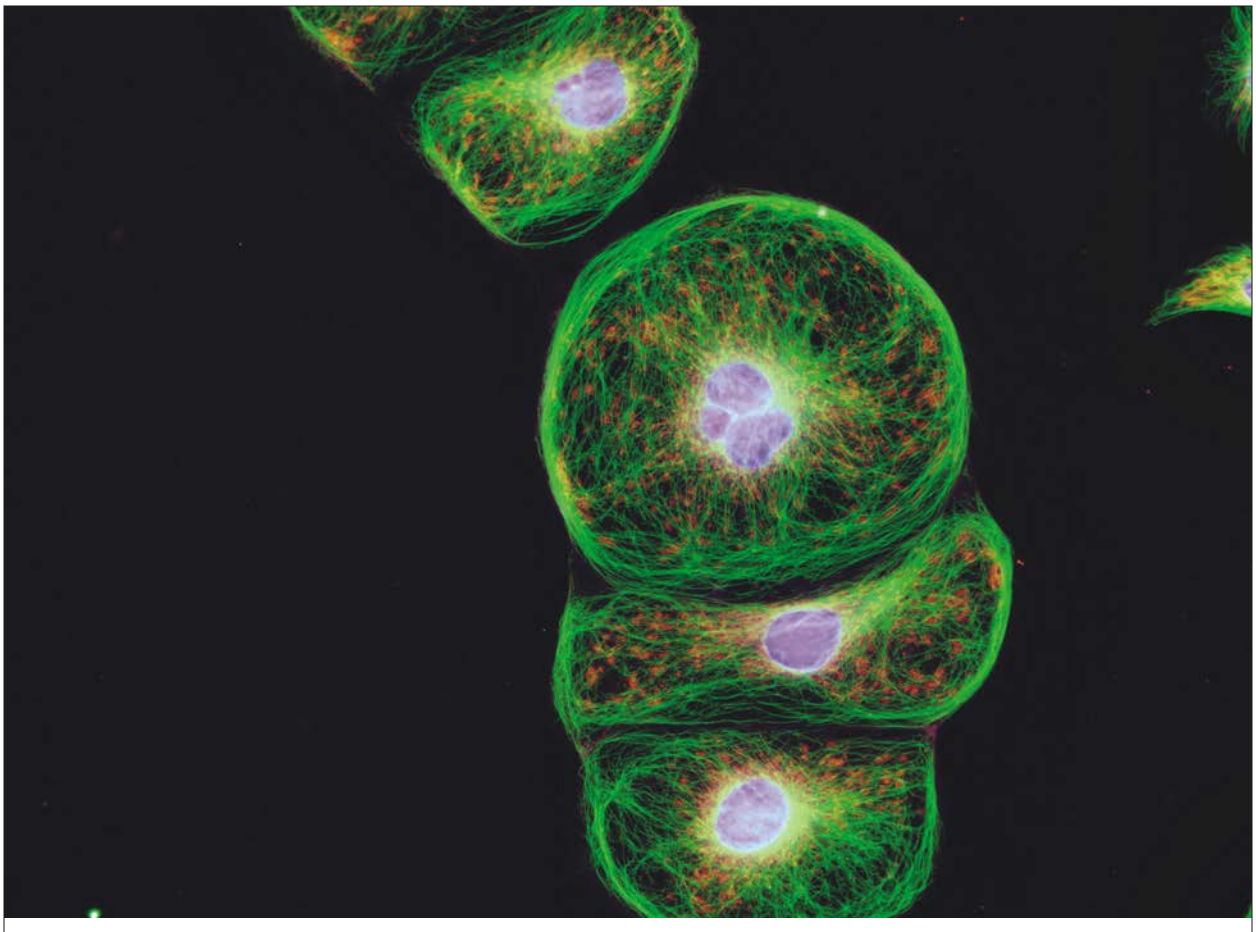

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Research Article
Selective Cytotoxic Potential of IFN-γ and TNF-α on Breast Cancer Cell Lines (T47D and MCF7)

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Abstract

Background: Breast cancer is one of the most common life threatening diseases worldwide and it is the leading cause of death from cancer in women. The effectiveness of current cancer therapies is low, even though it requires expensive cost. Therefore, the development of the more efficient therapy is highly needed. **Objective:** This research was conducted to evaluate the effect Tumour Necrosis Factor alpha (TNF-α) and Interferon gamma (IFN-γ) as anticancer agents against breast cancer cells (T47D, MCF7) and selectivity of cytotoxic effect towards human Wharton's Jelly Mesenchymal Stem Cells (hWJMSCs) to produce hWJMSCs-Conditioned Medium (CM), hWJMSCs-Cell Lysate (CL) and hWJMSCs which potential used as anticancer candidates after induced by TNFα or IFNγ. **Methodology:** The hWJMSCs were isolated from umbilical cord and characterized by its surface marker phenotype and its multipotent differentiation potential. The breast cancer cell lines (T47D and MCF7) were treated by TNF-α and IFN-γ and its cytotoxic activity was observed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium (MTS) viability assay. The cytotoxic effects of TNF-α and IFN-γ toward hWJMSCs were also observed using the same method. **Results:** Flow cytometry analysis showed that hWJMSCs for early passage (P4) were positive for hMSCs marker CD105, CD73 and negative for CD34, CD45, CD14, CD19 and HLA-II. It also showed the osteogenic, adipogenic and chondrogenic differentiation. The effect of TNF-α and IFN-γ against MCF7 cells exhibited that the cytokines decreased the cell viability in a dose dependent manner. The IC_{50} value of TNF-α and IFN-γ against breast cancer cell lines were found higher for T47D than MCF7. **Conclusion:** The TNF-α and IFN-γ inhibit the cell growth of breast cancer cells due to apoptotic or necrotic cells but it non-toxic against hWJMSCs.

