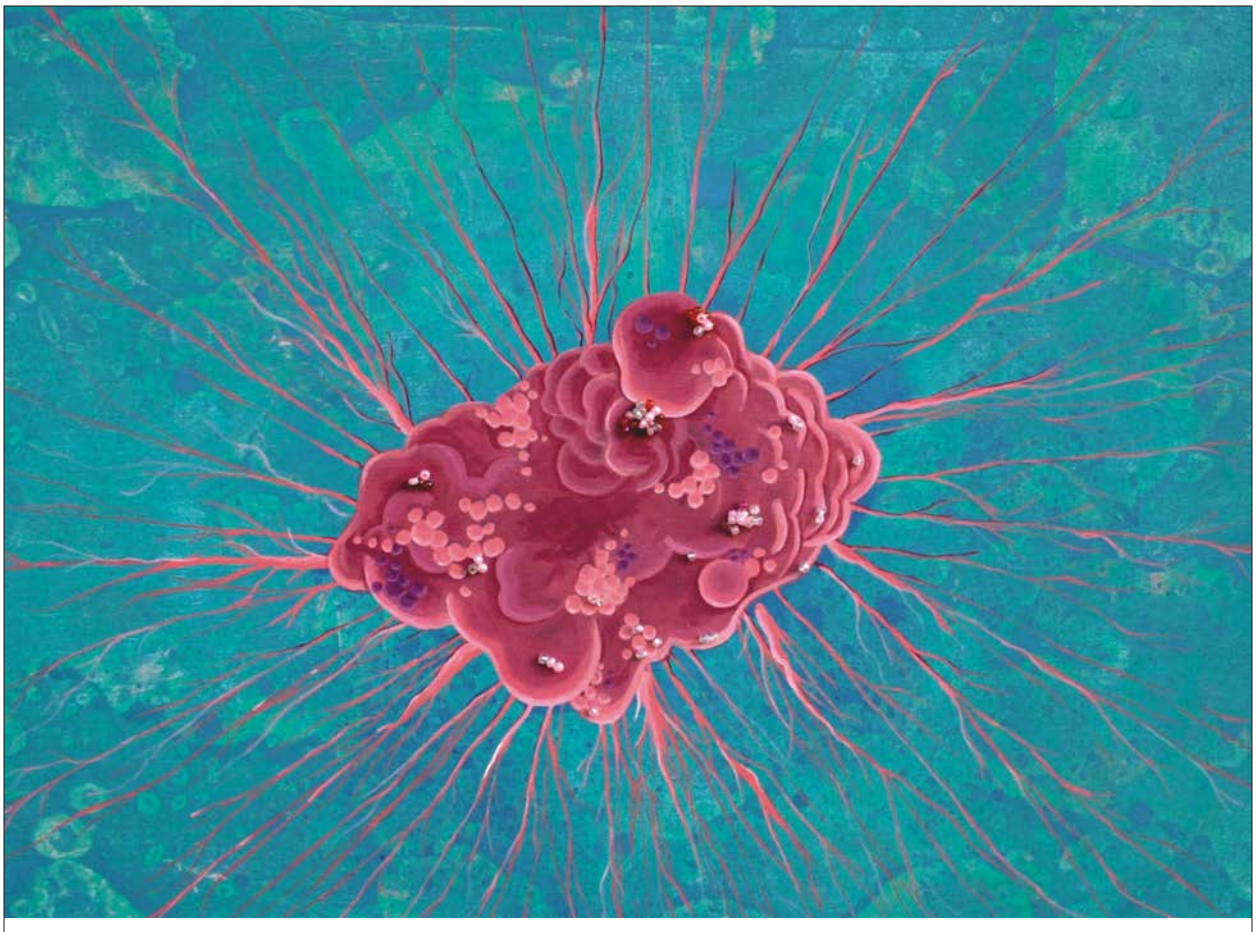

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# Tumoricidal Property of Normoxia and Hypoxia Cell-Free Lysate of Wharton's Jelly-Mesenchymal Stem Cells Toward Various Cancer Cells

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## ABSTRACT

Cancer is one of the leading causes of mortality and morbidity throughout the world. Since there are still some problems related to the conventional therapies for cancer treatment, it is critical to explore new more efficient therapy strategies. Mesenchymal Stem Cells (MSCs) are one of powerful tools for tissue engineering for regenerative medicine, as recent research aims to utilize MSCs for anti-cancer treatment. Our previous research demonstrated that Conditioned Medium from Whartons' Jelly MSCs (WJ-MSCs-CM) significantly lowered cancer proliferation of various cancer cell lines. This research was performed to evaluate the tumoricidal property of cell lysate from WJ-MSCs from normoxia (WJMSCs-norCL) and hypoxia-treated WJMSCs (WJMSCs-hypoCL) on the proliferation of human cancer cells, including cervical (HeLa), liver (HepG2), ovarian (SKOV3) and oral squamous (HSC3) cancer cell lines compared to normal cells including mouse fibroblast (NIH3T3), human Mesenchymal Stem Cells (hMSCs), human fibroblast. The WJMSCs-norCL and WJMSCs-hypoCL have cytotoxic activity, reduce proliferation of various cancer cell lines with minimum inhibitory concentration ($IC_{50}$) 21.094-95.928 μg $mL^{-1}$ and no cytotoxic to normal cells with $IC_{50}$ 409, 191-629, 799.738 μg $mL^{-1}$. The WJMSCs-norCL and WJMSCs-hypoCL inhibit proliferation in various cancer cell lines and are not toxic for normal cells.

