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Potential of Conditioned Medium of hATMSCs in Aging Cells Model

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ABSTRACT

Skin aging is caused by the exposure cumulative of ultraviolet radiation, it leads reactive oxygen species (ROS) production in the skin. The conditioned medium of human Adipose Tissue-derived Mesenchymal Stem Cells (hATMSCs) can scavenge free radicals and increase the survival rate of skin cells under oxidative stress. This study examined the protective effects of Conditioned Medium (CM) of hATMSCs in H_2O_2-induced human skin fibroblast cell line (BJ). The aging cells model using H_2O_2-induced BJ cells were added CM-hATMSCs in concentrations (0, 10, 30%) and incubated in various time, furthermore BJ cells induced by various H_2O_2 concentrations (0, 50, 100, 200 µM) incubated for 1 h. The anti-aging potential were measured including viability, ROS and collagen levels in BJ cells which treated CM-hATMSCs. The median inhibitory concentration (IC_{50}) of H_2O_2 on BJ cells for 1 h incubation was 107.87 µM and 91.25 µM for 10 min incubation. CM-hATMSCs increased the viability on aging model cells. CM-hATMSCs concentration 30% increased the viability of H_2O_2 50, 100, 200 µM-induced BJ cells. CM-hATMSCs concentration 25% decreased ROS, increased collagen level in H_2O_2 50, 100, 200 µM-induced BJ cells. CM-hATMSCs increase the viability cells, collagen level and decrease ROS level in aging model cells.

1. Introduction

Skin is the outermost protective layer of the human body and hence any damage caused to it is quite visible. Skin damage and skin aging may be caused due to a combination of various intrinsic factors, such as chronological aging or extrinsic factors, such as ultraviolet (UV) rays (photo-aging) and other environmental stressors (Chen *et al.* 2013; Amirkhani *et al.* 2016; Chandrasekaran *et al.* 2017). Skin damage and skin aging may be caused due to a combination of various intrinsic factors, such as chronological aging or extrinsic factors, such as ultraviolet (UV) rays (photo-aging) and other environmental stressors (Balasubramanian *et al.* 2017). The characteristics of skin aging includes wrinkles, dryness, laxity, thinning, irregular pigmentation, and loss of elasticity (Konno *et al.* 2013; Amirkhani *et al.* 2016), the development of sagging skin, appearance of blemishes or age spots, altered pigmentation or loss of skin tone and hydration (Gold *et al.* 2007).

Chronic exposure to UV radiation on human skin generates solar elastotic, degrades the extracellular matrix (ECM), forms wrinkle. UV radiation exposure leads to production of reactive oxygen species (ROS) in the skin (Widowati *et al.* 2018a). The excessive of reactive chemical molecules are the major cause of the aging process, which is caused by the oxidants–antioxidants imbalance or oxidative stress (Piotrowska and Bartnik 2014). The increasing ROS production by mitochondria and increased 8-Hydroxyguanine (8-oxo-dG) content in the mitochondrial DNA (mtDNA) are frequently detected in aged tissues, suggesting that progressive accumulation of oxidative DNA damage is a contributory factor to the aging process (Cui *et al.* 2012).

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Oxidative stress plays lead to cellular senescence and the associated secretory phenotype (Chandrasekaran *et al.* 2017), cause damage to biomolecules which ultimately results in decline of tissue functions and aging (Perez *et al.* 2009). Various approaches to evoke oxidative stresses, such as the exposures of cells to UV light, ethanol, tert-butyl hydroperoxide (t-BHP), and peroxide hydrogen (H_2O_2) have been used to study the onset of cellular senescence (Wang *et al.* 2013; Petrova *et al.* 2016), hyperoxia (Petrova *et al.* 2016) among which H_2O_2 is the most commonly used inducer (Miyoshi *et al.* 2006). An imbalance of the ROS scavenging system results in the accumulation of H_2O_2 in the skin (Pelle *et al.* 2005).

In recent years, cell therapies for rejuvenation have gained much importance. Stem cells are emerging science and progressively being used in regenerative medicine and offers hope to cure many critical diseases, especially in unmet medical needs (Amirthalingam and Seetharam 2016). Human Mesenchymal Stem Cells (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. Adipose Tissue-derived MSCs (ATMSCs) are a population of cells derived from adipose tissue that are relatively easy to obtain from adipose tissue and are more frequent than MSCs in bone marrow (Bieback *et al.* 2010). ATMSCs are a good alternative source for cell therapy because of their easily accessible, high amount of cells, ubiquitously available in large quantities, minimal invasive harvesting procedure (Kolaparthy *et al.* 2015). ATMSCs exhibit multi lineage development potential and are able to secrete various factors, which influence adjacent cells. Previous studies have reported the effectiveness of ATMSCs Conditioned Medium (CM-ATMSCs) in wound healing, anti melanogenesis, wrinkle improvement and hair growth (Lee *et al.* 2015). MSCs Conditioned Medium (CM-MSCs media having a variety of growth factors; cytokines etc. acts as chemo-attractant and recruiting macrophages and endothelial cells for the wound healing (Ansari *et al.* 2013), increased collagen synthesis and suggest that CM-MSCs might be a potential candidate for preventing UV-induced skin damage (Kwon *et al.* 2016), CM consisting of various growth factors and cytokines is known to promote regeneration of damaged tissue. Thus, the stem cell conditioned medium is an innovative technology, which can be used in skin care, hair care, and dark circles under the eye (Pawitan 2014; Amirthalingam and Seetharam 2016). ATMSCs and their soluble factors have been reported to increase the proliferation and migration of normal keratinocytes and fibroblasts (Kim *et al.* 2007). Therefore, in this study we evaluated CM from ATMSCs (CM-ATMSCs) in reducing ROS, increasing collagen level and cell viability in H_2O_2-induced skin fibroblast cells.

