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Hypoxic and Normoxic-Human Wharton's Jelly Mesenchymal Stem Cell-Free Lysate for Anticancer Therapies

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Abstract

Cancer can be defined as a disease in which a group of uncontrolled cells growth by ignoring the rules of normal cell division. Mesenchymal Stem Cells (MSCs) are one of the promising cell-based therapies for anticancer therapies. Preceding research had disclosed that conditioned medium from hypoxic-treated human Wharton's jelly mesenchymal stem cells (hWJMSCs-hypoCM) and normoxic-treated hWJMSCs (hWJMSCs-norCM) can inhibit the proliferation of various cancer cell lines and have no cytotoxic effect on normal cells. The present study, hence, measured the effect of a human Wharton's jelly stem cells cell-free lysate (hWJMSCs-CL), cultured with hypoxic condition (hWJMSCs-hypoCL), and normoxic condition (hWJMSCs-norCL) towards several cancer cell lines including ovarian (SKOV3), cervical (HeLa), tongue squamosa (HSC3), and liver (HepG2) proliferation. Human mesenchymal stem cells (hMSCs), human fibroblast cells, and mouse fibroblast cells (NIH3T3) were used as control. Both hWJMSCs-CL grown in hypoxic and normoxic conditions could inhibit the proliferation of various cancer cell lines with median inhibitory concentration (IC_{50}) 21.09 - 95.92 µg/mL and could cause low inhibition of the normal cells with IC_{50} 409.19 - 799.74 µg/mL. The hWJMSCs-hypoCL and hWJMSC-norCL could inhibit various cancer and were not harmful to normal cells.

Keywords: Anticancer, Cell-free lysate, Hypoxia, Mesenchymal stem cells, Wharton's jelly

Introduction

Cancer can be defined as a disease caused by the uncontrolled growth of a cluster of cells [1]. Various types of therapies for cancer have been found, such as chemotherapy, surgery, and radiotherapy [2]. However, treatment-related side effects and drug resistance limit the efficacies of many therapeutic options. Researchers are working to develop new therapies with low toxicity to normal cells but effective to kill/ inhibit cancer cells. Cell-based therapy has now become one of the most explored treatments. One of the pledging cell-based therapies in stem cells is the application of Mesenchymal Stem Cells (MSCs).

The MSCs are easily isolated and propagated in vitro and applied widely in the treatment of different cancers [3]. One pivotal source of MSCs is the umbilical cords' Wharton's jelly. The MSCs have several unique characteristics, including high expression of cluster of differentiation (CD) CD73, CD90, and CD105; short doubling time; low expression of CD34, CD14, CD45 and HLA-II; and able to differentiate into osteocytes, chondrocytes, and adipocytes [4].

Cell culture conditions, e.g., oxygenation, can affect MSCs proliferation [4]. Previous studies had shown that incubation on normoxic tension affected hWJMSCs characteristics at late and early passage, in contrast, hypoxic tension can increase the MSCs proliferation without affecting surface markers [4-6]. Previous studies discovered that hWJMSCs cell lysate (hWJMSCs-CL) could inhibit the growth of cancer cells such as ovarian carcinoma (TOV-112D), breast adenocarcinoma (MDA-MB-231), and osteosarcoma (MG-63) cells [7]. In the current study, the aim was to measure the hWJMSCs, cell-free lysate cultured with hypoxia-treated hWJMSCs (hWJMSCs-hypoCL), and normoxia (hWJMSCs-norCL) for anticancer therapies toward cancer cell lines, involving HSC3, HepG2, HeLa, SKOV3, hMSCs, human fibroblast cells (normal cell), and NIH3T3.

Materials and methods

Isolation and cultivation of hWJMSCs

Isolation: Umbilical Cords (UC) were collected freshly from conceiving women (age: 25 - 40) in Immanuel Hospital, Bandung, Indonesia. This research had been approved by the Institutional Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia, and from the Institutional Ethics Committee collaboration between Immanuel Hospital Bandung, Bandung, Indonesia, and Maranatha Christian University, Bandung, Indonesia number 12/KEP FK UKM-RSI/III/2013 [4,6].