**Key words:** Cancer cells, cell-free lysate, hypoxia, normoxia, WJMSCs

## INTRODUCTION

Cancer is one of the leading causes of mortality and morbidity throughout the world. It is one of the most common life-threatening disease, as in some countries such as United States it is estimated that one in four deaths is due to cancer (Siegel *et al.,* 2013). Radiotherapy, chemotherapy, surgery and oncolytic viruses recently reported as current promising treatments for human cancer (Komarova *et al*., 2006; Vile *et al*., 2002; Xia *et al*., 2011). Despite these improved therapy strategies, many cancers remain unresponsive to the treatments. Hence, the new ideal and promising therapeutic strategies are still urgently needed (Siegel *et al*., 2013; Xia *et al*., 2011; Jemal *et al*., 2007). Directly target the tumors in both primary and metastatic sites as well as able to act locally over a sustained period of time, cell-based therapies have great potential as the new prosperous cancer treatment (Kucerova *et al.,* 2007).

One of promising cell-based therapies is utilization of Mesenchymal Stem Cells (MSCs), which are a population of stem cells with self-renewal, extensive proliferation, multilineage differentiation ability *in vitro* and *in vivo,* transplantable cells, ease of sourcing and *in vitro* expandability (Siegel *et al*., 2013; Kim and Cho, 2013; Gjorgieva *et al*., 2013). The MSCs are able to migrate and incorporate within malignant tumors (Komarova *et al*., 2006; Xia *et al*., 2011; Gjorgieva *et al*., 2013) have strong chemotactic response to injury (Ho *et al*., 2013) and an intrinsic property for homing towards tumor sites and can be used as tumortropic vectors for tumor therapy (Siegel *et al*., 2013). Numerous *in vivo* studies reporting animal cancer models showed that MSCs are able to migrate preferentially to tumors with various delivery methods, including intravenously (Gjorgieva *et al*., 2013; Ho *et al*., 2013), intraperitoneal (Komarova *et al*., 2006; Gjorgieva *et al*., 2013) and intracerebral (Gjorgieva *et al*., 2013).

The therapeutic application of MSCs need a large number of *ex vivo* expansion. A fast and efficient protocol for generation of large quantities of MSCs is required for clinical, therapeutic application and research requirements (Siegel *et al*., 2013; Lavrentieva *et al*., 2010; Nekanti *et al*., 2010). Oxygen concentration plays a role in maintaining the MSCs proliferation (Widowati *et al*., 2014). Hypoxic incubation can increases the MSCs proliferation without affecting surface markers and differentiation of WJ-MSCs at early and late passage compared to the normoxic tension (Nekanti *et al*., 2010; Widowati *et al*., 2014, 2015).

Various cytokines can be secreted by MSCs, such as growth factors, proinflammatory and anti-inflammatory cytokines and other cytokines. They play a role in the various diseases improvement (Pawitan, 2014). In the tumor milieu, MSCs secrete various cytokines that have both paracrine and autocrine functions (Ho *et al*., 2013). The MSCs including MSCs-derived Umbilical Cord (UC-MSCs), MSCs-derived Bone Marrow (BM-MSCs) and MSCs-derived Adipose Tissue (AT-MSCs) are capable to attenuate the proliferation of tumor cells by *in vitro* and *in vivo* assays (Siegel *et al*., 2013; Tamura *et al*., 2011).

Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) possess many potential advantages as stem cell based therapies agent for various diseases, including cancer, chronic liver disease, cardiovascular diseases, nerve, cartilage and tendon injury (Kim and Cho, 2013). Un-engineered human and rat umbilical cord MSCs (hUC-MSCs, rUC-MSCs) could significantly attenuated and inhibited the proliferation multiple various cancer cell lines by *in vivo* and *in vitro* assays through multiple mechanisms (Siegel *et al*., 2013; Ayuzawa *et al*., 2009). Cell lysate isolated from human Whartons' Jelly Stem Cells (hWJSCs-CL) 15 μg $mL^{-1}$ have ability to inhibit the growth of cancer cells, namely breast adenocarcinoma (MDA-MB-231), ovarian carcinoma (TOV-112D) and osteosarcoma (MG-63) cells (Gauthaman *et al*., 2012).

The previous research showed that Conditioned Medium from normoxic WJMSCs (WJMSCs-norCM) and hypoxic WJMSCs (WJMSCs-hypoCM) could inhibit proliferation of various cancer cell lines and caused low inhibition or have no cytotoxic effect on normal cells including mouse fibroblast cells, hMSCs, human fibroblast cells (Widowati *et al*., 2014). In this study, we performed the continuing research to measure the tumoricidal property of cell-free lysate of normoxia-treated WJMSCs (WJMSCs-norCL) and hypoxia-treated WJMSCs (WJMSCs-hypoCL) towards cancer cell lines including cervical (HeLa), liver (HepG2), ovarian (SKOV3) and oral squamosa (HSC3) cancer compared to human fibroblast cells, mouse fibroblast cells (NIH3T3) and human Mesenchymal Stem Cells (hMSCs).