2. Materials and Methods

2.1. Isolation and Expansion of hATMSCs

Adipose tissue resulted from liposuction of three different healthy woman volunteer donors was put into schott bottle 250 ml fulfilled with transport medium including 80% Minimum Essential Medium Eagle-α (MEM-α, Gibco, A1049001), 1% Antibiotic Antimycotic (ABAM) (Gibco, 15240062) and 20% Fresh Frozen Platelet (FFP, Indonesia Red Cross, Bandung) in ice-bag with informed consent using the guidelines approved by the Institutional Ethics Committee of Padjadjaran University, Bandung, West Java, Indonesia (No: 1062/UN6.C1.3.2/KEP/PN/2016). The fats were filtered using cell strainer 100 µm and washed with phosphate buffered saline (PBS) (Gibco, 14200075), then transferred into 15 ml tube. Briefly, 30 ml of Collagenase type I 0.075% (Gibco, 17100017) was added into the tube and centrifuged (MPW-2000) at 1,200 rpm, 10 min at room temperature. Then, the cells pellet was put into flask with completed consist of 80% MEM-α, 20% FFP, 1% Antibiotic and Antimycotic and 1% heparin (Inviclot, IH2983) (Widowati *et al.* 2014, 2015, 2019).

2.2. hATMSCs Characterization

MSCs from passage 3 (P3) cultured at density of 2 x 10^6 cells in T-flask 25 cm^3 (Corning, 430168) reached 80% confluent were harvested (P4) to analyze positive and negative markers using flowcytometry (Macsquant Analyzer 10, Miltenyi). The cells was stained with some components (CD90 FIT C, CD105 PerCP-Cy5, CD73 APC, CD34 PE, CD116 PE, CD19 PE, CD45 PE, HLA-DR PE, and CD44 PE) in hMSCs analysis kit and the procedure was according to manufacture protocol (BD stem flowTM kit, 562245). The experiments and measurement of surface marker were performed from three donors (Widowati *et al.* 2014, 2015, 2019).

2.3. hATMSCs Differentiation

For osteogenic differentiation, ATMSCs were seeded at density 5 x 10^3 cells in 24 well plate (Nunc, 72296-18) using StemPro Osteogenesis Differentiation Kit (Gibco A10072-01) for 3 weeks. Calcium deposits were visualized using Alizarin

red S (Sigma Aldrich, A5533). For chondrogenic differentiation, ATMSCs were seeded at density 5 x 10^3 cells in 4 well plate (Nunc, 176740) using StemPro Chondrogenesis Differentiation Kit (Gibco A10071-01) for 3 weeks. Chondrocytes were visualized using Alcian blue (Sigma Aldrich, A5268). Adipogenic differentiation of AT-MSCs was done using StemPro Adipogenesis Differentiation Kit (Gibco A10070-01) for 3 weeks. Oil Red O (Sigma Aldrich, O0625) to confirm lipid droplets (Widowati *et al.* 2015; Balasubramanian *et al.* 2017; Widowati *et al.* 2019).

2.4. Preparation of CM-hATMSCs

hATMSCs Passage 4 (P4) were seeded on plastic-surfaced culture disks with MEM-α 80%, 1% Antibiotic and Antimicotic, FFP 20%, incubated in 37°C, 5% CO_2 to obtain CM-hATMSCs. After cells were confluence 80-90%, cells were detached using 0.25% trypsin EDTA solution (Gibco, 25200072). Detached cells were cultured in another flask until confluence was achieved. Briefly, the conditioned medium (CM) was collected and centrifuged at 1,600 rpm, 5 min at room temperature, and the supernatant was filtered by a Syringe Filter Pes 0.22 µm (TPP, 99722), stored in -80°C and used as CM-hATMSCs (Widowati *et al.* 2015, 2018b).

2.5. Human Skin Fibroblast Cell Culture and Cells Viability Assay

Human skin fibroblast cell line (BJ cells, ATCC® CRL-2522) was obtained from Aretha Medika Utama Biomolecular and Biomedical Research Center, Bandung, Indonesia. The cells were cultured in MEM (Gibco, 11095080), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, 10270106), 1% ABAM and incubated at 37°C, 5% CO_2 and 95% humidity. Cells (5 x 10^3 cell/well) were seeded into 96-well plate, incubated for 24 h at 37°C, 5% CO_2. After cells were 80% confluent, growth medium changed with fresh growth medium, and added CM-hATMSCs (0, 10, and 30%), incubated for 2 h. Cells were added with varied level of H_2O_2 (0, 50, 100, 200 µM), Tert-butyl hydroperoxide 100 µM (TBHP, Sigma Aldrich 416665) and incubated for 1 h. Briefly, 20 µl MTS3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Abcam, ab22388) was added and incubated at 37°C, 5% CO_2 for 3 h, the absorbance was read at 490 nm wavelength (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific). The cells viability was calculated according the absorbance value (Widowati *et al.* 2015, 2019).

