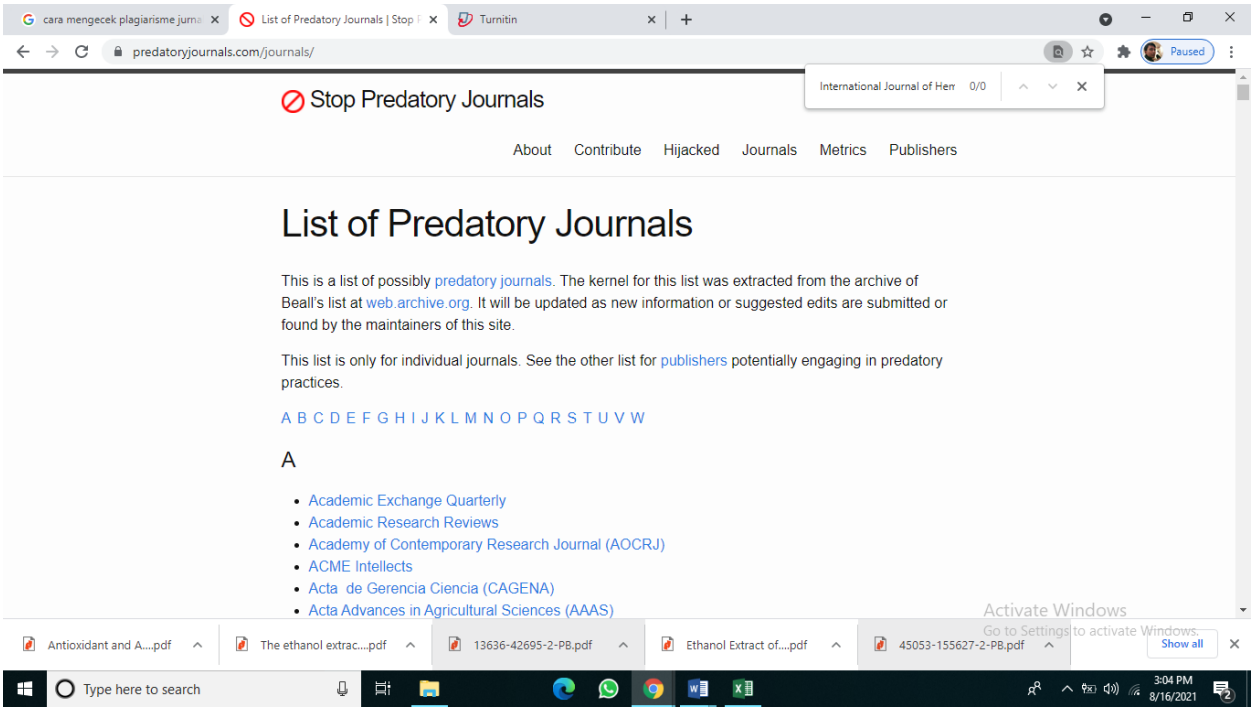
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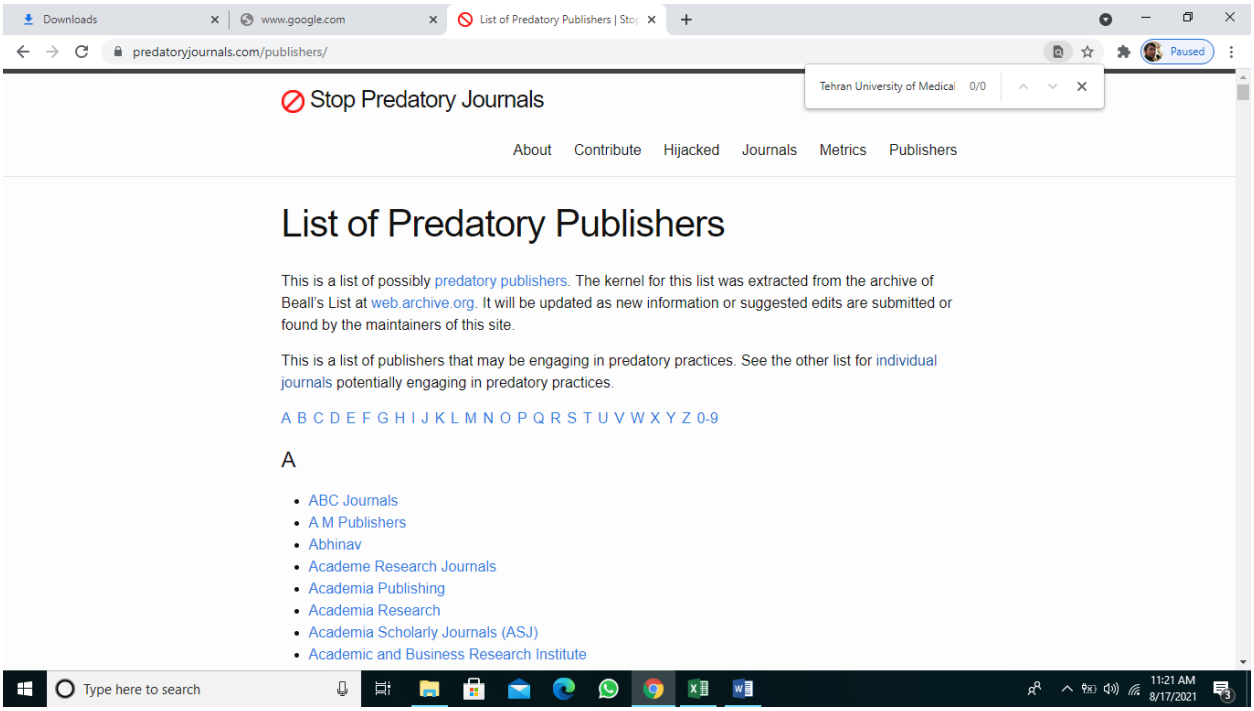
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