## MATERIALS AND METHODS

**Isolation and culture of WJ-MSCs:** The MSCs were isolated and cultivated from fresh umbilical cord of women aged 25-40 years after full-term births (normal vaginal delivery) (Widowati *et al*.,

2014, 2015). A previous informed consent was applied to all donors. Informed consent approved by the Institutional Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia and from the Institutional Ethics Committee collaboration between Maranatha Christian University, Bandung, Indonesia and Immanuel Hospital Bandung, Bandung, Indonesia.

The WJ-MSCs were isolated from UC, after rinsing with normal saline (0.9% w/v sodium chloride), UC were cut into very small pieces explants (approximately 1-2 mm). The explants were plated and cultured in MEM-α with 2 mM GlutaMAX (Invitrogen, Carlsbad, CA, USA), supplemented with 20% fetal bovine serum (FBS, Invitrogen) and penicilline streptomycin amphotericin B (100 U $mL^{-1}$, 100 and 0.25 mg $mL^{-1}$, Invitrogen) in a humidified atmosphere with 5% $CO_2$ at 37°C. Medium was replaced every 5 days until 21 days. The cells were harvested and replated at a density $8\times10^3$ cells $cm^{-2}$ when cells reached 80-90% confluence. For normoxic studies, WJ-MSCs were cultured at 95% air (21% $O_2$) 5% $CO_2$ and for hypoxic cells were cultured at 5% $O_2$ (Nekanti *et al*., 2010; Widowati *et al*., 2014, 2015).

**Immunophenotyping and multipotent differentiation of WJ-MSCs:** The WJ-MSCs were evaluated using the phenotypic characterization of CD45, CD34, CD145, CD105, CD73, CD90, CD19 and HLA-II. The analysis was conducted using a flow cytometer at passage 4 (P4) to confirm the effect of oxygen concentration (hypoxia and normoxia) on MSCs characterization. The WJ-MSCs at 80% confluence were harvested and dissociated with trypsin-EDTA and centrifuged at 300× g for 10 min. The pellet was resuspended with PBS +2% FBS and cells were counted with a hemocytometer. Between 100 and 200 cells in 25 mL PBS were introduced into flowcytometer FACS (BD FACSCalibur™) tubes. Antibody was then added to each FACS tube: isotype mIgG2a-PE, CD105-PE, HLA class II-PE; isotype mIgG1-PE, CD73-PE, CD19-PE; isotype mIgG1-FITC, CD 34-FITC, CD45-FITC, CD14-FITC followed by incubation at 4°C for 15 min. The cells were analyzed by flow cytometry with a FACSCalibur™ 3 argon laser 488 nm (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest Pro Acquisition on the BD FACStation™ software. The experiments and measurement of surface marker were performed in triplicate (Widowati *et al*., 2014, 2015).

For osteogenic differentiation, WJMSCs (P4) were seeded at density $1\times10^4$ cells $cm^{-2}$ in culture dishes and then cultured in StemPro Osteogenesis Differentiation Kit (Gibco A10072-01, Invitrogen, Carlsbad, CA, USA) for 3 weeks. Alizarin red S (Amresco 9436) were used to visualize calcium deposits. In chondrogenic differentiation of WJMSCs, cells were cultured at $1\times10^4$ cells $cm^{-2}$ in StemPro Chondrogenesis Differentiation Kit (Gibco A10071-01, Invitrogen, Carlsbad, CA, USA) for 2 weeks and Alcian blue (Amresco, 0298) were used to visualize the chondrocyte. For adipogenic differentiation, WJMSCs were cultured at $1\times10^4$ cells $cm^{-2}$ in StemPro Adipogenesis Differentiation Kit (Gibco A10070-01, Invitrogen, Carlsbad, CA, USA) for 2 weeks, then to confirm lipid droplets Red O (Sigma 00625) were used (Dominici *et al*., 2006; Zheng *et al*., 2012; Jun *et al*., 2014).

**Preparation of cell free lysate from normoxia (WJMSCs-norCL) or hypoxia-treated WJMSCs (WJMSCs-hypoCL):** The WJMSCs of P4 were used for the experiments. The WJMSCs were seeded at density $8\times10^3$ cells $cm^{-2}$. Cell lysate was prepared by incubating the cells under normoxia (20% $O_2$, 5% $CO_2$) and hypoxia (5% $O_2$, 5% $CO_2$) for 72 h, when cells reached 80-90% confluence the cells were harvested. Subsequently, Trypsin-EDTA were added to the cells and then cells were centrifuged at 500× g for 4 min at room temperature. The cell pellets were prepared by

adding mix solution (ReadyPrep Protein Extraction Kit, BioRad, no. 163-2086) with the comparison of 5-10×$10^6$ cells for 1 mL 2-D Rehydration and 10 μL TBP reducing agent. Cells were placed in the ultrasonic tank 28°C for 4 times, between the interval cells were maintained in an ice box (frequency 40,000 Hz, Skymen JP040S). Cells then centrifuged at 16,000× g for 30 min and supernatant was collected. The supernatant cells as aliquot were stored at -80°C (Gauthaman *et al.,* 2012).