2.6. Measurement of ROS Level

The intracellular ROS levels were measured using a DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Ab113851) protocol with slight modification by flow-cytometry (MacsQuant Analyzer 10, Miltenyi). BJ cells were harvested and suspended into buffer DCFDA in FACS tube at final concentration of 2.5 x 10^5 cells/500 µl. Stained cells with 25 µM DCFDA on each tube, then incubated for 30 min in 37°C, 5% CO_2 (dark room). CM-hATMSCs (0, 10, and 25%) were added to each tube and incubated for 2h in 37°C, 5% CO_2. Then, the various level of H_2O_2 (0, 50, 100, and 200 µM) were added as ROS models, H_2O_2-induced BJ cells and treated with CM-hATMSCs were incubated for 1h in 37°C, 5% CO_2. Treated BJ cells were analyzed using flow cytometer (Gilmore *et al.* 2017; Prahastuti *et al.* 2019; Girsang *et al.* 2021).

2.7. Measurement of Collagen Content

The BJ cells with density 2 x 10^5/well were seeded with complete growth medium in plate with 6 well. After cells reached 80% confluence, cells were treated with various concentrations of CM-hATMSCs (0, 10, and 25%) and incubated for 2 h in 37°C, 5% CO_2. Then, various level of H_2O_2 (0, 50, 100, and 200 µM) were added to well as ROS models, and incubated for 1 h in 37°C, 5% CO_2. The medium of treated BJ cells (growth medium which treated with CM-hATMSCs and H_2O_2) were collected. Briefly, 500 µl medium was added with 200 µl Sirius Red 1% (Sigma Aldrich, 365548) incubated at room temperature for 1-2 h, furthermore centrifuged at 13,000 rpm for 30 min. Supernatants were discarded. Pellets were suspended with 200 µl NaOH 1N (Merck, 1091371000). Absorbance was read at 540 nm wavelength. The collagen content of cells were calculated according the collagen type I standard curve (Keira *et al.* 2004).

3. Results

3.1. Characteristic of hATMSCs

We measured the surface marker of hATMSCs P4 which can be seen at the Table 1. Based on the data (Table 1) show that hATMSCs of P4 have surface markers expression CD90 (98.24%), CD44 (99.53%), CD105 (99.60%), CD73 (90.70%), and lineage negative (0.39%).

3.2. Effect CM-hATMSCs Towards Proliferation of Aging Cells Model

We measured the potency of CM-hATMSCs to improve the human skin fibroblast proliferation after inducing by free radicals (H_2O_2 and TBHP) (Figure 1). The data (Figure 1A) showed that H_2O_2 kill and inhibit

Table 1. hATMSCs characteristic with surface marker lineage-positive (CD90, CD44, CD105, and CD73), and lineage-negative (CD11b, CD19, CD34, CD45, and HLA-DR)

Replication	CD90 (%)	CD44 (%)	CD105 (%)	CD73 (%)	Lineage-negative (%)
1	99.55	99.49	90.60	90.60	0.45
2	95.56	99.53	90.27	90.27	0.41
3	99.62	99.57	91.23	91.23	0.30
Mean ± STD	98.24±2.32	99.53±0.04	90.70±0.49	90.70±0.49	0.39±0.08