Key words: Breast cancer, TNF-α, IFN-γ, hMSCs, cytotoxic, umbilical cord

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

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

INTRODUCTION

Breast Cancer (BC) is the leading cause of death from cancer in women and one of the important contributors to the global health burden[1]. It is the second leading cause of death by cancer, which is 1 in 3 cancers diagnosed among women in the United States[2] and also the second most frequent among women in Mexico. In 2008, breast cancer was the most incident of cancer and a major cause of mortality from cancer among women in ASEAN[3]. Cancer has a severe impact on individuals and community which leads to disability and death. Its high treatment costs, which are associated with higher income loss can quickly undermine family finances[3]. The development of antineoplastic drugs have been based on the conviction that cancer would constitute a cell-autonomous genetic and epigenetic disease[4]. Even though there are improved treatment models, many tumours remain unresponsive to the current conventional cancer therapies including surgery, chemotherapy and radiotherapy[5]. The effectiveness of current cancer therapies is low although it requires high cost[6]. Therefore, the development of the more efficient therapy is highly needed. Strategic therapy can target tumours directly, both in primary and metastatic side[7].

Tissue engineering has also become the new promising therapies. Human Mesenchymal Stem Cells (hMSCs) are the sources of adult stem cells for cell therapy and tissue engineering[8]. The hMSCs are the powerful source for tissue repair, because it has the multi-potency differentiation capability, easy to acquire, easy harvesting process and culture, fast *in vitro* expansion, the feasibility of autologous and allogenic therapy and a powerful paracrine function[9]. The hMSCs are capable to migrate, incorporate into the tumour site both *in vitro* co-culture and *in vivo* xenograft and survive in cancer tissue[5,7,8]. The hMSCs can be delivered successfully for various treatments to treat multiple metastatic cancers, including lung, breast, squamous cell, colon, pancreas and cervical cancers[10,11]. The hMSCs can be isolated from adult tissues, including bone marrow, peripheral blood, adipose tissue, skeletal muscle, synovium, dental pulp and neonatal birth-associated tissues including umbilical cord blood, umbilical cord, placenta, decidua, chorion villi, chorion membrane, amniotic membrane, amniotic fluid and Wharton's Jelly (WJ)[12]. Wharton's Jelly (WJ) is part of the umbilical cord, as one source of hMSCs has many advantages including low risk of infection, non-carcinogenesis, multipotency and low immunogenicity[13]. The hWJMSCs have anticancer potential which mediated via cell-to-cell and/or non-cellular contact mechanism. The hWJMSCs, Conditioned Medium (CM) and cell free lysate (CL) of hWJMSCs demonstrate anticancer potential against solid tumour and become attractive candidates for future cancer therapies[14,15]. The CM of hWJMSCs (hWJMSCs-CM) incubated in normoxic and hypoxic conditions can inhibit proliferation of various cancer cell lines, including cervical (HeLa), liver (HepG2), prostate (PC-3), ovarian (SKOV3) and oral squamous (HSC3) cancer cell lines and were safe for normal cells in human fibroblast, mouse (NIH3T3) and human Mesenchymal Stem Cells (hMSCs)[15]. The hMSCs are the attractive vehicles to deliver therapeutic agents in various tumour sites to introduce gene expression or to secrete the therapeutic factor for cancer and to enhance the anticancer activity[16]. Anticancer agents produced by engineered MSCs are classified into: (a) Immunostimulation including chemokine C-X3-C motif ligand 1 (CX3CL1), interferon (IFN) and interleukins (IL2, IL7 and IL12), (b) Pro-drug conversion, such as cluster of differentiation (CD) and Herpes Simplex Virus Thymidine Kinase (HSV-tk) and (c) Apoptosis induction such as IL-8, Natural Killer 4 (NK4) and tumour necrosis factor-related apoptosis inducing ligand (TRAIL)[5,10,17].

The TNF-α as an anticancer drug is limited to local treatments because of its dose-limiting systemic toxicity[18]. The TNF-α can induce apoptosis but also supports cell survival pathways[19]. Although TNF-α is cytotoxic to certain tumour cell lines, it does not trigger apoptosis in normal cells but instead stimulates the proliferation of normal fibroblasts[20]. The type II cytokine Interferon (IFN-γ) is an essential cytokine for immunity against intracellular pathogens and in controlling tumour cell growth[21] but IFN-γ may also facilitate melanoma progression[22]. Continuing research was conducted to evaluate the effect TNF-α and IFN-γ as anticancer agents against breast cancer cells (T47D, MCF7) and selectivity of cytotoxic effect towards hWJMSCs to produce CM (hWJMSCs-CM) and CL (hWJMSCs-CL) and hWJMSCs as anticancer candidate induced by TNF α or IFNγ.

MATERIALS AND METHODS

Isolation and cell culture of hWJMSCs: Isolation and cultivation of hWJMSCs from umbilical cord was as described by Widowati *et al.*[15,23]. Fresh human Umbilical Cords (UC, n = 3) from women aged 25-40 years after full-term births (normal vaginal delivery), all donors signed an informed consent and research guidelines using UC were approved by Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia and from Ethics Committee collaboration between Maranatha Christian University, Bandung, Indonesia and Immanuel Hospital Bandung, Bandung, Indonesia.

The hWJMSCs from UC were rinsed in normal saline (0.9% w/v sodium chloride) and cut into very small explants (1-2 mm), then plated on tissue culture plastic plates. The explants were cultured in Minimum Essential Medium-α (MEM-α) with 2 mM GlutaMAX (Invitrogen, Carlsbad, CA, USA), supplemented with 20% Fetal Bovine Serum (FBS, Invitrogen) and penicillin, streptomycin amphotericin B (100 U mL^{-1}, 100 and 0.25 mg mL^{-1}; Invitrogen). Cell cultures were incubated in a humidified atmosphere with 5% CO_2 at 37°C and medium was replaced every 5 days for 21 days. The cells were harvested and replated at a density 8×10^3 cells cm^{-2}, when cells reached 80-90% confluence. The hWJMSCs were cultured in 95% relative humidity (21% O_2) and 5% CO_2[15,23].