Cultivation: UC were washed by phosphate buffer saline (PBS) and cut into 1 - 2 mm. The explants were cultured in MEM-α with 2 mM GlutaMax (Gibco, 32561037, Massachusetts, USA), supplemented with 20 % fetal bovine serum (FBS) (Gibco,10270106, Massachusetts, USA) and added with penicillin-streptomycin (Gibco, 15140122, Massachusetts, USA), amphotericin B (Gibco, 15290018, Massachusetts, USA). Cultures were incubated in a humidified atmosphere with 5 % CO_2 at 37 ºC for 3 weeks. The mediums were replaced every 5 days. When cultures had reached 80 - 90 % confluency, cells were harvested and divided into 2 plates at 8×10^3 cells/cm^2 density. First plate was cultured in normoxic condition (95 % air (21 % O_2)/ 5 % CO_2) and 2nd plate in hypoxic condition (5 and 2.5 % O_2) [4,6].

Phenotype characterization of hWJMSCs

Passage 4 of the hWJ-MSCs was characterized for CD19, CD34, CD45, CD73, CD90, CD105, CD145, and HLA-II expression using a flow cytometer. The hWJMSCs were harvested at 80 % confluence and dissociated using trypsin-EDTA and centrifuged at 300 g for 10 min. The pellet was resuspended using 2 % FBS + PBS and cells were counted using a hemocytometer. Between 100 - 200 cells in 25 mL PBS were put into flow cytometer FACS (BD FACS Calibur^{TM}) tubes. The antibody was added to each FACS tube pursued by incubation at 4 ºC for 15 min. Results were analyzed in Cell Quest Pro Acquisition on the BD FACStation^{TM} software. Each measurement was done with 3 replications [4,6].

Trilineage differentiation character of hWJMSCs was done with the following steps. Osteogenic differentiation: hHWJMSCs (P4) at 1×10^4 cells cm^{-2} density were cultured in StemPro Osteogenesis Differentiation Kit (Gibco, A10072-01, Massachusetts, USA) for 3 weeks. Calcium deposits were visualized with Alizarin red S (Amresco, 9436, Pennsylvania, USA). Chondrogenic differentiation: hWJMSCs at 1×10^4 cells cm^{-2} density were cultured in StemPro Chondrogenesis Differentiation Kit (Gibco A10071-01, Massachusetts, USA) for 2 weeks. The extracellular matrix deposits were visualized with Alcian blue (Amresco, 0298, Pennsylvania, USA). Adipogenic differentiation: hWJMSCs were cultured at 1×10^4 cells cm^{-2} density in Adipogenesis Differentiation Kit (Gibco, A10070-01, Massachusetts, USA) for 2 weeks. Lipid droplets were confirmed using Red O (Sigma Aldrich, 00625, Missouri, USA) [8-10].

Preparation of cell free lysate from hypoxia-treated hWJMSCs (hWJMSCs-hypoCL) and normoxia-treated hWJMSCs (hWJMSCs-norCL)

Cell-free lysates were obtained using 2-D Rehydration and Tributylphosphine (TBP) reducing agent (BioRad, 163-2086, California, USA). Harvested hWJMSCs were centrifuged at 500 g for 4 min and the pellets were suspended in a mix solution (1 mL 2-D Rehydration + 10 µL TBP). The cells were, then, sonicated at 28 ºC for 30 sec 4 times. Then, the mixture was centrifuged at 16,000 g and 20 ºC for 30 min. The supernatant, pellets were stored at –80 ºC for further use [9].

Cultivation of cancer and normal cells

Liver (HepG2-ATCC® HB-8065™), cervical (HeLa-ATCC® CCL-2™), tongue squamous (HSC3-ATCC, Manassas, VA), ovarian (SKOV3-ATCC® HTB-77™) cancer cell lines and normal cells such as mouse fibroblast (NIH3T3-ATCC® CRL-1658™), hMSCs (primary cells from Wharton's Jelly), human fibroblast (primary cells) were obtained from Stem Cell and Cancer Institute, Jakarta Indonesia. Liver and cervical cells were maintained, and grown in Eagle's Minimum Essential Medium (MEM, Biowest, L0416-500, France), ovarian cells were maintained and grown in McCoy's 5a Medium Modified (Biowest, L0210-500, France), mouse fibroblast cells were maintained and grown in Dulbecco modified Eagle's medium (DMEM, Biowest, L0101-500, France) with 10 % FBS (Sigma Aldrich, F9665, Missouri, USA), penicillin-streptomycin (Gibco, 15140122, Massachusetts, USA). The cells were incubated in a humidified atmosphere at 37 ºC and 5 % CO_2 [4,6,11].