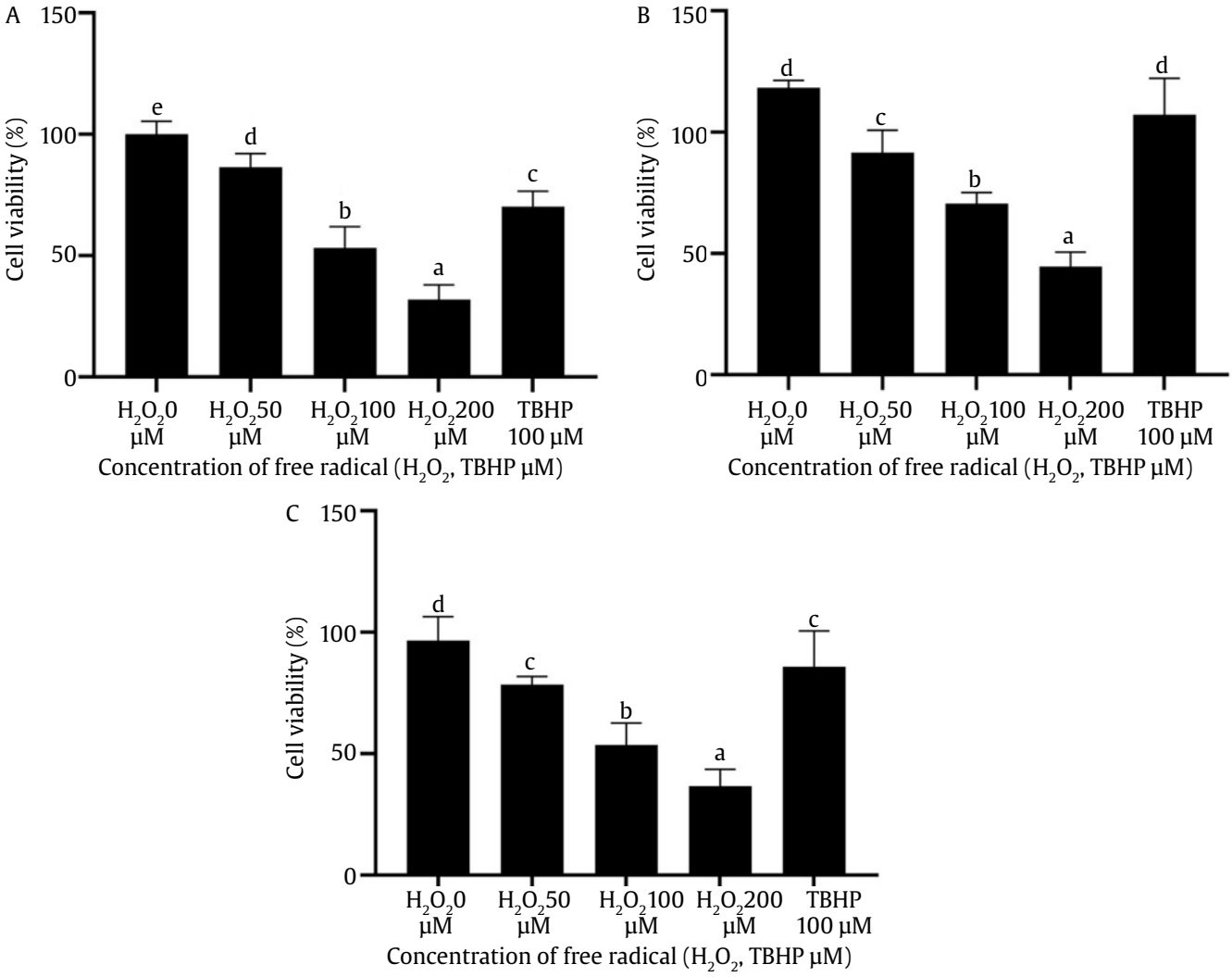

Figure 1. Effect various concentrations of CM-hATMSCs treatment on H_2O_2-induced human skin fibroblast cells as aging cells model toward cells proliferation capacity. (A) The histogram are presented as mean ± standard deviation of 0% CM-hATMSCs treatment toward aging cells proliferation. Different small letters (a, b, c, d, e) in the histogram (among concentrations of H_2O_2 inducer on BJ cells) were significantly different at $p<0.05$ (Tukey post hoc test), (B) the histogram are presented as mean ± standard deviation of 10% CM-hATMSCs treatment toward aging cells proliferation. Different small letters (a, b, c, d) in the histogram (among concentrations of H_2O_2 inducer on BJ cells) were significantly different at $p<0.05$ (Tukey post hoc test), (C) the histogram are presented as mean ± standard deviation of 30% CM-hATMSCs treatment toward aging cells proliferation. Different small letters (a, b, c, d) in the histogram (among concentrations of H_2O_2 inducer on BJ cells) were significantly different at $p<0.05$ (Tukey post hoc test)

skin fibroblast proliferation, higher concentration of H_2O_2 increase cell death. TBHP 100 µM inhibit BJ cells proliferation lower compared to H_2O_2 (100 µM, 200 µM). Treatment using CM-hATMSCs (10 %) decrease BJ cells death, the most active of CM-hATMSCs (10 %) toward BJ cells is treated with TBHP 100 µM then followed by H_2O_2 50 µM, 100 µM, 200 µM respectively (Figure 1B). Treatment using CM-hATMSCs (10%) improves H_2O_2 induced-cells proliferation (Figure 1B) but CM-hATMSCs (30 %) has not ability to improve H_2O_2 induced-cells (Figure 1C).

3.3. Effect CM-hATMSCs Towards ROS Level of Aging Cells Model

This research was very important to measure the ROS level of H_2O_2 induced-human skin fibroblast which treated by CM-hATMSCs in various concentrations (Figure 2). The CM-hATMSCs (30%) have lower ability to improve cells proliferation compared to 10 % on H_2O_2-induced cells, the further step to measure the ROS level of aging cells treated with CM-hATMSCs in level 10%, 25%. Based on the data (Figure 2A) show that H_2O_2 increase ROS level in H_2O_2-induced skin fibroblast cells, higher concentration of H_2O_2 increase ROS level. Treatment using CM-hATMSCs (10%, 25%) increase ROS level in normal cells (Figure 2B and C). The CM-hATMSCs (10%, 25%) decrease ROS level in aging cells model (induced by H_2O_2 100, 200 µM) (Figure 2D). The representative ROS level in dot plot for each treatment can be seen in Figure 3.

