

Regenerative Effects of Wharton's Jelly Stem Cells-Conditioned Medium in UVA-Irradiated Human Dermal Fibroblasts

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ABSTRACT

Background: Ultraviolet A radiation (UVA) can photo-age skin by suppressing the proliferation, migration, and collagen deposition of human dermal fibroblasts (HDFs). This process occurs because UVA light can inhibit the gene expression of the TGF- β receptor in HDFs. Moreover, Wharton's Jelly Stem Cells-Conditioned Medium (WJSC-CM) is hypothesized to release microvesicles that contain short m-RNA with regenerative properties.

Objectives: This study aimed to determine the regenerative properties of WJSC-CM on UVA-Irradiated Human Dermal Fibroblasts (UVA-HDFs)

Methods: Passaged fourth of HDFs obtained from the foreskin of six (11- to 13-year-old) boys were repeatedly irradiated with a total of 10 J/cm² UVA and treated with various concentrations of WJSC-CM. We used non-irradiated HDFs as positive control. After that, the consumption of TGF- β , cellular proliferation, cellular migration, and collagen deposition of each group were measured and compared.

Results: Compared to the non-irradiated groups, the proliferation rates, migration rates, and collagen deposition of UVA-HDFs significantly decreased ($p < 0.05$). WJSC-CM can improve the consumption of TGF- β , proliferation, and cellular migration of UVA-HDFs. However, WJSC-CM failed to improve the collagen deposition of UVA-HDFs ($p > 0.05$).

Conclusions: WJSC-CM has regenerative properties and is a candidate material for the treatment of prematurely ageing skin induced by UVA-irradiation.

Key words: collagen depositions, migration, photo-ageing fibroblasts, proliferations, TGF- β , UVA, Wharton's jelly stem cells conditioned-medium

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INTRODUCTION

Solar radiation, especially ultraviolet A light (UVA), can prematurely age skin. Various studies have shown that the collagen content of exposed human skin is lesser than that of unexposed skin (Yamauchi et al, 1991; Chung et al, 2001; Schwartz et al, 2008) based on the levels of procollagen-1 expression and procollagen-1 m-RNA (Chung et al, 2001). These phenomena may be caused by UVA light, which produces reactive oxygen species (ROS) that affect human dermal fibroblasts (HDFs) in several ways. Among HDFs, ROS induce DNA damage that prolongs G1 arrest for DNA repair (Pignolo et al, 1998; Auclair et al, 2010) and AP-1 expression that results in the gene expression of matrix metalloproteinase (MMP) to lead to collagen degradation (Wlaschek et al, 1995; Choi et al, 2007). Moreover, ROS induce AP-1 expression can also inhibit membrane TGF- β 1 receptor gene expression (Quan et al, 2001; Fisher et al, 2002; Quan et al, 2002; Quan et al, 2004; Quan et al, 2005). Also, degradable collagen materials may inhibit new collagen synthesis (Varani et al, 2006), and the decreasing dermal collagen contents may shrink HDFs and change their TGF- β 1 receptor structure to disrupt TGF- β 1 signalling (Quan et al, 2013). All of these processes impair the function of photo-aged HDFs.

Various methods have been developed to treat wrinkles in prematurely aging skin, ranging from fillers of autologous young fibroblasts isolated from gingiva (Watson et al, 1999; Burgess, 2005) and Botox injection (Burgess, 2005) to the utilisation of secretory factors of adipose-derived stem cells (Kim et al, 2009). Conversely, Wharton's jelly from the umbilical cord has been considered a source of mesenchymal stem cells or WJSCs (Wang et al, 2004; Wu et al, 2007), and these stem cells have been proven to accelerate complete wound healing with the formation of the epithelium on the skin of a sheep's back (Azari et al, 2011). This happens because these cells have ability to differentiate into various cells, including differentiate into sweat gland-like cells which are important components of skin epithelial (Xu et al, 2012). Biancone et al. (2012), in a study supported by Bruno and Bussolati (2013), hypothesised that WJSCs may release microvesicles that contain short m-RNAs into their conditioned-medium, which can interfere with the local genetic programmes of cells cultured in this medium to induce cells to self-repair. Because the pathologic conditions of ageing fibroblasts in prematurely aging skin are similar to those of ageing fibroblasts in chronic ulcers (Watson and Griffiths, 2005), culturing UVA-irradiated HDFs (UVA-HDFs) in WJSCs conditioned-medium (WJSC-CM) may restore the activities of UVA-HDFs.