Cell surface phenotype and multipotent differentiation: The surface marker characterization, including CD105, CD73, CD90, CD34, CD45, CD14, CD19 and Human Leukocyte Antigen II (HLA-II) was performed to confirm the phenotype characteristic of hWJMSCs of passage (P4) using a flow cytometry. The hWJMSCs at 80% confluence were harvested and dissociated with trypsin-EDTA, then centrifuged at 300 g for 10 min. The pellet was resuspended with Phosphate Buffered Saline (PBS)+2% FBS and cells were counted with a hemocytometer. Around 100-200 cells in 25 mL PBS were stained with the appropriate surface monoclonal antibodies. Isotype controls were used to determine background staining. These antibodies were as follows: PE (Phycoerythrin) conjugated: CD105 (abcam 53321-100), CD73 (BD550257), CD90 (abcam 226), CD34 (BD 348053), CD45 (BD 555482), CD14 (abcam 28061-100), CD19 (abcam 1168-500) and HLA-DR (abcam 23901), FITC conjugated: mIgG1 (BD349041 and BD 349043) and mIgG2 (BD349053) 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, CD34-FITC, CD45-FITC, CD14-FITC after incubation at 4°C for 15 min. The cells were analyzed by flow cytometry with a FACS Calibur TM 3 argon laser 488 nm (Becton Dickinsok Biosciences, Franklin Lakes, NJ, USA) using CellQuest Pro Acquisition on the BD FACS tation TM Software. The experiments and measurement of surface marker were performed in triplicate[15,23].

For osteogenic differentiation, hWJMSCs (P4) were seeded at density 1×10^4 cells cm^{-2} in culture dishes using StemPro Osteogenesis Differentiation Kit (Gibco A10072-01, Invitrogen) for 3 weeks. Calcium deposits were visualized using Alizarin red S (Biochemicals and Life Science Research Products, Amresco 9436). For chondrogenic differentiation, hWJMSCs were seeded at density 1×10^4 cells cm^{-2} in culture dishes using Stem Pro Chondrogenesis Differentiation Kit (Gibco A10071-01, Invitrogen) for 2 weeks. Chondrocytes were visualized using Alcian blue (Amresco, 0298). Adipogenic differentiation of hWJMSCs was done using StemPro Adipogenesis Differentiation Kit (Gibco A10070-01, Invitrogen, Carlsbad, CA, USA) for 2 weeks. We used Oil Red O (Sigma 00625, St Louis, USA) to confirm the lipid droplets[23].

Cell viability assay of cancer cells-treated TNF-α and IFN-γ: Breast cancer cell lines MCF7 (ATCC® HTB22™) and T47D (ATCC®HB133™) were obtained from Stem Cell and Cancer Institute, Jakarta Indonesia. Medium consist of Roswell Park Memorial Institute (RPMI) (Biowest L0495) for T47D and Dulbecco's Modified Eagle's Medium (DMEM) (Biowest L0104) for MCF7 supplemented with FBS 10% (Biowest S1810) and 1% penicillin-streptomycin (Biowest L0018), then incubated in 5% CO_2, 37°C incubator. Cells were seeded at density of 5×10^3 in 96 well plate for 24 h incubation after cells reached 80% confluence. Cells were supplemented with TNF-α recombinant (Biolegend 570106) and IFN-γ recombinant (Biolegend 570206) in various concentrations (0, 60, 180, 260 and 520 ng mL^{-1}) then incubated for 24, 40 and 72 h. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyme-thoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, Madison, WI, USA) was added at 10 µL to each well. The plate was incubated at 5% CO_2, 37°C for 4 h. The absorbance of the cells was measured at 490 nm using a micro-plate ELISA reader (MultiSkan Go). The data were presented as the number of viable cells, the percentage of viable cells (%), the inhibition of cell proliferation (%) and the median Inhibitory Concentration (IC_{50}) were calculated from the data[15,24,25].

Cell viability assay of hWJMSCs-treated TNF-α and IFN-γ: Cells viability of hWJMSCs was determined by using MTS (Promega, Madison, WI, USA). Cells were maintained at MEM-α (Biowest L0475) with 2 mM GlutaMAX (Invitrogen, Carlsbad, CA, USA), supplemented with 20% FBS (Biowest S1810) and penicillin streptomycin-amphotericin B (100 U mL^{-1}, 100 and 0.25 mg mL^{-1}; Invitrogen). Cell cultures were incubated in an incubator (humidified atmosphere, 5% CO_2 at 37°C). Cells were seeded at density of 5×10^3 in 96 well plate for 24 h incubation after cells reached 80% confluence. Cells were supplemented with TNF-α recombinant (Biolegend 570106) and IFN-γ recombinant (Biolegend 570106) in various concentrations (0, 60, 180, 260, 520 ng mL^{-1}) then incubated for 72 h. The MTS was added at 10 µL to each well and incubated at 5% CO_2, 37°C for 4 h. The absorbance of the cells was measured at 490 nm using a micro-plate ELISA reader (MultiSkan Go). The data were presented as number of viable cells, the percentage of viable

cells (%), the percentage of proliferative inhibition (%) and the median Inhibitory Concentration (IC_{50}) were calculated from the data[15,24,25].

Statistical analysis: The data of cell number, cell viability and proliferative inhibition of hWJMSCs, MCF7 and T47D cells were calculated and expressed in means and standard deviation (M±SD). Analysis of variance (ANOVA) was used to compare among treatments with $p<0.05$ as statistically significant and continued with Tukey honestly significant difference *post hoc* test and 95% confidence interval. The median Inhibitory Concentration (IC_{50}) was analyzed by using probit analysis to determine the cytotoxic effect of TNF-α and IFN-γ on cells (hWJMSCs, MCF7 and T47D cells). Statistical analysis was performed in SPSS version 20.0 program (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp, SPSS Inc., Chicago, IL, USA)[15].

RESULTS

MSC markers by cell surface phenotype: Flow cytometry analysis showed that hWJMSCs for P4 were positive for hMSCs marker CD105, CD73 and negative for CD34, CD45, CD14, CD19 and HLA-II. The surface marker of hWJMSCs can be seen in Table 1 and Fig. 1. The surface marker of hWJMSCs from three donors show the characteristic of hWJMSCs by high expression of CD105, CD73 markers (>94%) and low expression of CD34, CD45, CD14, CD19 and HLA-II markers (<2%)[15,23].