3.4. Effect CM-hATMSCs Towards Collagen Level of Aging Cells Model

This research was done to evaluate the effect various concentrations of CM-hATMSCs treatment on free radicals-induced human skin fibroblast cells as aging cells model toward collagen level (Figure 4). Based on the data (Figure 4) shows that skin fibroblast cells without and with H_2O_2 treatment didn't contain collagen. The CM-hATMSCs contained collagen which higher concentration of CM-hATMSCs increased collagen level, the CM-hATMSCs 10% contained collagen 39.80 µg/ml and the CM-hATMSCs 25% contained 242.87 µg/ml. The H_2O_2-induced skin fibroblast in low level of H_2O_2 inducer (50 µM) resulted highest collagen level both in CM-hATMSCs 10% (99.81 µg/ml) and CM-hATMSCs 25% (604.98 µg/ml). Higher H_2O_2 (100 µM) as aging inducer decreased collagen level both in CM-hATMSCs 10% (92.68 µg/ml) and CM-hATMSCs 25% (495.97 µg/ml). The highest H_2O_2 (200 µM) inducer decreased collagen level were lower than H_2O_2 (100 µM) inducer.

4. Discussion

The cumulative exposure to UV light causes skin aging, which resulted the development of ROS in the skin. The CM-hATMSCs can scavenge free radicals and boost the survival rate of skin cells under oxidative stress.

Based on Figure 1 showed that H_2O_2 inhibit skin fibroblast proliferation and increase cell death. This result was consistent with previous research that treatment with 0.5 mM H_2O_2 reduced cell viability to 27.3% and with 1.0 mM decreased the viability to 9.07% (Dash *et al.* 2008). H_2O_2 increased the number of dead cells, 50–500 µM H_2O_2 significantly decreased the population of viable human pulmonary fibroblast (HPF) cells in a concentration-dependent manner, H_2O_2 induced cell death via apoptosis in HPF cells (Park and Bae 2016). Proliferation of normal human dermal fibroblasts was reduced by H_2O_2 (600 µM) treatment (Sohn *et al.* 2018).

Treatment using CM-hATMSCs (10%) improves normal skin fibroblast (H_2O_2 uninduced-cells) proliferation (Figure 1B), but CM-hATMSCs (30%) has not ability to improve H_2O_2 induced- cells (Figure 1C). The CM-hATMSCs has ability to improve cells viability on aging cells model, this result was consistent with previous research that CM-BMMSCs promoted the proliferation of human foreskin fibroblast (HFF) with concentration of CM-BMMSCs 5%, significant differences in proliferation potential at 25% and 50% compared to control medium (Balasubramanian *et al.* 2017). The CM-MSCs can increase the survival rate of dorsal root ganglia cells under oxidative stress of H_2O_2 and presents neuroprotective properties (Campisi 2013; Jones *et al.* 2013). The CM-MSCs have a variety of growth factors; cytokines which acts as chemo-attractant and recruiting macrophages and endothelial cells for the wound healing (Ansari *et al.* 2013). The application MSCs-derived growth factors and cytokines (GF/CKs) has been implicated for the repair and regeneration of the damaged skin that occurs due to aging and exposure to environmental stress factors (Balasubramanian *et al.* 2017). These growth factors and cytokines present in the CM, are believed to be beneficial in reducing signs of skin aging, owing to their capacity to promote dermal fibroblast and keratinocyte proliferation. CM was able to induce proliferation, migration, the Extracelllular Matrix (ECM) synthesis etc., in damaged fibroblasts by extraneous elements, thus suggesting that it may be useful for skin repair and rejuvenation (Balasubramanian *et al.* 2017). MSCs produce an array of mediators such as cytokines and