MATERIAL AND METHODS

Isolation and culture of HDFs

We collected HDFs from the dermal part of the foreskins of six voluntary human subjects (11-13 years old) with informed consent. After mechanically removing of epidermal tissues from the skin, we cut the dermal parts into 2-4 mm³ pieces, placed in a culture flask, immersed in a small amount of growth medium (GM) consisting of high glucose Dulbecco's minimal essential medium (DMEM) (Gibco) supplemented with 10 % foetal bovine serum (FBS-Gibco) and 1 % penicillin/streptomycin (Gibco). Incubation was performed at 37°C until the tissues attached to the bottom of the flask. The medium was then exchanged with ten mL of fresh medium every 72 hours until 60 % of the HDFs were outgrowing. The HDFs were then harvested and sub-cultured until passage 4th.

Isolation and culture of WJSCs

The isolation and culture of WJSCs were based on the explant technique of Ishige et al. (2009) with slight modifications. Briefly, 2-3 cm of umbilical cord obtained with informed consent from a full-term Caesarean section patient was rinsed with PBS three times for 10 minutes each, while blood was squeezed out and cord torsion was straightened. Subsequently, the cord was cut perpendicular to the long axis into 2-3 mm thick pieces. The pieces were then placed on the bottoms of 75 cm² culture flasks that were already coated with 1 mg/mL type-1 collagenase (Sigma) and incubated for 30 minutes at 37°C. After the collagenase has been aspirated, the explants were carefully rinsed with PBS, immersed in 1 mL of low glucose DMEM with two ng/ mL b-FGF (Sigma), and incubated in 37°C with 5 % CO₂. Medium replacement was done every day until explants attached to the flask's bottom. Furthermore, the medium was then replaced with growth medium that consisted of low glucose/Ham-F12 DMEM (Gibco) supplemented with 10 % PBS, 2 ng/mL b-FGF, 2.5µg/mL amphotericin B (Gibco), and 1 % penicillin/streptomycin. This last step was repeated every 72 hours until 60 % of fibroblast-like cells were observed to be outgrowing (fig.1A and B).

Collection of WJSC-CM

WJSC-CM was collected from the supernatant of spheroid-bodies of a high-density WJSCs culture according to a method published by Kurosawa (2007). Briefly, 7.5 x 10⁵/mL passage-4 WJSCs were cultivated in growth medium in 24 well culture-plates until spheroid- bodies formed (fig.1C and D). At this condition, the growth medium was then replaced with low glucose/Ham-F12 DMEM containing 1 % FBS for 72 hours as previously performed by Potapova et al. (2007). Subsequently, the supernatant then was collected, centrifuged at 200 g for 10 minutes, and maintained at -20°C until use.

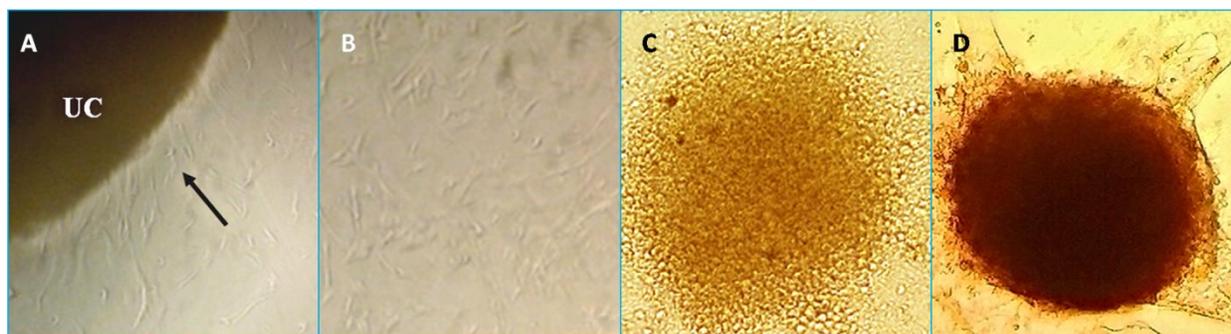


Figure 1: Isolation, cultivation, and spheroid body formation of WJSCs

A. An outgrowth of fibroblasts-like cells from explant, B. Culture of WJSCs, C. High- density cultivation of WJSCs, D. Spheroid-body of WJSCs