Multilineage differentiation capacity of hWJ-MSCs: The multilineage differentiation of hWJMSCs cultured in MEM-α was evaluated by culturing hWJMSCs in the differentiation medium (chondrogenic, osteogenic and adipogenic lineages).The hWJMSCs were cultured in osteogenesis differentiation medium (StemPro Osteogenesis Differentiation Kit, Gibco A10072-01, Invitrogen) for approximately 3 weeks with the osteocytes morphology were assayed. The differentiated cells exhibited calcium deposits based on the staining of Alizarin red S (Biochemicals and Life Science Research Products, Amresco 9436). The hWJMSCs were cultured in chondrogenesis differentiation medium (StemPro Chondrogenesis Differentiation Kit, Gibco A10071-01, Invitrogen) for 2 weeks and the differentiated hWJMSCs exhibited chondrocyte expression using Alcian blue (Amresco, 0298). After 2 weeks, hWJMSCs were cultured in adipogenesis differentiation (StemPro Adipogenesis Differentiation Kit, Gibco A10070-01, Invitrogen), the differentiated cells exhibited lipid droplets was observed by using Oil Red O (Sigma 00625, St Louis, USA) (Fig. 2). Cell staining showed that hWJMSCs from three donors were able to differentiate into chondrocytes, osteocytes and adipocytes (Fig. 2)[15].

Cytotoxic effect of TNF-α and IFN-γ in breast cancer cells: The viability assay was performed to evaluate the effect of TNF-α and IFN-γ against breast cancer cells (MCF7 and T47D). The breast cancer cells were cultured in a 96-well plate with density 5×10^3 cells cm^{-2}. The cytotoxic effect of TNF-α, IFN-γ were determined by the cells number, the cell viability according to the MTS assay. Human recombinant TNF-α and IFN-γ demonstrated reducing cancer cell proliferation in a dose dependent manner. The effect of TNF-α and IFN-γ against T47D cells in cells number, cells viability and proliferation inhibition can be seen in Table 2. The number of T47D cells increased at low concentration but at higher concentration of TNF-α and IFN-γ killed and inhibited the cells proliferation. The effect of TNF-α and IFN-γ against MCF7 cells in cells number, cells viability and proliferative inhibition can be seen in Table 3. The effect of TNF-α and IFN-γ against MCF7 cells exhibited that the cytokines decreased the cell

Table 1: Surface marker of hWJMSCs for passage 4

Surface marker	Donor 1	Donor 2	Donor 3
CD73	96.62±2.12	97.62±0.96	98.35±0.29
CD105	93.73±0.36	95.14±1.35	95.13±2.50
HLA-DR	-3.68±0.45	-2.90±2.01	-3.64±1.88
CD14	0.01±0.01	0.00±0.01	0.00±0.01
CD19	-1.54±1.38	-0.81±0.75	-0.61±0.07
CD34	0.00±0.01	0.00±0.01	0.00±0.00
CD45	0.00±0.01	0.00±0.01	0.00±0.01

Data was presented as Mean±SD of surface markers of hWJMSCs

Table 2: Effect of TNF-α and IFN-γ against breast cancer cells (T47D) for 72 h incubation

	TNF-α			IFN-γ		
Concentrations (ng mL^{-1})	No. of cells	Cells viability (%)	Cells inhibition (%)	No. of cells	Cells viability (%)	Cells inhibition (%)
0	3230±443^{b}	100.00±0.00^{c}	0.00±0.00^{a}	3230±443^{d}	100.00±0.00^{bc}	0.00±0.00^{ab}
60	3318±331^{b}	103.14±4.78^{c}	-3.14±4.78^{a}	3987±175^{e}	124.92±17.08^{c}	-24.92±17.08^{a}
180	2484±448^{b}	77.13±10.48^{b}	22.87±10.48^{b}	2491±85^{c}	78.21±12.37^{b}	21.79±12.37^{b}
260	871±46^{a}	27.38±4.79^{a}	72.62±4.79^{c}	1395±143^{b}	43.82±8.25^{a}	56.18±8.25^{c}
520	633±125^{a}	20.09±6.09^{a}	79.91±6.09^{c}	553±146^{a}	16.91±2.44^{a}	83.09±2.44^{c}

Data are expressed as Mean±SD, different superscripts of small $^{a, ab, b, bc, c, d, e}$letters in the same column (among concentrations of TNF-α and IFN-γ treatment on T47D cells) were significantly different at $p<0.05$ (Tukey honestly significant difference *post hoc* test), TNF-α: Tumour necrosis factor alpha, IFN-γ: Interferon gamma