**Cultivation cancer and normal cells:** The cancer cell lines of cervic (HeLa-ATCC® CCL-2™), liver (HepG2-ATCC® HB-8065™), ovarian (SKOV3-ATCC® HTB-77™), oral squamous (HSC3-ATCC, Manassas, VA), mouse fibroblast (NIH3T3-ATCC® CRL-1658™), human fibroblast (primary cells) and hMSCs (primary cells from Wharton's jelly) were obtained from Stem Cell and Cancer Institute, Jakarta Indonesia. The cells were grown and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U $mL^{-1}$ penicillin (Invitrogen) and 100 μg $mL^{-1}$ streptomycin (Invitrogen) and incubated at 37°C in a humidified atmosphere and 5% $CO_2$ (Widowati *et al*., 2013a, 2014, 2015).

**Cell viability assay:** This assay used an optimized reagent containing resazurin which can be converted to fluorescent resorufin by viable cells that absorbs the light at 490 nm. Briefly, cells were seeded at density of 5×$10^3$ in 96 well plate for 24 h incubation (Widowati *et al.,* 2013a, b), after that cells were supplemented by WJMSCs-norCL and WJMSCs-hypoCL in various concentrations (0, 5, 10, 15 μg $mL^{-1}$) then incubated for 72 h. To analyze tumoricidal property of WJMSCs-CL toward cancer cell lines including HeLa, HepG2, SKOV3, HSC3, some cells such as NIH3T3, human fibroblast, MSCs were used as control to know cytotoxic effect of WJMSCs-CL in normal cells. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI, USA) was used in order to determine the cell viability. The MTS was added at 10 μL to each well. The plate was incubated at 5% $CO_2$, 37°C for 4 h. Absorbance of the cells was measured at 490 nm using a microplate ELISA reader (Multiskan Go). The data was presented as number of viable cells and the percent of viability (Widowati *et al*., 2013a, b, 2014).

**Statistic analysis:** The cell number data in cytotoxic effect both in cancer cell lines and in normal cells were calculated, expressed in means and standard deviation (M±SD). To compare among treatments, analysis of variance (ANOVA) were used, with p-values of less than 0.05 were considered as statistically significant and continued Tukey HSD post-Hoc test 95% confidence interval. The median inhibitory concentration ($IC_{50}$) value of cytotoxic effect was analyzed using probit analysis. Statistical analysis was conducted using SPSS 20.0 program.

## RESULTS

The WJ-MSCs markers were detected using flow cytometry analysis showed characterization of cultured cells under normoxia and hypoxia (5% $O_2$) for P4. The WJ-MSCs were positive (more than 95%) for MSCs marker CD105, CD73, CD90 and negative (less than 2%) for CD34, CD45, CD14, CD19 and HLA-II. Surface marker expression of WJ-MSCs (P4) on hypoxia and normoxia were not significantly different (Widowati *et al*., 2014, 2015) and WJ-MSCs cells were differentiated into osteocytes, chondrocytes, adipocytes (Widowati *et al*., 2014).

**Tumoricidal property:** To determine the tumoricidal property of WJMSCs-norCL, WJMSCs-hypo 5% CL from P4 on various cancer cell lines (HeLa, HepG2, SKOV3, HSC3), first we evaluated the number of cells, viability, inhibition and median inhibitory concentration ($IC_{50}$) on HeLa, HepG2, SKOV3, HSC3 cell lines by MTS assay. The number of HeLa, HepG2, SKOV3, HSC3 cells decreased compared with control in a concentration dependent-manner (Table 1). Effect of WJMSCs-norCL and WJMSCs-hypoCL toward the viability of cancer cells can be seen at Table 2. The cells viability decreased compared with control in a concentration dependent-manner. Effect of WJMSCs-norCL and WJMSCs-hypoCL toward inhibition of cancer cells can be seen in Table 3. The $IC_{50}$ value of WJMSCs-norCL and WJMSCs-hypoCL (concentration of anticancer candidate, which could inhibit 50% cell proliferation) was found to be 21.094-95.928 μg $mL^{-1}$ (Table 4).

Based on Table 1, the number of HeLa, HepG2, SKOV3, HSC3 cells significantly decreased compared with control in a concentration dependent-manner. Both normoxic and hypoxic cell lysate could decrease cell number of HeLa, HepG2, SKOV3, HSC3. The effect of WJMSCs-CL toward proliferation inhibition in various cancer cells were determined. The WJMSCs-norCL and WJMSCs-hypoCL could inhibit proliferation of cell significantly compared with the control.

Each sample (WJMSCs-norCL and WJMSCs-hypoCL) was done in triplicate and inhibition data were analyzed using probit analysis to obtain the $IC_{50}$. The selective cytotoxic effect of WJMSCs-norCL and WJMSCs-hypoCL were done in NIH3T3, human fibroblast, hMSCs. Effect of WJMSCs-norCL and WJMSCs-hypoCL toward number of normal cells can be seen at Table 5.