Experiments

UVA-irradiated HDFs: UVA irradiations onto HDFs were prepared based on a procedure of UVA-induced aging of HDFs that published by Naru et al. (2005). Briefly, three replicates of 200 μ L of 2×10^3 / mL passage-4 HDFs from different sources were cultured in each well of a 96-well plate in GM in 5 % CO₂ at 37°C for 24 hours. The HDFs were then washed with PBS and exposed to UVA with 3–4 drops of PBS. UVA irradiation provided by **PasSun A** lamps (**LIPI Indonesia**) and the irradiance was determined using a photometer (**Davlin X-96 detector-USA**). The UVA irradiation dosage was 3.33 J/cm² for each radiation treatment. Immediately after the irradiation, the PBS was aspirated, replaced with complete medium, and the cells were returned to the incubator. Irradiation was repeated every 72 hours until the total dose approached 10J/cm². Also, non-UVA-irradiated HDFs or normal fibroblasts were cultured in different plates with similar washing and medium replacement procedures.

Treatment with WJSC-CM: Immediately after the final UVA irradiation, UVA-irradiated HDFs were treated with 200 μ L of 100 %, 50 %, or 25 % WJSC-CM diluted in DMEM supplemented with 1 % FBS or control medium (CM) that consisted of DMEM plus 1 % FBS. NFs were co-cultured in GM and CM. All cells were cultured for 72 hours in 37°C and 5 % CO₂, and the medium was replaced once every 72 hours. Each treatment was replicated three times. The WJSC-CM concentration that resulted in the best proliferation rate was used in subsequent experiments to assess the migration ability, collagen deposition, and TGF- β consumption of UVA-irradiated HDFs.

Measurements

Proliferation index: Cellular viability was measured using a MTT (-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The medium removed, the cells were washed with PBS, and 200 μ L complete medium plus 50 μ L of (5 mg/mL) MTT (**MP biomedical-France**) was then added to each well. The plates were then wrapped with aluminium foil and incubated at 37°C in 5 % CO₂ for 4 hours. The medium and MTT then removed and the remaining MTT-formazan was then dissolved by adding 200 μ L DMSO (Dimethyl sulfoxide). The optical density resulting from the formazan in each well then was measured at 570 nm using a spectrophotometer.

$$\text{Determination of proliferation rates were as} = \frac{\text{The OD of experimental groups}}{\text{The OD of Normal HDFs}} \times 100\%$$

Cellular migration assay: A cellular migration assay was performed based on a method by Yarrow et al. (2004) and computed based on a method by Liang et al. (2007) with little modification. Briefly, after the final UVA irradiation, all wells were linearly scratched with the blunt tip of a 32G sterile needle through the centre of the well bottom. After treatment followed by 3 incubations of 72 hours each, the cells were stained with Meyer haematoxylin and microscopic photo images were taken using a **Moticam-350 (China)** camera in JPG format. The capacity for migration was determined via the computerised counting of blue fibroblast pixel-numbers compared to the non-blue pixel-numbers of the remaining free space along the scratching lines.

Cellular collagen deposition rate: The collagen deposition assay was based on an insoluble collagen of Sirius red assay according to Taskiran et al. (1999). Briefly, the wells were washed with PBS after treatment, and the cells were fixed with Bouin solution for one hour. The wells were then washed off with tap water and allowed to dry at room temperature overnight. Two hundred microliters of 0.1 % Sirius red in saturated picric acid (Sigma-Aldrich, USA) were added to each well for one hour. The unbound Sirius red was washed away using four washes with 200 μ L 0.1 N HCl. The Sirius red bound to collagen was dissolved using 200 μ L of 0.5 N NaOH, and the optical density was read using a spectrometer at 570 nm. Collagen deposition rates among experimental groups were calculated based on equation:

$$\text{Collagen deposition was} = \frac{\text{OD of Experimental groups}}{\text{OD of Normal HDFs}} \times 100\%$$

TGF- β Consumption: The levels of TGF- β in the complete medium, 50 % WJSC-CM, and cell culture supernatants were determined by direct ELISA using anti-human anti TGF- β (Abcam cat.ab66043), HRP-conjugated secondary antibody (Abcam cat.ab6721), and 3,3',5,5'-tetra-methylbenzidine (Sure-BlueTMB microwell peroxidase substrate-KPL The USA). The optical densities were measured at 450nm using a spectrophotometer. The optical densities were converted to TGF- β concentrations based on an ELISA standard curve of serial dilutions of known levels of TGF- β starting from 1000 pg/mL.

$$\text{Consumption of TGF-}\beta \text{ was} = \frac{\text{Basic-level of TGF-}\beta \text{ in medium} - \text{TGF-}\beta \text{ supernatant of culture}}{\text{Basic-level of TGF-}\beta \text{ in medium}} \times 100\%$$

Statistics

All the results are shown as an average \pm standard error. Comparisons among groups were analysed with an ANOVA test followed by LSD for a post hoc test. $P < 0.05$ was considered significant.