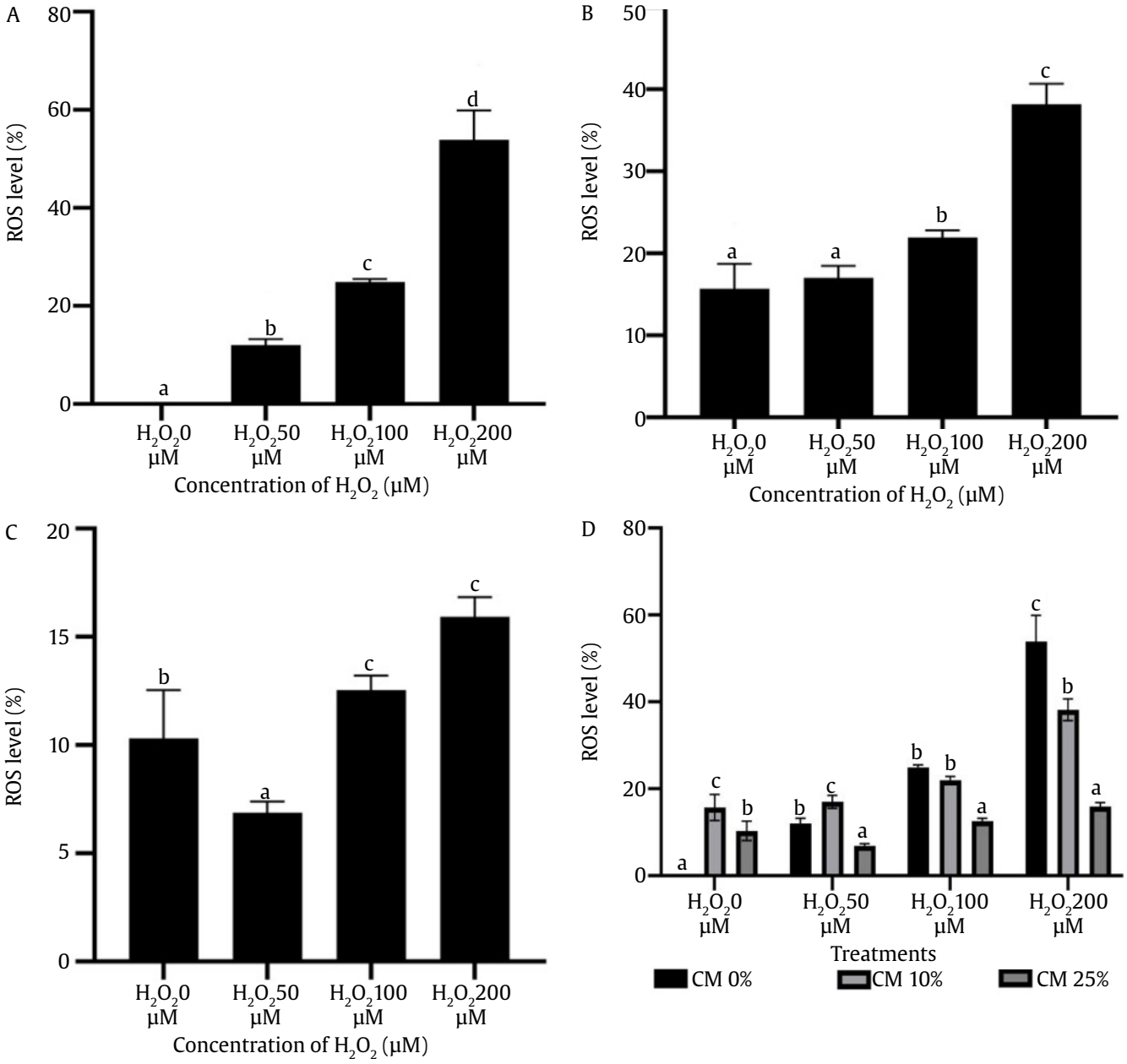

Figure 2. Effect various concentrations of CM-hATMSCs treatment on free radicals-induced human skin fibroblast cells as aging cells model toward ROS level. (A) The histogram are presented as mean ± standard deviation of 0% CM-hATMSCs treatment toward ROS level. Different small letters (a, b, c, d) in the histogram (among concentrations of H_2O_2 inducer on BJ cells) were significantly different at $p<0.05$ (Tukey post hoc test), (B) the histogram are presented as mean ± standard deviation of 10% CM-hATMSCs treatment toward ROS level. Different small letters (a, b, c) in the histogram (among concentrations of H_2O_2 inducer on BJ cells) were significantly different at $p<0.05$ (Tukey post hoc test), (C) the histogram are presented as mean ± standard deviation of 25% CM-hATMSCs treatment toward ROS level. Different small letters (a, b, c) in the histogram (among concentrations of H_2O_2 inducer on BJ cells) were significantly different at $p<0.05$ (Tukey post hoc test), (D) the histogram are presented as mean ± standard deviation of 0, 10, 25% CM-hATMSCs treatment toward ROS level. Different small letters were significant different among concentrations 0, 10, 20, 30 % of CM-hATMSCs. Different letter (a, b, c) were significant different CM-hATMSCs concentrations in 0 µM H_2O_2. Different letter (a, b, c) were significant different among CM-hATMSCs concentrations in 50 µM H_2O_2. Different letter (a, b) were significant different among CM-hATMSCs concentrations in 100 µM H_2O_2. Different letter (a, b, c) were significant different among CM-hATMSCs concentrations in 200 µM H_2O_2 at $p<0.05$ (Tukey post hoc test)