Cell treatments and viability assay

Cells were seeded at 5×10^3 density in 96-well plates with growth mediums according to the cell types as mentioned before and incubated at 37 ºC 5 % CO_2 condition for 24 h. hWJMSCs from normoxic and hypoxic condition cell lysates (hWJMSCs-norCL and hWJMSCs-hypoCL) in various concentrations (0, 5, 10, and 15 µg/mL) were later added. Oxygen level for the normoxic and hypoxic condition were 2.5 and 5 %. Cultures were later incubated for 72 h [6,11,12]. Anticancer activity as indicated with viability was measured using tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, G3581, Madison, USA). MTS was added at 10 µL to each well. The plate was incubated at 5 % CO_2, 37 ºC for 4 h. The 490 nm absorbance was measured using microplate reader (Multiskan Go, Thermo Scientific Inc, USA) [6].

Statistical analysis

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) statistics version 20.0 software. The cell number, viability, and proliferation inhibition in cancer cell lines and in normal cells were calculated, expressed in means and standard deviation (Mean±STD). Analysis of variance (ANOVA) was performed to the significant difference among treatments at 95 % confidence interval. Tukey's HSD post hoc test was performed on different treatment concentrations.

Results and discussion

The hWJMSCs under both hypoxic and normoxic condition displayed the negative expression for CD14, CD19, CD34, CD45, and HLA-II and positive expression for CD73, CD90, CD105 in early (P4) and later (P8) passage (**Table 1**). The hWJMSCs also depicted tri-lineage differentiation (chondrogenic, adipogenic, osteogenic) in both hypoxic and normoxic condition (data/photo not shown). The hypoxic or normoxic condition did not affect hWJMSCs characteristics, which was also stable in a later passage.

Table 1 Effects of the oxygen level and type of passage toward the surface marker of hWJMSCs.

Passage	O_2 level	CD14 (%)	CD19 (%)	CD34 (%)	CD45 (%)	CD73 (%)	CD90 (%)	CD105 (%)	HLA-II (%)
P4	Normoxia	0.00±0.01	–0.39±1.12	0.00±0.00	0.00±0.00	96.67±2.25	96.17±1.00	96.00±1.39	0.23±0.40
	Hypoxia 5 %	0.01±0.01	0.12±0.11	0.03±0.05	0.04±0.06	97.30±2.25	96.70±0.71	96.88±0.11	0.00±0.00
	Hypoxia 2.5 %	–0.43±0.54	0.51±0.1	–0.06±0.51	–0.07±0.14	97.47±3.10	96.42±4.13	94.98±5.70	–1.88±2.56
P8	Normoxia	0.00±0.01	–0.79±1.24	0.00±0.00	0.00±0.0	96.40±3.11	96.55±1.76	95.65±1.77	0.00±0.00
	Hypoxia 5 %	0.00±0.01	0.15±0.21	0.00±0.01	0.00±0.0	97.63±0.45	96.57±1.95	95.07±0.45	0.03±0.05
	Hypoxia 2.5 %	–0.51±0.76	0.50±0.87	–0.26±0.32	0.02±0.09	96.21±4.11	91.74±0.00	93.23±4.11	–2.84±2.82

hWJMSCs-hypoCL and hWJMSCs-norCL could inhibit the proliferation of cancer cells significantly compared to control. Based on **Figure 1**, the concentration of cell lysate 15 µg/mL had the highest proliferation inhibition to HepG2 cells. The most active of hWJMSC-hypoCL and hWJMSC-norCL inhibited liver cancer cells (HepG2). Both hWJMSC-hypoCL and hWJMSC-norCL have similar anticancer activity.