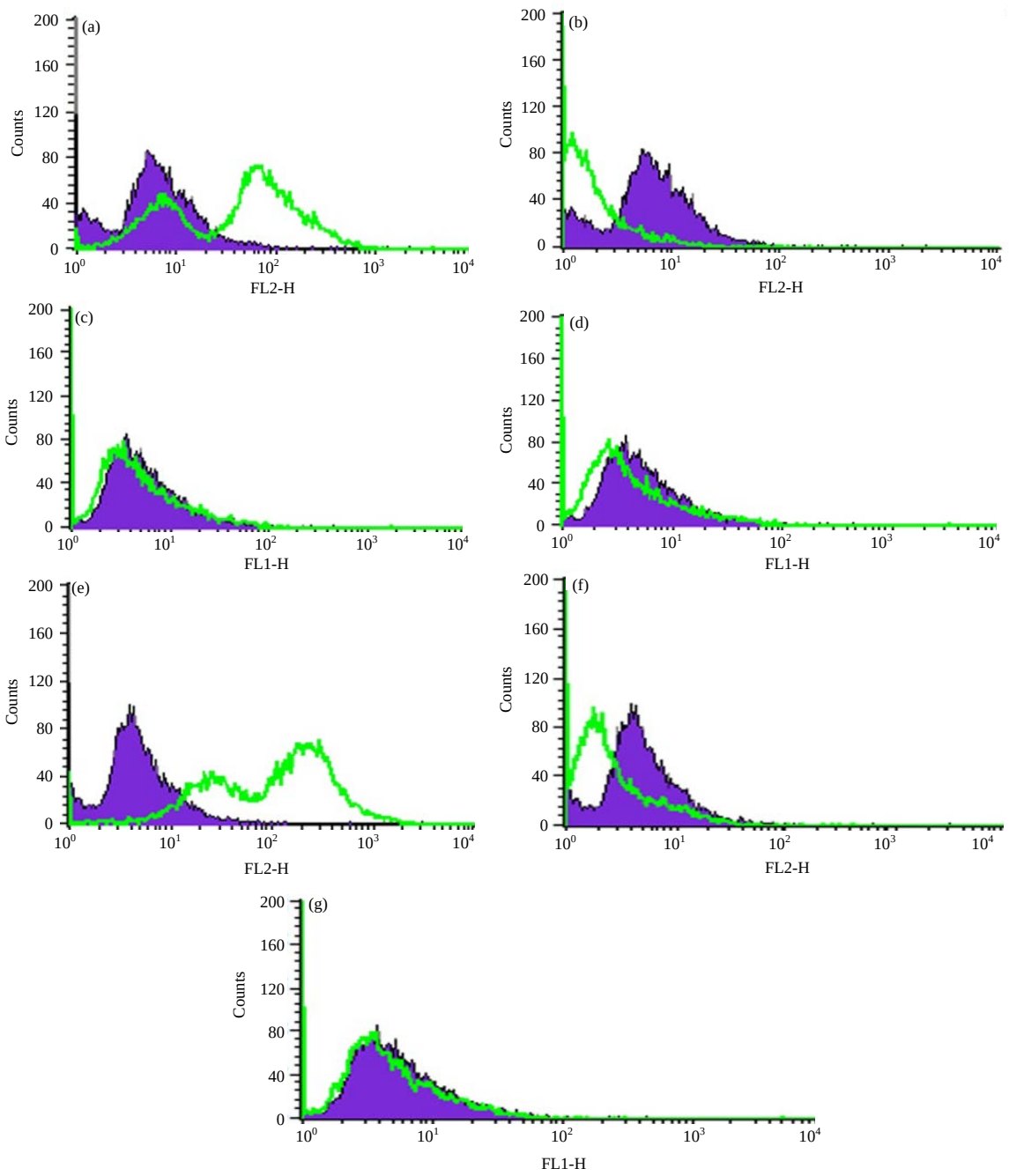

Fig. 1(a-g): Representation of flow cytometry measurement on (a) CD105, (b) HLA-II, (c) CD34, (d) CD45, (e) CD73, (f) CD19 and (g) CD14, hWJMASCs: Human Wharton's jelly mesenchymal stem cells

Table 3: Effect of TNF-α and IFN-γ against breast cancer cells (MCF7) for 72 h incubation

	TNF-α			IFN-γ		
Concentrations (ng mL^{-1})	No. of cells	Cells viability (%)	Cells inhibition (%)	No. of cells	Cells viability (%)	Cells inhibition (%)
0	15380±1.168^{d}	100.00±0.00^{d}	0.00±0.00^{a}	15380±1.168^{c}	100.00±0.00^{c}	0.00±0.00^{a}
60	11951±96^{c}	79.53±4.84^{c}	20.47±4.84^{b}	9050±1.643^{b}	58.18±6.80^{b}	41.82±6.80^{b}
180	9550±213^{b}	63.78±2.27^{b}	36.22±2.27^{c}	7221±129^{ab}	48.09±2.57^{a}	51.91±2.57^{bc}
260	7691±892^{a}	52.46±8.56^{ab}	47.44±8.56^{cd}	7135±801^{ab}	45.88±3.94^{a}	54.12±3.94^{c}
520	6900±375^{a}	45.35±2.86^{a}	54.65±2.86^{d}	5832±333^{a}	38.58±3.40^{a}	61.42±3.40^{c}

Data are expressed as Mean±SD, different superscripts of small $^{a, ab, b, bc, c, cd, d}$letters in the same column (among concentrations of TNF-α and IFN-γ treatment on MCF7 cells were significantly different at p<0.05 (Tukey honestly significant difference *post hoc* test), TNF-α: Tumour necrosis factor alpha, IFN-γ: Interferon gamma

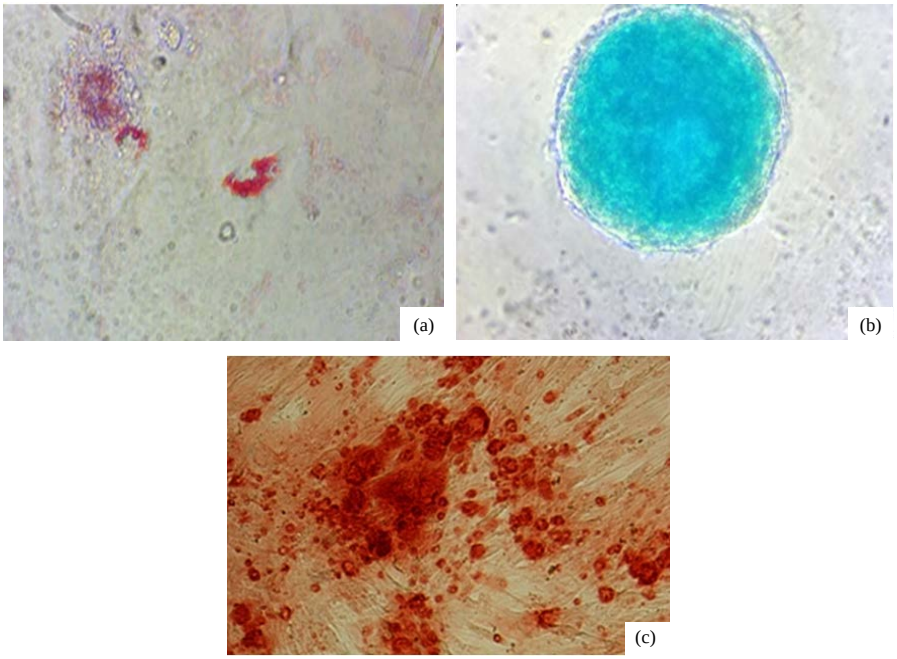

Fig. 2(a-c): Morphological appearance of (a) Osteogenic (b) Adipogenic and (c) Chondrogenic differentiation of hWJMSCs from three donors at P4, hWJMSCs: Human Wharton's jelly mesenchymal stem cells