Table 1: Effect of WJMSCs-norCL and WJMSCs-hypoCL toward number of HeLa, HepG2, SKOV3, HSC3 cancer cells

| | Cell lysate (μg $mL^{-1}$) | | | |
|---|---|---|---|---|
| No. of cells | 0 | 5 | 10 | 15 |
| **WJMSCs-hypoCL** | | | | |
| HeLa | 24.985±1,465$^{c}$ | 20.495±546$^{b}$ | 19.675±472$^{ab}$ | 17.615±318$^{a}$ |
| HepG2 | 23.980±1,448$^{c}$ | 22.413±1,763$^{bc}$ | 19.837±237$^{b}$ | 14.997±92$^{a}$ |
| SKOV3 | 20.024±220$^{d}$ | 17.988±320$^{b}$ | 17.044±148$^{b}$ | 14.631±191$^{a}$ |
| HSC3 | 20.872±698$^{d}$ | 19.136±127$^{c}$ | 15.756±95$^{b}$ | 14.356±310$^{a}$ |
| **WJMSCs-norCL** | | | | |
| HeLa | 24.985±1,465$^{c}$ | 22.345±387$^{b}$ | 19.338±125$^{a}$ | 18.615±318$^{a}$ |
| HepG2 | 23.980±1,448$^{c}$ | 22.353±1,702$^{bc}$ | 20.410±1,031$^{b}$ | 14.987±691$^{a}$ |
| SKOV3 | 20.024±220$^{d}$ | 18.047±452$^{c}$ | 16.914±221$^{b}$ | 15.808±543$^{a}$ |
| HSC3 | 20.872±698$^{c}$ | 20.519±210$^{c}$ | 18.359±330$^{b}$ | 15.602±0$^{a}$ |

Data is expressed as Mean±SD, different letters in the same row (number of cell in each cell lysate variation) are significant differences at $p<0.05$ (Tukey HSD Post Hoc test). HeLa: Cervical cancer, HepG2: Liver cancer, SKOV3: Ovarian cancer, HSC3: Oral squamous cancer cell lines

Table 2: Effect of WJMSCs-norCL and WJMSCs-hypoCL toward cell viability of HeLa, HepG2, SKOV3, HSC3 cancer cells

| | Cell lysate (μg $mL^{-1}$) | | | |
|---|---|---|---|---|
| Cell viability (%) | 0 | 5 | 10 | 15 |
| **WJMSCs-hypoCL** | | | | |
| HeLa | 100.00±5.86$^{c}$ | 82.03±2.18$^{b}$ | 78.75±1.89$^{ab}$ | 70.50±1.27$^{a}$ |
| HepG2 | 100.00±6.04$^{c}$ | 93.47±7.35$^{bc}$ | 82.72±0.99$^{b}$ | 62.54±0.39$^{a}$ |
| Skov3 | 100.00±1.10$^{d}$ | 89.83±1.60$^{c}$ | 85.12±0.74$^{b}$ | 73.07±0.95$^{a}$ |
| HSC3 | 100.00±3.34$^{d}$ | 91.68±0.61$^{c}$ | 75.49±0.46$^{b}$ | 68.78±1.49$^{a}$ |
| **WJMSCs-norCL** | | | | |
| HeLa | 100.00±5.86$^{c}$ | 89.43±1.55$^{b}$ | 77.40±0.50$^{a}$ | 74.50±1.27$^{a}$ |
| HepG2 | 100.00±6.04$^{c}$ | 93.22±7.10$^{bc}$ | 85.11±4.30$^{b}$ | 62.50±2.88$^{a}$ |
| Skov3 | 100.00±1.10$^{d}$ | 90.12±2.26$^{c}$ | 84.47±1.10$^{b}$ | 78.94±2.71$^{a}$ |
| HSC3 | 100.00±3.34$^{c}$ | 98.31±1.00$^{c}$ | 87.96±1.58$^{b}$ | 74.75±0.27$^{a}$ |

Data is expressed as Mean±SD, different letters in the same row (cell viability in each cell lysate variation) are significant differences at $p<0.05$ (Tukey HSD post hoc test). HeLa: Cervical cancer, HepG2: Liver cancer, SKOV3: Ovarian cancer, HSC3: Oral squamous cancer cell lines

Table 3: Effect of WJMSCs-norCL and WJMSCs-hypoCL toward proliferation inhibition of HeLa, HepG2, SKOV3, HSC3 cancer cells

| | Cell lysate (μg mL$^{-1}$) | | | |
|---|---|---|---|---|
| Proliferation inhibition (%) | 0 | 5 | 10 | 15 |
| **WJMSCs-hypoCL** | | | | |
| HeLa | 0.00±5.86$^{a}$ | 17.97±2.18$^{b}$ | 21.25±1.89$^{bc}$ | 29.50±1.27$^{c}$ |
| HepG2 | 0.00±6.04$^{a}$ | 6.53±1.35$^{ab}$ | 17.28±0.99$^{b}$ | 37.46±0.39$^{c}$ |
| SKOV3 | 0.00±1.10$^{a}$ | 10.17±1.60$^{b}$ | 14.88±0.74$^{c}$ | 26.93±0.95$^{d}$ |
| HSC3 | 0.00±3.34$^{a}$ | 8.32±0.61$^{b}$ | 24.51±0.46$^{c}$ | 31.22±1.49$^{d}$ |
| **WJMSCs-norCL** | | | | |
| HeLa | 0.00±5.86$^{a}$ | 10.57±1.55$^{b}$ | 22.60±0.50$^{c}$ | 25.50±1.27$^{c}$ |
| HepG2 | 0.00±6.04$^{a}$ | 6.78±1.10$^{ab}$ | 14.89±4.30$^{b}$ | 37.50±2.88$^{c}$ |
| SKOV3 | 0.00±1.10$^{a}$ | 9.88±2.26$^{b}$ | 15.53±1.10$^{c}$ | 21.06±2.71$^{d}$ |
| HSC3 | 0.00±3.34$^{a}$ | 1.69±1.00$^{a}$ | 12.04±1.58$^{b}$ | 25.25±0.27$^{c}$ |