RESULTS

The results can be observed in the following figures.

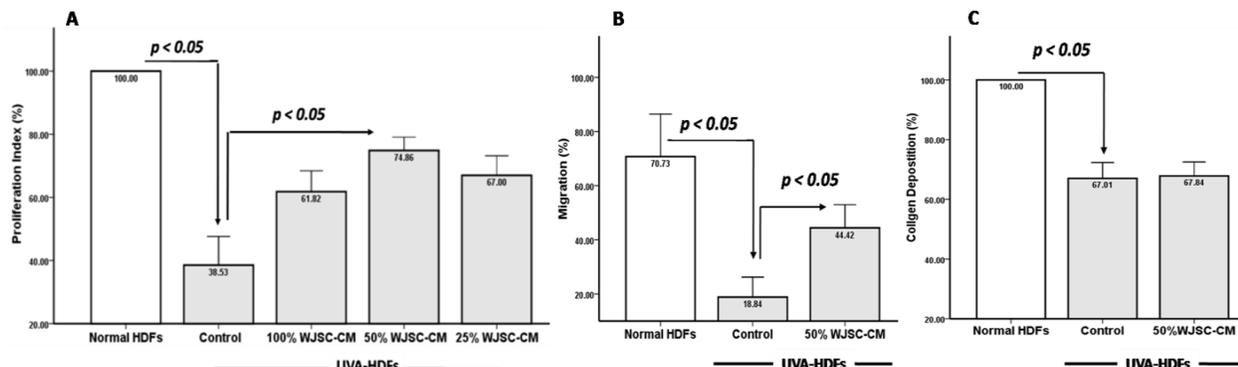


Figure 2: The effect of WJSCs-CM on activities of UVA-HDFs: (A) on cellular proliferation, (B) on cellular migration, (C) on collagen deposition

On this figure, it can be observed that repeatedly UVA-irradiation in total dosage of 10 KJ.cm⁻² suppresses HDFs activities in cellular proliferation indexes, migration rates, and collagen deposition. Moreover, culture UVA-HDFs in 50% WJSC-CM can ameliorate their cellular activities in proliferation indexes and migration rates significantly ($p < 0.05$) but fail in collagen deposition activities. The change in TGF- β receptor expressions of various groups in this experiment was measured by TGF- β consumptions. On the following figure, they can be observed.

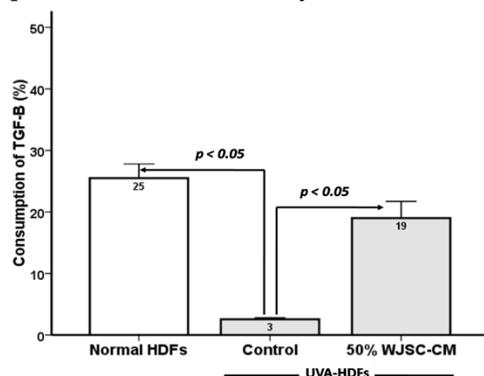


Figure 3: Consumption of TGF- β

On this figure, Consumption of TGF- β among UVA-HDFs was significantly lower than normal HDFs ($p < 0.05$) and cultured of UVA-HDFs in 50% WJSC-CM had significantly higher. However, the improvement of TGF- β consumption was still under normal HDFs's consumption ($p > 0.05$; data was not shown).

DISCUSSION

Stem cells still express MHC on their membranes, which lead to a risk of donor rejection if they are used as transplanted foreign materials (Rossignol et al, 2009). Therefore, many researchers now focus on stem cells secretory factors for regenerative medicine. Various experiments have shown that factors secreted by stem cells into the conditioned medium have regenerative properties. For example, adipose-derived stem cells conditioned medium can induce proliferation, type I collagen synthesis, and inhibit MMP-1 expression in UVB-irradiated HDFs (Kim et al, 2009; Song et al, 2011; Kim et al, 2008). These effects may be associated to the antioxidant action of the conditioned medium of adipose-derived stem cells, as previously reported by Kim et al. (2008).