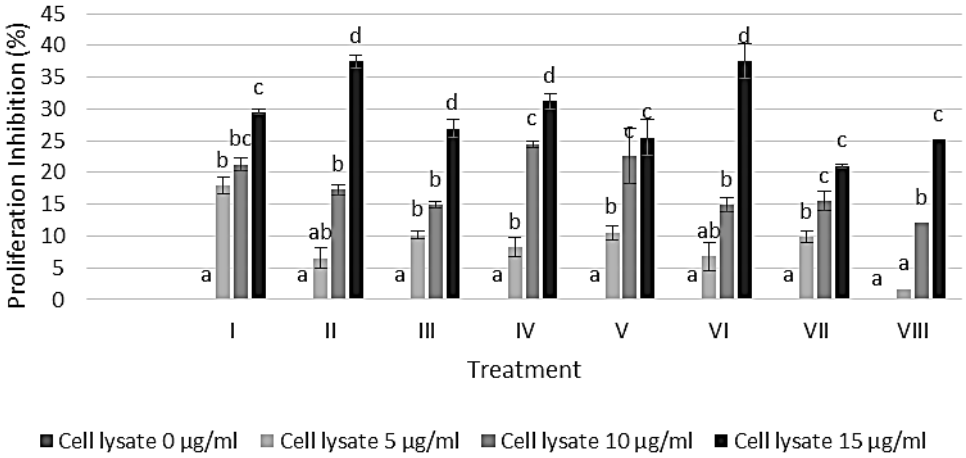

Figure 1 Effect of hWJMSCs-hypoCL and hWJMSCs-norCL toward proliferation inhibition of HeLa, HepG2, SKOV3, HSC3 cancer cells.
*(I) Hypoxia-HeLa, (II) Hypoxia-HepG2, (III) Hypoxia-SKOV3, (IV) Hypoxia-HSC3, (V) Normoxia-HeLa, (VI) Normoxia-HepG2, (VII) Normoxia-SKOV3, (VIII) Normoxia-HSC3. The data of cell proliferation inhibition are presented as a histogram of Mean±STD, different letters in the same group treatment with various levels of hWJMSCs-CL are significant differences among concentrations at $p < 0.05$ (Tukey HSD Post Hoc test). HeLa: Cervical cancer, HSC3: Tounge squamous cancer, HepG2: Liver cancer, SKOV3: Ovarian cancer.

The most active of cell lysate were hWJMSCs-hypoCL and hWJMSCs-norCL toward HepG2 cells, HSC3 cells. The hWJMSCs-norCL was lowest anticancer activity toward SKOV3 (IC_{50} = 95.93 µg/mL) and hWJMSCs-hypoCL was lowest anticancer activity toward HeLa cells (IC_{50} = 86.90 µg/mL) can be seen on **Table 2**.

Table 2 The IC_{50} of hWJMSCs-hypoCL and hWJMSCs-norCL in various cancer cell lines.

Cancer cell lines	IC_{50} value (µg/mL)	
	hWJMSCs-norCL	hWJMSCs-hypoCL
HeLa	22.69	86.90
HepG2	21.71	21.09
SKOV3	95.93	46.79
HSC3	24.80	33.46

The effect of hWJMSCs-hypoCL and hWJMSCs-norCL toward human, mouse fibroblast cells, hMSCs can be seen at **Figure 2**. **Figure 2** described that normal cells, such as NIH3T3, hMSCs, and human fibroblast treated with hWJMSC-hypoCL and hWJMSC-norCL with various concentrations (0, 5, 10, 15 µg/mL), resulted low (< 10 %) proliferation inhibition compared with control. Cell lysate both hWJMSC-hypoCL and hWJMSC-norCL were not toxic toward normal cells.