Table 4: IC_{50} of TNF-α and IFN-γ against breast cancer cells (T47D and MCF7)

T47D	IC_{50} (ng mL^{-1})	IC_{50} (μg mL^{-1})
TNF-α	242.77	0.24
IFN-γ	266.88	0.27
MCF7	IC_{50} (ng mL^{-1})	IC_{50} (μg mL^{-1})
TNF-α	364.78	0.36
IFN-γ	295.03	0.30

IC_{50} is the median inhibitory concentration of TNF-α and IFN-γ against the T47D and MCF7, TNF-α: Tumour necrosis factor alpha, IFN-γ: Interferon gamma

viability in a dose dependent manner. The IC_{50} value of TNF-α and IFN-γ against breast cancer cell lines were found 242.77-266.88 ng mL^{-1} for T47D and 295.03-364.78 ng mL^{-1} for MCF7 can be seen in Table 4. Based on the Table 4 both TNF-α and IFN-γ are more active as anticancer in T47D cells compared to MCF7.

Cytotoxic effect of TNF-α and IFN-γ in mesenchymal stem cells: The cells viability assay of hWJMSCs after TNF-α and IFN-γ treatment were performed to determine the selective cytotoxic effect of TNF-α and IFN-γ against only to cancer cells and non-toxic against to normal cells. The TNF-α and IFN-γ are non-toxic, safe and can induce or increase the anticancer potency of mesenchymal stem cells. This research was carried out to evaluate the cytokines TNF-α and IFN-γ as an anticancer drug directly toward cancer cells but these cytokines are safe against hWJMSCs. The effect of TNF-α and IFN-γ against hWJMSCs were carried out in various concentrations of cytokines (0, 60, 100, 180, 260 and 520 ng mL^{-1}), which hWJMSCs were treated with cytokines for 1, 2, 3 days. The selective cytotoxic effect of TNF-α, IFN-γ against hWJMSCs were determined, including the cells number, the cell viability by MTS assay, it can be seen in Table 5 and 6. The IC_{50} value of TNF-α and IFN-γ against hWJMSCs after 72 h incubation were found infinite (Table 7). Based on the Table 7 both TNF-α and IFN-γ are non-toxic toward hWJMSCs.

DISCUSSION

The surface marker of hWJMSCs from 3 donors showed positive results for CD105, CD73 and negative for CD34, CD45, CD14, CD19 and HLA-II (Table 1). These data were validated by the previous result that hWJMSCs highly expressed CD105, CD73 and low expression of CD34, CD45, CD14, CD19 and HLA II[15,22]. The hWJMSCs were able to differentiate into chondrocyte, osteocyte and adipocyte (Fig. 1) as previous research[15]. The TNF-α and IFN-γ inhibited the growth rate of breast cancer cells both in T47D and MCF7 in a dose dependent manner, higher concentration of cytokines would decrease number of cells, cell viability and increase proliferation inhibition (Table 3). The TNF-α and IFN-γ inhibited the breast cancer cells induced by apoptosis or cell death.

Table 5: Effect of TNF-α against hWJMSCs in various incubations and concentrations

	Concentrations (ng mL^{-1})					
Incubation	0	60	100	180	260	520
Number of cells hWJMSCs-TNF-α						
Day 1	9422±135^{aA}	11217±48^{bA}	9133±496^{aA}	9268±536^{aA}	9388±589^{aA}	9320±411^{aA}
Day 2	15435±456^{bcC}	15425±264^{bcC}	15503±588^{cC}	14054±188^{abB}	14071±51^{abcB}	13227±995^{aB}
Day 3	11948±699^{bB}	12898±3.11^{cB}	10919±199^{abB}	9648±188^{aA}	9958±91^{aA}	9943±49^{aA}
Viability of hWJMSCs-TNF-α (%)						
Day 1	100.00±0.00^{aA}	119.08±2.20^{bA}	96.96±5.68^{aA}	98.43±6.96^{aA}	99.61±5.08^{bA}	98.89±3.12^{aA}
Day 2	163.89±6.93^{cdC}	163.74±3.21^{bcdC}	164.51±3.97^{dC}	149.17±0.84^{abB}	149.37±2.10^{abcB}	140.35±9.52^{aB}
Day 3	126.77±5.97^{cB}	136.89±2.54^{dB}	115.93±3.48^{bB}	102.42±1.36^{aA}	105.70±1.43^{aA}	105.54±1.00^{aA}
Inhibition of hWJMSCs-TNF-α (%)						
Day 1	0.00±0.00^{bC}	-19.08±2.20^{aC}	3.04±5.68^{bC}	1.57±6.96^{bA}	0.39±5.08^{bB}	1.11±3.12^{bB}
Day 2	-63.89±6.93^{abA}	-63.74±3.21^{abcA}	-64.51±3.97^{aA}	-49.17±0.84^{cdB}	-49.37±2.10^{cdA}	-40.35±9.52^{dA}
Day 3	-26.77±5.97^{bB}	-36.89±2.53^{aB}	-15.93±3.48^{cB}	-2.42±1.36^{dA}	-5.70±1.43^{dB}	-5.54±1.00^{dB}

Data are presented as Mean±SD, $^{a, ab, abc, b, bc, c, cd, d}$Different superscript of small letter are significant differences among the means of groups (among concentrations of TNF-α treatment) in same row, ^{A,B,C}Significant differences among the means of groups (among long incubation day 1, 2 and 3) in number of cells, cells viability, cells proliferation inhibition in same column at p<0.05 based on Tukey honestly significant difference *post hoc* test, TNF-α: Tumour necrosis factor alpha, hWJMSCs: Human wharton's Jelly mesenchymal stem cells