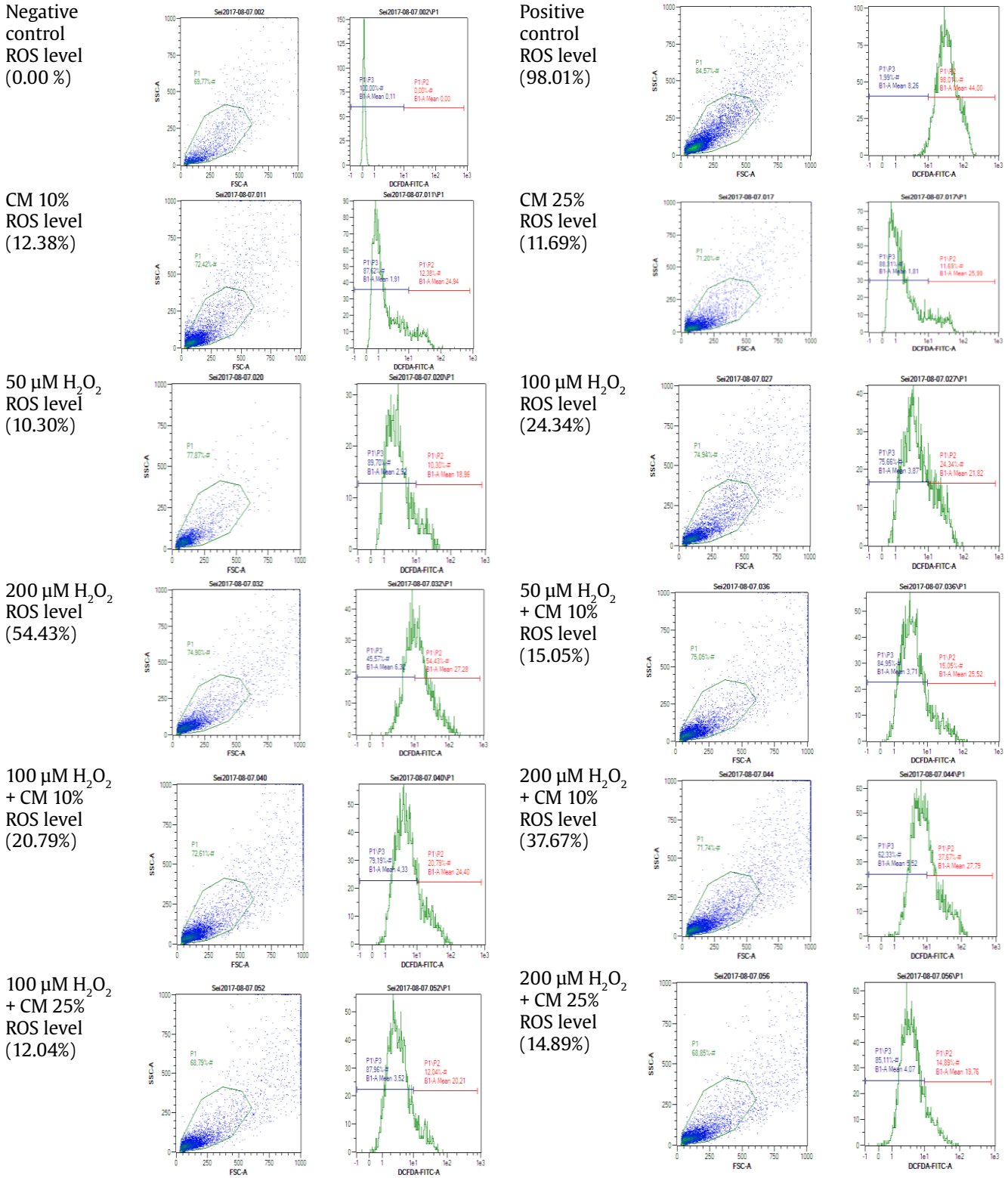

Figure 3. Dot plot of various concentrations of CM-hATMSCs treatment on H_2O_2 -induced human skin fibroblast cells as aging cells model toward ROS level. This figure is the dot plot representative of ROS level in skin fibroblast cells which induced by H_2O_2 and treated using CM-hATMSCs

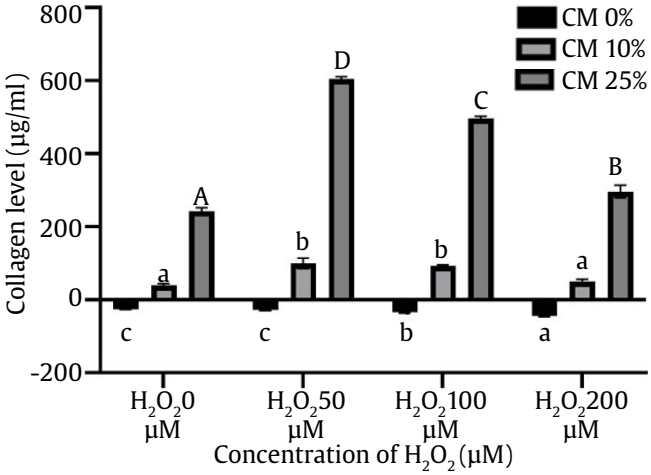

Figure 4. Effect various concentrations of CM-hATMSCs treatment on H_2O_2 -induced human skin fibroblast cells as aging cells model toward collagen level. *The histogram are presented as mean ± standard deviation of 0%, 10%, and 25 % CM- hATMSCs treatment toward collagen level. Different letters (a.b,c) in the histogram (among concentrations of H_2O_2 inducer on BJ cells in CM 0%), different small letters (a, b) in the histogram (among concentrations of H_2O_2 inducer on BJ cells in CM 10%), different capital letter (A, B, C, D) in the histogram (among concentrations of H_2O_2 inducer on BJ cells in CM 25%), were significantly different at $p<0.05$ (Tukey post hoc test)

growth factors (Caplan and Correa 2011) that promote tissue repair mainly by activating endogenous repair mechanisms, and by acting as temporal immune suppressants (Le Blanc and Pittenger 2005; Giordano and Galderisi 2006).