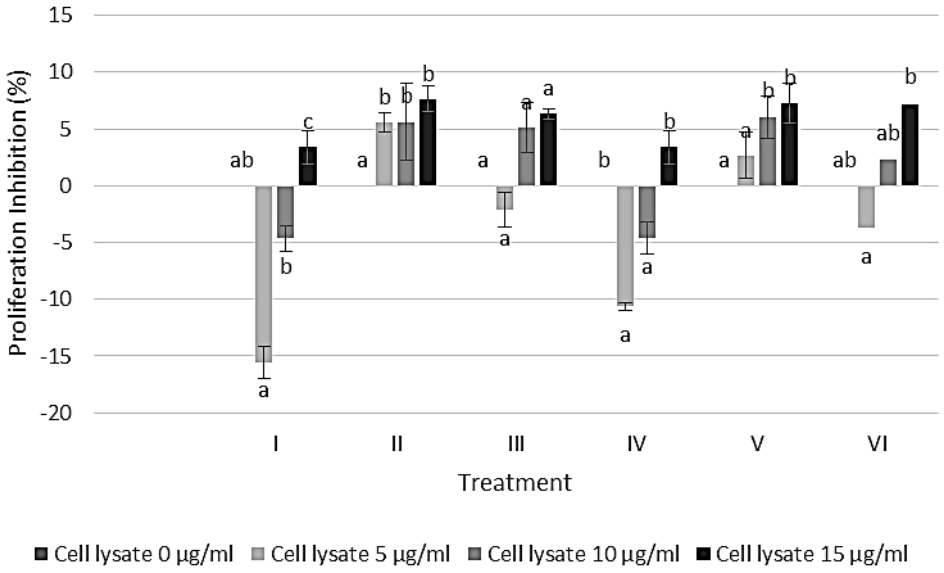

Figure 2 Effect of hWJMSCs-hypoCL and hWJMSCs-norCL toward proliferation inhibition of normal cells.
*(I) Hypoxia-NIH3T3, (II) Hypoxia-Human Fibroblast, (III) Hypoxia-hMSCs, (IV) Normoxia-NIH3T3, (V) Normoxia-Human Fibroblast, (VI) Normoxia-hMSCs. The data of cell proliferation inhibition are presented as a histogram of Mean±SD, different letters in the same group treatment with various levels of hWJMSCs-CL are significant differences among concentrations at $p < 0.05$ (Tukey HSD Post Hoc test). NIH3T3: Mouse fibroblast, hMSCs: human Mesenchymal Stem Cells.

Based on the high value of IC_{50}, it was considered that cell lysate was safe, not toxic toward normal cells. The unlimited value of IC_{50} toward NIH3T3 cells can be seen in **Table 3**.

Table 3 The IC_{50} of hWJMSCs-hypoCL and hWJMSCs-norCL in various normal cells.

Normal cells	IC_{50} value (µg/mL)	
	hWJMSCs-norCL	**hWJMSCs-hypoCL**
NIH3T3	-	-
Human fibroblast	409.19	629,799.74
hMSCs	468.28	4,860.85

*IC_{50} = median inhibitory concentration; hWJMSCs: human Wharton's Jelly Mesenchymal Stem Cells; hWJMSCs-hypoCL: hypoxia-treated hWJMSCs cell lysate, hWJMSCs-norCL: normoxia-treated hWJMSCs cell lysate.

The hMSCs have a high potential that is used as a therapeutic agent for clinical studies. The hWJMSCs is the best source of hMSCs that can be used clinically. Besides, recent research findings revealed that WJ is the best source of stem cells among other compartments of the UC [13]. The surface markers of hWJMSCs in hypoxic and normoxic conditions were not significantly different [4,6].

The cell lysate of hWJMSCs (hWJMSCs-CL) in the treatment of hypoxia 5 % (hWJMSCs-hypoCL) and normoxia (hWJMSCs-norCL) from passage 4 (P4) has anticancer activity in various cancer cells including HeLa, SKOV3, HSC3, HepG2 cancer cells and not toxic to normal cells including NIH3T3, human fibroblasts, and hMSCs.

The MSCs have an antitumor activity intermediator by paracrine resulted in damaged Endothelial Progenitor Cells (EPCs) downregulation proangiogenic factor including Platelet-Derived Growth Factor (PDGF-BB), Fibroblast Growth Factor-2 (FGF-2), Insulin Growth Factor (IGF-1), and interleukin-1β (IL-1β) [14]. Paracrine secretions of hWJMSCs-CL, contain various factors, including cytokines which were able to trigger tumor cell apoptosis [6].

Human WJMSCs (hWJMSCs) inhibits the proliferation of solid tumors such as osteosarcoma, ovarian carcinoma, and mammary carcinoma [13]. Cell lysate significantly down-regulated the expression of TAF (TBP-associated factor) in keloid cells [14]. Cell lysate was collected from hWJMSCs culture at early passages (P3 to P7) resulted in increasing cell death in breast adenocarcinoma cells (MDA-MB-231), osteosarcoma cells (MG-63) and while no toxicity was observed in the case of a normal human fibroblast cell line (CDE-112sk) [7]. The secretome in hWJMSCs was measured including IL-1α, IL-6, IL-8, and Vascular Endothelial Growth Factor (VEGF) in both of normoxic and hypoxic oxygen tension, in early (P4) and late passage (P8) [15], which the secretome of hWJMSCs have anticancer activity toward various cancer cell line [6].