Table 6: Effect of IFN-γ against hWJMSCs in various incubations and concentrations

	Concentrations (ng mL^{-1})					
Incubation	0	60	100	180	260	520
Number of cells hWJMSCs-IFN-γ						
Day 1	10210±607^{abA}	12817±1.960^{bA}	9814±687^{aA}	9851±621^{aA}	9793±953^{aA}	9744±627^{aA}
Day 2	10922±1.122^{aA}	19175±629^{cB}	16246±205^{bB}	14890±984^{bC}	10218±547^{aA}	9749±15^{aA}
Day 3	10093±278^{aA}	15340±1.060^{cA}	12198±422^{bB}	12046±523^{bB}	11232±257^{abA}	10005±344^{aA}
Viability of hWJMSCs-IFN-γ						
Day 1	100.00±0.00^{aA}	126.370±25.09^{aA}	96.130±3.72^{aA}	96.560±4.96^{aA}	96.170±11.07^{aA}	95.420±1.02^{aA}
Day 2	107.68±17.78^{aA}	188.160±10.55^{cB}	159.460±8.79^{bcC}	146.120±11.48^{bB}	100.540±11.72^{aA}	95.710±5.75^{aA}
Day 3	98.98±3.44^{aA}	150.740±15.73^{bAB}	119.710±7.16^{aB}	118.330±10.05^{aA}	110.180±4.45^{aA}	98.190±45.78^{aA}
Inhibition of hWJMSCs-IFN-γ						
Day 1	0.00±0.00^{aA}	-26.370±25.09^{aB}	3.870±3.72^{aC}	3.440±4.96^{aB}	3.830±11.07^{aA}	4.580±1.02^{aA}
Day 2	-7.68±17.78^{cA}	-88.160±10.55^{aA}	-59.460±8.79^{abA}	-46.120±11.48^{bA}	-0.540±11.72^{cA}	4.290±5.75^{cA}
Day 3	1.02±3.44^{bA}	-50.740±15.73^{aAB}	-19.710±7.16^{bB}	-18.330±10.05^{bB}	-11.300±1.81^{bA}	0.820±4.16^{bA}

Data are presented as Mean±SD, $^{a, ab, b, bc, c}$Different supercript of small letters are significant differences among the means of groups (among concentrations of TNF-α treatment) in same row, ^{A,B,C}Significant differences among the means of groups (among long incubation day 1, day 2, day 3) in number of cells, cells viability, cells proliferation inhibition in same column at p<0.05 based on Tukey honestly significant difference *post hoc* test, TNF-α: Tumour necrosis factor alpha, hWJMSCs: Human Wharton's Jelly mesenchymal stem cells

Table 7: IC_{50} of TNF-α, IFN-γ against hWJMSCs

Incubation	TNF-α	IFN-γ
Day 3 (72 h)	~	~

~: Infinite, TNF-α: Tumour necrosis factor alpha, IFN-γ: Interferon gamma, hWJMSCs: Human Wharton's jelly mesenchymal stem cells, IC: Inhibitory concentration

Tumour Necrosis Factor (TNF) super family is a group of cytokines that have many important functions in process of immunity, inflammation, cell differentiation, cell survival, cell proliferation and apoptosis[26,27]. The binding of TNF-α to its receptor, which is Tumour Necrosis Factor Receptor (TNFR1) can alternatively induce cell death or survival and differentiation through the formation of two sequential complexes[28]. Complex I triggers rapid activation of the transcription factors Nuclear Factor-kappa B (NF-kB) and activator protein-1, complex II is formed after the former is released from the membrane into the cytosol by receptor internalization and endosomal trafficking[29,30]. Complex II leads to the death-inducing signalling complex formation and ultimately, caspase-dependent apoptosis[31].

The direct targeting potential of TNF-α in tumour cells has been clinically used recently in therapy designs such as for soft tissue sarcoma and melanomas with an acceptable safety profile[32,33]. The TNF-α was reported as anticancer therapy due to its cytotoxic effect against a number of tumour cells[32,34,35]. Blocking the intracellular cell survival pathways that activated by TNF-α definitely leads tumour cells to apoptosis[33,36]. The apoptotic process is complex and mainly triggered through the activation of death receptors[37], among these the Tumour Necrosis Factor Receptor1 (TNFR1) and its ligand TNF-α play a crucial role[38]. The binding of TNF-α to TNFR1 triggers a series of intracellular events initiated by the recruitment of a key adaptor protein TNFR1-associated with death domain protein (TRADD) to the receptor complex[26,39].

The TNF-α induces caspase-dependent (apoptotic) and caspase-independent (necrosis-like) cell death in different types of cells[40]. Caspases play key roles in mediating Fas or TNF-α-induced apoptosis and are divided into two classes based on the lengths of their N-terminal prodomains: upstream caspases such as caspase 8 and 10 and downstream caspases[41] such as -3, -6 and -7. The activation of caspases are required for apoptosis[41]. The FAS Associated Death Domain Protein (FADD) is essential for TNF-induced apoptosis as it recruits caspase-8[42,43]. Caspase-8 is activated, thereby initiating a caspase cascade, which results in apoptosis[26,44-46]. The BID is a target of caspase-8[47,48]. The TNF-α is able to induce apoptosis via two distinct caspase-8 activation pathways, which are differentially regulated by Cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2) and Cellular FLICE-like inhibitory protein (c-FLIP)[49]. The TNF-induced necrotic cell death through NF-kB activation are the anti-apoptotic RIP-, TRAF2- and FADD-mediated Reactive Oxygen Species (ROS) accumulation[46]. Reactive Oxygen Species was found to be required for necrotic cell death in L929 cells[47]. The TNF-α can also induce cell death in a context-dependent manner[50]. The TNF-induced cell death involves the BCL2/Adenovirus E1B 19 kDa interacting protein 3 (BNIP3), ROS production and activation of the lysosomal death pathway[40]. Apoptosis induced by TNF-α is also associated with the generation of ROS. Induction of ROS production or an inhibition of the NF-kB pathway by cyclooxygenase-2 (COX-2) inhibitors is reported to be successful in sensitizing tumour cells to TNF-α-induced apoptosis[51].