The MSCs secretome can be modulated to boost the beneficial actions of these cells, so that they can respond even more effectively to inflammatory conditions (Redondo-Castro *et al.* 2017). The secretome of MSCs contain a large array of growth factors, cytokines, chemokines, enzymes, extracellular matrix proteins and small molecules (Balasubramanian *et al.* 2017). These factors possess the ability to trigger a cascade of biological reactions such as cell proliferation and migration, tissue granulation and epithelialization and angiogenesis, modulate the local environment, affecting differentiation and functional recovery of resident cells (Chen *et al.* 2014). The MSCs can secrete over 200 different types of factors that encompasses a large array of growth factors, cytokines and chemokines, and ECM of proteins and enzymes, many of which may work in conjunction or independently and essentially trigger tissue repair and possibly rejuvenation (Ranganath *et al.* 2012; Flower *et al.* 2015; Konala *et al.* 2016). It is evident that while majority of factors responsible for fibroblast proliferation including Fibroblast Growth Factor-7 (FGF-7)/ Keratinocyte Growth Factor (KGF), Platelet Derived Growth Factor (PDGF), angiogenesis such as Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), Insulin-Like Growth Factor-1 (IGF-1), Angiopoietin-1 (Ang-1), epithelialization including Transforming Growth Factor-β1 (TGF-β1), Laminin, Tissue Inhibitor of MetalloProteinases-1 (TIMP-1) and TIMP-2 are present in varying amounts ranging from 53 pg/ml to 2218000 pg/ml (Balasubramanian *et al.* 2017). Paracrine factors such as PDGF, bFGF, KGF, TGF-β1, HGF, VEGF, Type-1 collagen (COL-1) and fibronectin secreted by ATMSCs have been shown to restore dermal thickness, improve skin texture and reduce wrinkles in human skin (Jayaraman *et al.* 2013). MSCs-derived Growth Factor and Cytokines (GF/CKs) are important regulatory molecules which mediate the signaling pathways between and within the cells and play an important role in maintaining the skin structure and function (Jayaraman *et al.* 2013). BM-MSCs secreted VEGF-A, bFGF, and other factors, these growth factors and cytokines function together modulate the local environment, affecting the proliferation, migration, differentiation and functional recovery of resident cells (Chen *et al.* 2014).

ROS are highly reactive molecules such as superoxide (O_2^{*-}), H_2O_2, and hydroxyl radicals (*OH) contribute in the initiation and progression of the aging process (Widowati *et al.* 2018a). Increased levels of ROS can damage various cellular processes. The *OH generated from H_2O_2 reacting with different transition metals is particularly damaging to DNA (Dash *et al.* 2008). ROS cause oxidative deterioration of DNA, protein, and lipid, ROS as one of the causative factors of aging (Cui *et al.* 2012). H_2O_2 can be diffused within the cell and be removed by cytosolic antioxidant systems such as catalase (CAT), glutathione peroxidase (GPX), and thioredoxin peroxidase (TPx) (Cui *et al.* 2012). Oxidative stress contributes to cellular aging (Widowati *et al.* 2018a), pathogenesis (Macnee 2005), oxidative stress altered differentiation of aged-MSCs. The aged mice MSCs display decreased antioxidant power (Kasper *et al.* 2009; Broekman *et al.* 2016). The CM from WJMSCs has ability to scavenge effectively free radicals and increase the survival rate of skin cells under oxidative stress. The cosmetic composition contain CM can be used to improve the undesired skin conditions as anti-oxidation and anti-aging (US 9284528 B2). The present study showed that CM-hATMSCs (10%, 25 % has ability suppressing of ROS level in cells aging model (induced by H_2O_2 100, 200 µM) (Figure 2D). In other study was reported that the contribution of human GF/CKs in

reducing the levels of ROS most effectively at 5% and 10% of CM-BMMSCs (Balasubramanian *et al.* 2017). The CM protected the activities of antioxidant enzymes including Superoxide Dismutase (SOD), CAT, and GPx which were reduced by H_2O_2 exposure (Sohn *et al.* 2018).

Fibrous collagen is very important for the strength and elasticity of skin, and the amount of this protein is generally decreased with aging. The Adipose Derived Stem Cells (ADSCs) increase the dermal collagen synthesis and even vascularity via production of many cytokines and growth factors (Kim *et al.* 2007; Rubina *et al.* 2009). Based on the result, CM-hATMSCs contained collagen which higher concentration of CM-hATMSCs increased collagen level, CM-hATMSCs 25% contained the higher collagen level (242.87 µg/ml) compared to hATMSCs-CM 10% (39.80 µg/ml) (Figure 4). It has been shown that ADSCs secrete growth factors that have an effect on fibroblast and keratinocyte proliferation (Moon *et al.* 2012; Lee *et al.* 2012; Amirkhani *et al.* 2016; Noverina *et al.* 2019). The CM of hMSCs consisting of various GF and CKs is known to promote regeneration of damaged tissue (Kamprom *et al.* 2016). Growth factors and cytokines in CM promote the collagen, elastin, and hyaluronic acid synthesis, which helps in keeping the skin elasticity and durability skin, making it appear younger (Amirthalingam and Seetharam 2016). CKs stimulate fibroblasts that secrete collagen and elastin (Amirthalingam and Seetharam 2016). GF and CKs of stem cells promote dermal fibroblast proliferation, deposition and reorganization collagen (Gold *et al.* 2007; Balasubramanian *et al.* 2017). Percentage restoration of collagen was 8.4%, 8.8%, 28.1% higher in CM treated group at the dilutions of 5, 10, and 50% respectively compared to control medium, suggesting that human GF/CKs contributed to collagen synthesis in a dose dependent manner (Balasubramanian *et al.* 2017).

In conclusion, in this study, adipose tissue from liposuction could be source hATMSCs which secreted Conditioned Medium, and the anti-aging properties of Conditioned Medium of hATMSCs were evaluated in increasing proliferation and collagen synthesis, effectively protected free radical induced-fibroblast cells from oxidative stress by *in vitro* study.

Conflicts of Interest

The authors declare that they have no competing interests.

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