Similar to adipose-derived stem cells, WJSCs also release various secretory factors into their conditioned medium, and the most important secretory factors are microvesicles that contain short m-RNAs that may be transferred into injured cells, interfere with local genetic programming, and induce resident cells to re-enter the cell cycle, which leads cells to self-repair, as previously supposed by Biancone et al. (2012). This theory is based on the fact that WJSCs can reverse acute and chronic kidney injury in different experimental models via paracrine mechanisms (Bruno and Bussolati, 2013).

In the process of photo-aging skin, UVA light may generate ROS to prolong cell cycle G1 arrest for DNA repair DNA and stimulate AP-1 expression. AP-1 expression causes matrix metalloproteinase (MMP) gene expression, which leads to collagen degradation, and AP-1 expression blocks membrane TGF- β 1 receptor to inhibit the TGF- β 1 signalling. All of these behaviours may affect the proliferation, migration, and collagen deposition rates of UVA-

HDFs, as shown in fig.2. In our experiments, the cultivation of UVA- HDFs in 100 %, 50 %, and 25 % WJSC-CM improved the cellular proliferation indexes compared with control as diluent media (fig.2). The cultivation of UVA-HDFs in 50 % WJSCM showed the best proliferation rates. Because the decreasing activities of UVA-HDFs are due to the impairment of the TGF- β signalling pathway, these improved UVA-HDFs proliferation indexes might be caused by improved TGF- β consumption, as demonstrated in fig.3. The improved TGF- β signalling of UVA-HDFs might be caused by secretory factors that contain short-mRNAs released by WJSCs. These short-mRNAs might interfere with the genetic programming of UVA-HDFs to result in TGF- β receptor gene expression self-repair, as indicated by TGF- β consumption. This improvement in the TGF- β signalling of UVA-HDFs was also observed in the migration abilities of UVA-HDFs (fig.2). In this case, the improved UVA-HDFs migration rates were significantly higher than the migration abilities of normal-HDFs. The TGF- β -smad signalling pathway is well known to be important in the migration of HDFs (Montesano and Orci, 1988; Capelo, 2005; Hinz, 2007).

The collagen deposition assay showed an unexpected result (fig.2). Theoretically, improved TGF- β signalling should restore the collagen deposition ability of UVA-HDFs because either TGF- β signalling itself or its combination with other TGF- β signalling pathway members induce connective tissue growth factor gene expression, which can stimulate collagen synthesis. Failure to stimulate collagen deposition by UVA-HDFs on above might be caused by the fact that WJSC-CM contains MMP-2 and MMP-9, as previously reported by Mauro et al. (2010). Both MMPs are gelatinases and can degrade gelatin as the by-product of collagen degradation induced by UVA radiation (Wlaschek et al, 1995; Naru et al, 2005). These gelatinase activities result in soluble materials that could not be detected by the Sirius red insoluble collagen assay. Also, degraded gelatin can stimulate the migration abilities of HDFs while inhibiting the ability of HDFs to synthesise new collagen (Xue et al, 2012).

Quan et al. (2013) reported that the improved TGF- β signalling of dermal fibroblasts in an aged human skin is caused by the restoration the dermal microenvironment during skin aging. The increased collagen content in the microenvironment improves the mechanical force to elongate fibroblasts, couple with type I collagen synthesis, and upregulate TGF- β receptor gene expression. Based on the lack of increased collagen deposition in fig.2, the improved TGF- β signalling in UVA-HDFs shown in fig.3 was not caused by improved microenvironmental mechanical forces.

Sobolewski et al. (2005) reported that high-levels of TGF- β , b-FGF, EGF, PDGF, and IGF-1 can be extracted from Wharton's jelly materials. The presence of these growth factors in WJSC-CM is still unclear, but WJSC-CM 50 % in our experiments showed the presence of sufficient levels of TGF- β (fig.3). Because PDGF is well known to be an important growth factor for wound healing (Werner and Grose, 2003) and injection of WJSC-CM can stimulate wound healing in mice (Zhang et al, 2012), PDGF likely plays a role in the regenerative properties of WJSC-CM. Also, various experiments showed that the PDGF signalling pathway can restore the TGF- β signalling pathway in human dermal fibroblasts (Czuwara-Ladykowska et al, 2001) and mouse cardiac fibroblasts (Zhao et al, 2013). Therefore, the repair activities of UVA-HDFs cultured in WJSC-CM may be due to the high-level PDGF in WJSC-CM. Unfortunately, this assumption was not be evidenced in this experiment. We hope that this limitation study can be completed in further research.

CONCLUSIONS

Wharton's jelly stem cells-conditioned medium has regenerative properties that improve TGF- β signaling, as well as cellular proliferation and migration rates of UVA-HDFs.

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