Based on Table 2 and 3, TNF-α could induce proliferation and inhibition at 72 h of incubation period. This result was validated with previous research that TNF-α showed a dose- and time-dependent cytotoxic effect on cell survival. The longer the periods of time, the cell mortality increased even further. The MCF7 stimulated TNF-α increased the phosphorylated state of Akt (pAkt)[52]. Treatment using 20 ng mL^{-1} of TNF-α in HeLa and HepG2 cell lines for 24 h incubation could not inhibit the cell proliferation or no dead cell observed[32]. Low dose of TNF-α induced the cell survival while the high dose of TNF-α (100 ng mL^{-1}) inhibited the cell survival (10%)[49]. This previous research showed consistent with this data, Table 2 shows that low concentration of TNF-α (60 ng mL^{-1}) could induce cell proliferation in T47D cells.

The TNF-α also demonstrated to enhance the anti-tumour effect *in vitro*, when used in combination with other cytokines[34,53]. When combined with interferon alpha and gamma (IFN-α and IFN-γ), TNF-α induced synergistic growth inhibition toward pancreatic cancer cell lines[54]. The combination of TNF-α and IFN-γ on 23 cell lines *in vitro*, showed cytostatic and cytotoxic effects on cell lines previously resistant to TNF-α and IFN-γ individually[34]. The IFN-γ has an important role in protective mechanisms against viral, bacterial infections and tumour control[55,56], antiviral, anti-tumour and immunomodulatory activities[57]. The IFNs mediate anti-tumourigenic effects indirectly by modulating immunomodulatory responses or directly by regulating tumour cell proliferation and differentiation[58] and inhibition of tumour angiogenesis[59].

The IFN-γ treatment induced phosphorylation of ATR (ataxia-telangectasia and Rad3-related protein) and Chk1 (Serine/threonine-specific protein kinase that in humans), which activated the ataxia-telangectasia by ATR and Chk1 signalling following the treatment. The ATR-Chk1 signalling might be associated with IFN-γ-induced responses. Phosphorylation of both ATR and Chk1 increased in the presence of IFN-γ in a dose-dependent manner[57]. Dose-dependent decrease of Chk1 protein was also observed following the IFN-γ treatment. The IFN-γ treatment decreased the half-life of Chk1 indicating that Chk1 protein was destabilized following IFN-γ treatment[57]. The direct effect of IFN-γ on tumour cell killing is inhibiting cellular proliferation mediated by Signal Transducers and Activators of Transcription 1 (STAT-1), Interferon Regulatory Factor-1 (IRF-1), p21 or p27[60-62]. The IFN-γ has anti-proliferative effects on cancer cells. The numerous anti-proliferative effects of IFN-γ in many cancers through G1 arrest involving down-regulation of G1/S cyclins (cyclins A and E)[63] and CDK2/4. Treatment using IFN-γ induces the up regulation of pro-apoptotic proteins such as Fas, Fasligand (FasL) and TRAIL[58,64,65]. These proteins can interact with FADD or TRAIL-receptor proteins, resulting in initiation of apoptosis through activation of caspase-8[66,67]. The IFN-induced apoptosis involves the FADD/caspase-8 signalling pathway, activation of the caspase cascade, release of mitochondrial cytochrome c, disruption of mitochondrial membrane potential, changes in plasma membrane symmetry and DNA fragmentation[58,68]. The IFN-γ exposure also increased caspase-8 expression in three of six cell lines[69]. Many of the inhibitory effects of IFNγ appeared to be mediated by IRF-1. The IRF-1 also regulates the expression of several genes involved in apoptosis, such as the pro-apoptotic protein BAX, the tumour suppressor p53, caspase-1 and interferon-induced protein kinase (PKR)[70]. The anti-proliferative action of IFN-γ depends in part on STAT1, which induces the expression of the cell cycle inhibitor, cyclin dependent kinase inhibitor 1A (CDKN1A) (p21CIP1), preventing entry into the S phase of the cell cycle[60]. The IFNγ-induced growth suppression can be rescued by blocking Ras GTPase[71] and reducing p53 or the Ataxia-Telangectasia Mutated (ATM) kinase protein levels[57,72].

The stimulation of TNF-α and IFN-γ did not inhibit the hWJMSCs proliferation. This data were consistent with previous research that MSCs-prestimulated IFN-γ and TNF-α significantly reduced expression of Transforming Growth Factor beta (TGF-β)[73], a secreted protein that controls cell proliferation, cellular differentiation and other functions in most cells. The TGF-β acts as an anti-proliferative factor in normal epithelial cells and at early stages of oncogenesis[74]. The IFN-γ stimulation (500 U mL^{-1}) in hMSCs elevated the Indolamine 2,3-dioxygenase (IDO) expression and increased the iNOS levels in mouse MSCs lysate[75]. The IDO in inhibiting alloantigen drives proliferation by stimulated MSCs[73].

The IFN-γ prestimulated MSCs (IMSCs) maintained their *in vitro* differentiation capacity, forming osteoblasts, adipocytes and chondroblasts[75]. Human MSCs cultured for 6 days in the presence of IFN-γ retained their capacity to adhere to plastic[75]. The pre-treatment of MSCs prior to transplantation with TNF-α increased adhesiveness and migration of MSCs *in vitro,* thus lead to increased expression of Bone Morphogenetic Protein (BMP)-2 by MSCs[72].

CONCLUSION

This preliminary research about the effect of TNF-α and IFN-γ directly in T47D and MCF7 cell lines have not been identified yet. Breast cancer cells treated directly by TNF-α and IFN-γ showed that these cytokines inhibit the cell growth due to apoptotic or necrotic cells. The hWJMSCs treated by TNF-α and IFN-γ showed these cytokines non-toxic against normal cells. The apoptotic mechanism by direct TNF-α and IFN-γ treatment in T47D and MCF7 should be continued and clarified especially by the apoptotic gene expression analysis and indirectly effect of TNF-α and IFN-γ in T47D and MCF7 by involving Natural Killer (NK) cell as immunemodulatory.

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