Data is expressed as Mean±SD, different letters in the same row (proliferation inhibition in each cell lysate variation) are significant differences at p<0.05 (Tukey HSD post hoc test). HeLa: Cervical cancer, HepG2: Liver cancer, SKOV3: Ovarian cancer, HSC3: Oral squamous cancer cell lines

Table 4: Inhibition concentration ($C_{50}$) WJMSCs-norCCL dan WJMSCs-hypoCL of HeLa, HepG2, SKOV3, HSC3 cancer cells for 72 h incubation

| | $IC_{50}$ (μg mL$^{-1}$) oxygen condition | |
|---|---|---|
| Cancer cell lines | WJMSCs-norCL | WJMSCs-hypoCL |
| HeLa | 22.685 | 86.898 |
| HepG2 | 21.705 | 21.094 |
| SKOV3 | 95.928 | 46.788 |
| HSC3 | 24.797 | 33.460 |

HeLa: Cervical cancer, HepG2: Liver cancer, SKOV3: Ovarian cancer, HSC3: Oral squamous cancer cell lines

Table 5: Effect of WJMSCs-norCL and WJMSCs-hypoCL toward number of cell on various normal cells

| | Cell lysate (μg mL$^{-1}$) | | | |
|---|---|---|---|---|
| Number of cells | 0 | 5 | 10 | 15 |
| **WJMSCs-hypoCL** | | | | |
| NIH3T3 | 25.222±255$^{ab}$ | 29.149±1,240$^{c}$ | 26.389±546$^{b}$ | 24.369±110$^{a}$ |
| Human fibroblast | 12.689±13$^{b}$ | 11.982±173$^{a}$ | 11.974±148$^{a}$ | 11.720±189$^{a}$ |
| hMSCs | 34.785±1.934$^{a}$ | 35.525±284$^{a}$ | 33.005±1.196$^{a}$ | 32.578±398$^{a}$ |
| **WJMSCs-norCL** | | | | |
| NIH3T3 | 25.222±255$^{a}$ | 27.902±380$^{b}$ | 27.075±450$^{b}$ | 24.282±707$^{a}$ |
| Human fibroblast | 12.689±13$^{b}$ | 12.352±45$^{b}$ | 11.919±181$^{a}$ | 11.767±185$^{a}$ |
| hMSCs | 34.785±1.934$^{ab}$ | 36.078±711$^{b}$ | 33.971±652$^{ab}$ | 32.305±601$^{a}$ |

Data is expressed as Mean±SD, different letters in the same row (number of cell in each cell lysate variation) are significant differences at p<0.05 (Tukey HSD post hoc test). NIH3T3: Mouse fibroblast, hMSCs: Human mesenchymal stem cells

Table 6: Effect of WJMSCs-norCL and WJMSCs-hypoCL toward cell viability on various normal cells

| | Cell lysate (μg mL$^{-1}$) | | | |
|---|---|---|---|---|
| Cell viabilities (%) | 0 | 5 | 10 | 15 |
| **WJMSCs-hypoCL** | | | | |
| NIH3T3 | 100.00±1.01$^{ab}$ | 115.57±4.92$^{c}$ | 104.63±2.16$^{b}$ | 96.62±0.44$^{a}$ |
| Human fibroblast | 100.00±0.10$^{b}$ | 94.43±1.37$^{a}$ | 94.37±1.16$^{a}$ | 92.37±1.49$^{a}$ |
| hMSCs | 100.00±5.56$^{a}$ | 102.13±0.82$^{a}$ | 94.88±3.44$^{a}$ | 93.66±1.15$^{a}$ |
| **WJMSCs-norCL** | | | | |
| NIH3T3 | 100.00±1.01$^{a}$ | 110.63±1.51$^{b}$ | 107.35±1.78$^{b}$ | 96.27±2.80$^{a}$ |
| Human fibroblast | 100.00±0.10$^{b}$ | 97.35±0.35$^{b}$ | 93.93±1.43$^{a}$ | 92.74±1.46$^{a}$ |
| hMSCs | 100.00±5.56$^{ab}$ | 103.72±2.04$^{b}$ | 97.66±1.87$^{ab}$ | 92.87±1.73$^{a}$ |

Data is expressed as Mean±SD, different letters in the same row (cell viability in each cell lysate variation), significant differences at p<0.05 (Tukey HSD post hoc test). NIH3T3: Mouse fibroblast, hMSCs: Human mesenchymal stem cells

Effect of WJMSCs-norCL and WJMSCs-hypoCL toward cells viability can be seen at Table 6. The WJMSCs-norCL and WJMSCs-hypoCL toward cells inhibition can be seen at Table 7. The $IC_{50}$ value of WJMSCs-norCL and WJMSCs-hypoCL was found to be 136.290-185.339% (Table 8).