The hWJMSCs secretome can upregulate pro-apoptotic genes. The increased cell death was observed at sub-G1/S and proliferation was observed at the G2/M phase of the mitotic cycle [13]. Tumor suppressor cells and upregulation of several pro-apoptotic in hWJMSCs involving several cytokines in cell (IL-12a), which inducing apoptosis and thus mediate the anticancer effects of hWJMSCs-CL [16,17]. Anti-inflammatory IL-6 observed in the conditioned medium of hWJMSCs (hWJMSCs-CM) has promise anticancer. Anti-inflammatory agents can alter the tumor themselves, the tumor microenvironment, induce apoptosis, decrease cancer cell migration, and increase sensitivity to other therapies or simultaneous therapies [6,15]. Tumor-producing factors (chemokines, cytokines, growth factors, and angiogenic) have overlapping functions in promoting tumor growth [18]. Bone Marrow Mesenchymal Stem Cells (BMMSCs) under hypoxic conditions and their fractions showed that the stem cells expressed and secreted significant IL-6 and IL-8 [19], hWJMSCs both hypoxic and normoxic condition secreted IL-6, IL-8 which can kill cancer cells [6,7,15]. Survival of mice containing lymphoma through induction of endothelial cell (EC) apoptosis could be increased after intraperitoneal MSCs injection [12,20]. The most effective cell lysate treatment for cancer inhibition was treated with normoxia (hWJMSCs-norCL)

compared to hypoxia (hWJMSCs-hypoCL), in the previous study, it was shown that hWJMSCs-norCM had higher anticancer compared to hWJMSCs-hypoCM [6].

The hWJSC-CL treatment to osteosarcoma (SKES-1 and MG-63) cells showed significant decreases in cell proliferation. The pro-apoptotic gene (BAX) was increased, the autophagy-related ATG-5 and BECLIN-1 genes were increased, the anti-apoptotic BCL2 and SURVIVIN genes were decreased both in MG-63 and SKES-1 cells treated with hWJSC-CM and hWJSC-CL [21]. A previous study showed that a hypoxic environment could greatly improve the expression of chemokine receptors during *in vitro* expansion and eventually increased the efficiency of MSCs-based regenerative therapies [22]. The hWJMSCs-hypoCL and hWJMSCs-norCL were able to inhibit various cancer including HSC3, HeLa, SKOV3, HepG2, and not toxic for normal cells. The hWJMSCs-norCL and hWJMSCs-hypoCL could inhibit proliferation of various cancer cell lines with IC_{50} 21.09 - 95.93 µg/mL and exhibited low inhibition toward normal cells with IC_{50} 409.19 µg/mL up to unlimited value.

Conclusions

The experiments in this study had uncovered that hWJ-MSCs-hypoCL and hWJ-MSCs-norCL could inhibit various cancer cell lines and were not toxic for normal cells.

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Abbreviations

ATG-5	: Autophagy Related 5 gene
BMMSCs	: Bone marrow-derived Mesenchymal Stem Cells
BAX	: Bcl-2-associated X protein
BCL2	: B-cell lymphoma 2
CD	: Cluster of differentiation
EC	: Endothelial cell
EPCs	: Endothelial Progenitor Cells
FACS	: Fluorescence Activated Cell Sorting
FGF-2	: Fibroblast Growth Factor-2
HeLa	: human Cervical cancer cell line
HSC3	: human Tongue squamous carcinoma cell line
HepG2	: human Liver cancer cell line
hWJMSCs	: human Wharton's Jelly Mesenchymal Stem Cells
hWJMSCs-hypoCM	: Conditioned Medium from hypoxic-treated human Wharton's Jelly Mesenchymla Stem Cells
hWJMSCs-norCM	: Conditioned Medium from normoxic-treated human Wharton's Jelly Mesenchymla Stem Cells
hWJMSCs-hypoCL	: Cell Lysate from hypoxic-treated human Wharton's Jelly Mesenchymla Stem Cells
hWJMSCs-normoCL	: Cell Lysate from normoxic-treated human Wharton's Jelly Mesenchymla Stem Cells
hWJSC -CM	: Conditioned Medium from human Wharton's Jelly Mesenchymla Stem Cells
IGF	: Insulin Growth Factor
IC_{50}	: Median Inhibitory Concentration
IL-1α	: Interleukin 1-α
IL-6	: Interleukin-6
IL-8	: Interleukin-8

IL-12a : Interleukin 12-a
MDA-MB-231 : human Breast adenocarcinoma cell line
MSCs : Mesenchymal Stem Cells
MG 36 : human Osteosarcoma cell line
NIH3T3 : mouse Fibroblast cell line
PDGF : Platelet-Derived Growth Factor
SKOV3 : human Ovarian cancer cell line
SKES-1 : human Osteosarcoma cell line
TBP : Tributylphosphine
TOV-112D : Ovarian cancer cell line
UC : Umbilical Cord
VEGF : Vascular Endothelial Growth Factor

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