Table 7: Effect of WJMSCs-norCM and WJMSCs-hypoCM toward proliferation inhibition on various normal cells

| | Cell lysate (μg $mL^{-1}$) | | | |
|---|---|---|---|---|
| Proliferation inhibition (%) | 0 | 5 | 10 | 15 |
| **WJMSCs-hypoCL** | | | | |
| NIH3T3 | 0.00±1.01$^{ab}$ | -15.57±4.92$^{a}$ | -4.63±2.16$^{b}$ | 3.38±0.44$^{c}$ |
| Human fibroblast | 0.00±0.10$^{a}$ | 5.57±1.37$^{b}$ | 5.63±1.16$^{b}$ | 7.63±1.49$^{b}$ |
| hMSCs | 0.00±5.56$^{a}$ | -2.13±0.82$^{a}$ | 5.12±3.44$^{a}$ | 6.34±1.15$^{a}$ |
| **WJMSCs-norCL** | | | | |
| NIH3T3 | 0.00±1.01$^{b}$ | -10.63±1.51$^{a}$ | -7.35±1.78$^{a}$ | 3.38±0.44$^{b}$ |
| Human fibroblast | 0.00±0.10$^{a}$ | 2.65±0.35$^{a}$ | 6.07±1.43$^{b}$ | 7.26±1.46$^{b}$ |
| hMSCs | 0.00±5.56$^{ab}$ | -3.72±2.04$^{a}$ | 2.34±1.87$^{ab}$ | 7.13±1.73$^{b}$ |

Data is expressed as Mean±SD, different letters in the same row (proliferation inhibition in each cell lysate variation) are significant differences at $p<0.05$ (Tukey HSD post hoc test). NIH3T3: Mouse fibroblast, hMSCs: Human mesenchymal stem cells

Table 8: Inhibition concentration ($IC_{50}$) of WJMSCs-norCL and WJMSCs-hypoCL on various normal cells for 72 h incubation

| | $IC_{50}$ (μg $mL^{-1}$) | |
|---|---|---|
| Normal cells | WJMSCs-norCL | WJMSCs-hypoCL |
| NIH3T3 | >> | >> |
| Human fibroblast | 409.191 | 629,799.738 |
| hMSCs | 468.280 | 4,860.849 |

NIH3T3: Mouse fibroblast, hMSCs: Human mesenchymal stem cells

## DISCUSSION

The surface markers and multipotent differentiation of WJ-MSCs under normoxia and hypoxia (5% $O_2$) for P4 were not presented, the data were consistent with the previous research (Vile *et al*., 2002; Widowati *et al*., 2014) showed highly expressed (95%) for MSCs' marker of CD105, CD73 and CD90 and low expressed (<2%) for CD34, CD45, CD14, CD19 (Widowati *et al*., 2014, 2015). The surface marker of WJ-MSCs in both normoxia and hypoxia were not significantly different (Widowati *et al*., 2014, 2015). The MSCs differentiated to three main mesenchymal lineages which are osteocytes, chondrocytes, adipocytes in WJ-MSCs normoxia and hypoxia (5% $O_2$) at P4 were not presented, the data were consistent with previous research (Widowati *et al*., 2014, 2015). Both the nor-WJMSCs and hypo-WJMSCs are able to differentiated to osteocytes, chondrocytes and adipocytes (Widowati *et al*., 2015).

This study showed that WJ-MSCs-norCL and WJ-MSCs-hypoCL were able to inhibit various of cancer cells including HeLa, HepG2, SKOV3, HSC3. This data were validated with previous studies that cell lysate isolated from human Wharton's Jelly Stem Cells (hWJSCs-CL) 15 μg $mL^{-1}$ has ability to change cell morphology with cell shrinkage, blebbing, vacuolations, cell death, lower proliferation rate, increase in sub-G1 and G2/M phases, increase gene expression of pro-apoptotic BAX, downregulate of anti-apoptotic BCL2 and SURVIVIN, migration rate 31-46% on breast adenocarcinoma (MDA-MB-231), ovarian carcinoma (TOV-112D) and osteosarcoma (MG-63) cells (Gauthaman *et al*., 2012). The hWJSCs-CL or hWJSCs-CM could inhibit the proliferation of mammary carcinoma and osteosarcoma cells (Lin *et al*., 2014). The incubated hWJMSCs-CM in normoxia and hypoxia condition were able to inhibit various cancer cells, such as ovarian, cervical, prostate, oral squamosa liver cells (Widowati *et al*., 2014, 2015).

The co-culture of a small number of hUCMSCs and human breast carcinoma cells (MDA 231) significantly attenuated phosphorylation of Akt and ERK1/2 in MDA 231 cells (Ayuzawa *et al*., 2009). The BM-MSCs could exert anti-tumor effects on Kaposi's sarcoma, through cell to cell contact (Khakoo *et al.,* 2006; Tamura *et al*., 2011). The UC-MSCs suppressed mitogen-induced lymphocyte-proliferation, it have convenient properties as cytotherapeutic for various diseases, including cancer (Tamura *et al*., 2011; Prasanna *et al.,* 2010). Both human and rat UC-MSCs also

possess a strong tropism to various tumor tissues (Ayuzawa *et al*., 2009; Tamura *et al*., 2011; Doi *et al*., 2010a). The UC-MSCs-dependent growth attenuation is mediated by secretory proteins/peptides and associated with decreased MAP kinase activity. The UC-MSCs secreted proteins/peptides which could induced cell death of cancer cells and cell cycle arrest (Tamura *et al*., 2011). Various cytokines are also secreted by stem cells and they played a role in the various diseases improvement. Those cytokines are growth factors, proinflammatory and anti-inflammatory cytokines and other cytokines (Pawitan, 2014). Co-culture of hUC-MSCs and MDA 231 human breast cancer cells could caused G2 arrest (Ayuzawa *et al*., 2009). Rat UC-MSCs(rUC-MSCs) also could decreased CDK2 expression and caused G0/G1 arrest of Lewis lung carcinoma (LLC) cells and murine pancreatic carcinoma cells (Tamura *et al*., 2011; Doi *et al*., 2010a; Maurya *et al*., 2010; Zheng *et al.,* 2012). Another co-culture method such as co-cultured of UC-MSCs and rat mammary tumor cells (Mat B III) in a Transwell culture system showed over-expression of multiple Tumor Suppressor Genes (TSG) (Tamura *et al*., 2011). Profiles gene expression of co-cultured UC-MSCs and Mat B III resulted in identification of five up-regulated genes, including follistatin (FST), sulfatase1 (SULF-1), Glucose Phosphate Isomerase (GPI), HtrA serine peptidase (HTRA1) and Adipocyte Differentiation-Related Protein (ADRP). Two down-regulated genes were identified as well, which were growth factor, beta-induced, 68 kDa (TGFBI) and podoplanin (PDPN) (Doi *et al*., 2010b). The UC-MSCs could stimulate caspase activities and arrest the cell cycle, it have intrinsic ability to secrete factors that can result in cancer cell growth inhibition and/or apoptosis *in vitro* and *in vivo* assay (Tamura *et al*., 2011).

Intraperitoneal injection of MSCs improved the survival of lymphoma-bearing mice through induction of Endothelial Cells (EC) apoptosis (Ho *et al*., 2013; Otsu *et al*., 2009). The MSCs have antitumor activity, mediated by paracrine that resulted in impaired Endothelial Progenitor Cells (EPCs) recruitment and downregulation proangiogenic factor such as Platelet-Derived Growth Factor (PDGF-BB), Insulin Growth Factor-1 (IGF-1), Fibroblast Growth Factor-2 (FGF-2) and interleukin-1β (IL-1β) (Ho *et al*., 2013).

Human WJSCs (hWJSCs) possess tumoricidal properties and inhibit proliferation of solid tumours such as human mammary carcinoma, ovarian carcinoma and osteosarcoma (Lin *et al*., 2014). The hWJSCs or its extracts (hWJSCs-CM and hWJSCs-CL) could significantly inhibited lymphoma cell proliferation for 48 h exposure and did not transform to Tumor Associated Fibroblasts (TAFs), unlike hBM-MSCs. Human WJ-MSCs upregulated pro-apoptotic and tumor suppressor genes that were distinct from other MSCs. The increased cell death was observed at sub-G1 and S and the decreased proliferation was observed at G2/M phases of the mitotic cycle. Superoxide dismutase ($O_2^{-*}$) and hydrogen peroxide ($H_2O_2$) activities were significantly increased and glutathione peroxidase (GPx) significantly decreased in lymphoma cells. Increased cell death was observed at sub-G1 and S as well as decreased proliferation at G2/M phases of the mitotic cycle. The proliferation of lymphoma cells was inhibited by molecules secreted by hWJSCs which were used oxidative stress pathways to induced cell death and apoptosis (Lin *et al*., 2014). Human foetal dermal derived-MSCs (Z3hMSCs) could inhibited tumor progression in H7402 and HepG2 (human liver cells) by *in vitro* assay, showed downregulation of β-catenin, c-myc, BCL 2, Proliferating Cell Nuclear Antigen (PCNA) and SURVIVIN which were lead to decreased colony-forming ability and proliferation and increased apoptosis (Qiao *et al.,* 2008). The WJMSCs secrete dickkopf-1 (DKK-1) (Pawitan, 2014), a protein known to suppress the Wnt signaling pathway. The DKK-1 may mediate p53 tumor suppression by antagonizing the Wnt signaling pathway(Wang *et al*., 2000). The upregulation of several proapoptotic and tumor suppressor genes

in hWJSCs involving several cytokines in these cells such as IL-12a, this were thought to induce apoptosis as anticancer effects of hWJSCs, hWJSCs-CM and hWJSCs-CL (Kobayashi *et al*., 1989; Wolf *et al*., 1991).

Based on the data, hypoxic culture condition (5% $O_2$) inhibited the growth of various cancer cells. (Table 1-4). This data was consistent with previous studies that hypoxic environment could greatly improved expression of chemokine receptors during *in vitro* expansion and eventually increased efficiency of MSCs-based regenerative therapies (Haque *et al*., 2013). Hypoxic BM-MSCs and their fractions showed that the stem cells expressed and secreted significant interleukin 6 (IL-6) and interleukin 8 (IL-8) under hypoxic conditions (Chen *et al*., 2014), IL-8 of hWJSCs killed the cancer cell (Gauthaman *et al*., 2012).The WJMSCs-norCL and WJSCs-hypoCL may inhibit cells proliferation in various cancer cell lines and were not toxic for normal cells.

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