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Low Degree Hyaluronic Acid Crosslinking Inducing the Release of TGF-B1 in Conditioned Medium of Wharton's Jelly-Derived Stem Cells

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Keywords: Conditioned medium; Wharton's jelly stem cells; Hyaluronic acid crosslinking; Transforming growth factor-β1

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Abstract

BACKGROUND: Presently, the application of stem cells and their paracrine effect for anti-ageing therapy has commenced. Wharton's jelly-derived stem cell conditioned medium (WJSCs-CM) is renowned for increasing proliferation, migrate ageing skin fibroblasts and increase consumption of extracellular transforming growth factor-β (TGF-β). With more than 85% of frequently used dermal filler procedures are hyaluronic acid fillers (HA), a mixture of both with optimal HA crosslinking degree has not yet been identified.

AIM: This study aimed to determine the discrepancies in the results of various HA crosslinking degree in WJSCs-CM concerning various levels of growth factors (GF).

METHODS: Conditioned medium was obtained from mesenchymal stem cells Wharton's jelly of the newborn umbilical cord with caesarean section procedure, fabricated with hypoxia method (HCM). HA was obtained from preparations on the market with crosslinking degrees of 3%, 4%, and 10%. GF levels were measured using sandwich ELISA method based on the protocol provided by anti-TGF-β1, platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) antibody producers (Cloud-Clone Corp®, Texas, USA).

RESULTS: Low degree HA crosslinking (3% and 4%) elevated TGF-β1 release in WJSCs-CM. HA crosslinking did not provoke increased levels of PDGF and bFGF in WJSCs-CM, both at low and higher degrees.

CONCLUSION: Low degree HA crosslinking induced the increase of TGF-β1 release in WJSCs-CM.

Introduction

The intrinsic ageing process of the skin is accelerated by several extrinsic factors, with the most frequent is sun exposure. This process is known as photoaging skin, premature ageing skin, or ageing skin [1], [2]. Fibroblasts are the cells being most responsible for the occurrence of ageing skin manifestations [3]. One method in ageing skin therapy perceptible to be less invasive is filler injection. In filler injection, utilised materials range from autologous materials such as collagen, fat cells, fibroblasts, to synthetic materials [1], [4]. More than 85% of dermal filler procedures often used as ageing tissue fillers are hyaluronic acid (HA) [5].

Today experts are turning their attention away from stem cell transplants to stem cell products because the limitations of treatment for diseases using heterologous stem cells are rejection reactions from recipients [6]. Wirohadidjojo *et al.* found the benefit of Wharton's jelly-derived stem cell conditioned medium (WJSCs-CM) in the recovery of human ageing skin fibroblast activity [7]. It was reported that WJSCs-CM could increase proliferation, fibroblast migration and increase extracellular transforming growth factor-β (TGF-β) consumption.

This dermal filler injection with HA crosslinking would give immediate results in clinically

reducing wrinkles after being injected, but these results possess no durability as its bane, with occurrence possibility of biocompatibility, encapsulation and granuloma formation [8], [9]. While WJSCs-CM is used to provide indirectly visible improvement in relieving wrinkles clinically, after a certain period the results commence to appear. This is due to the time taken for the growth factors (GF) to stimulate autologous cell or fibroblasts regeneration [9], [10], [11].

If HA crosslinking combination with WJSCs-CM is envisioned to be promising to possess better outcomes and benefits compared to a sole HA crosslinking, clinicians can obtain additional biological material in the form of stem cell products, as a combination of skin rejuvenation methods to increase the effectiveness of the method. The risk of rejection due to the use of heterologous stem cells in photoaging skin sufferers can be avoided, and the necessity of using autologous material can be eliminated.

The optimal HA crosslinking degree has not yet been identified. This study aimed to determine the effect of crosslinking HA degrees on GF levels in WJSCs-CM.

Methods

This study was experimental in vitro with post-test control group design. This study was conducted at the Research Laboratory of the Department of Dermatology and Venereology, Faculty of Medicine, Universitas Gadjah Mada, Radiopoetro Building, Yogyakarta, Indonesia.

Inclusion criteria of mesenchymal stem cell donor were the newborn's umbilical cord from normal childbirth or cesarean section, term and healthy. This study had received approval from the Ethics Committee of the Faculty of Medicine, Universitas Gadjah Mada (Ref: KE/FK/0845/EC/2018). Mesenchymal stem cell (MSC) culture samples were subcultured to passage > 4. Conditioned medium (CM) was obtained from the stem cell laboratory, Institute of Tropical Disease (ITD), Universitas Airlangga. CM was taken from mesenchymal stem cells Wharton's jelly of newborn's umbilical cord with caesarean section and fabricated with the hypoxic method (hypoxic conditioned medium = HCM) with 1% nitrogen content and harvested at 72 h.

HA was obtained from preparations in the market with crosslinking degrees of 3%, 4% and 10%, from the fermentation of *Staphylococcus equine* bacteria (NASHA = non-animal stabilised hyaluronic acid). WJSCs-CM was isolated from the embryoid body of Wharton's jelly mesenchymal stem cell culture with the content of 50% dissolved in DMEM ± 1% FBS (Gibco™, Massachusetts, USA). HA and WJSCs-CM were mixed using the three-way connecting syringe method which was mixed repeatedly until homogeneous. The HA used in this combination was of 30% preparation concentration in WJSCs-CM. Comparison of HA and WJSCs-CM was 0.3 ml HA:0.7 ml WJSCs-CM. GF levels were measured in a solution or medium by sandwich ELISA method based on the protocol provided by anti-transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) antibody producers (Cloud-Clone Corp®, Texas, USA). Data were presented as mean ± SD.

Results

In HCM without HA crosslinking, the level of growth factor for TGF-β1 was 28.51 ± 9.41 pg/ml, PDGF-BB 144.79 ± 67.57 pg/ml, and bFGF 0.00 pg/ml. The HA group of low degree crosslinking (3% and 4%) resulted in the release of TGF-β1 in WJSCs-CM much higher compared to the group without HA crosslinking and crosslinking of 10%. TGF-β1 level in 3% HA crosslinking was 170.89 ± 128.36 pg/ml and 4% HA crosslinking was 105.26 ± 18.44 pg/ml. Whereas for the 10% HA crosslinking group, TGF-β1 level was only 19.62 ± 15.20 pg/ml, even lower than the group without HA crosslinking.

Table 1: Growth factor levels in cross-linked HA and HCM

Group	TGF-β1 (pg/ml) Mean ± SD	PDGF-BB (pg/ml) Mean ± SD	bFGF (pg/ml) Mean ± SD
HCM	28.51 ± 9.41	144.79 ± 67.57	0.00 ± 0.00
HCM + HA 3%	170.89 ± 128.36	141.89 ± 25.64	0.00 ± 0.00
HCM + HA 4%	105.26 ± 18.44	101.05 ± 19.15	0.00 ± 0.00
HCM + HA 10%	19.62 ± 15.20	102.02 ± 13.10	0.00 ± 0.00

HCM: hypoxic conditioned medium; HCM+HA 3%: conditioned medium + hyaluronic acid crosslinking grade 3%; HCM+HA 4%: conditioned medium + hyaluronic acid crosslinking grade 4%; HCM+HA 10%: conditioned medium + hyaluronic acid crosslinking grade 10%.

As for PDGF-BB levels, GF levels were reduced in all degree HA crosslinking groups. For bFGF, no release of GF was perceptible, with or without HA crosslinking. Contrary to TGF-β1, low degree HA crosslinking (3% and 4%) did not elevate PDGF-BB and bFGF levels in WJSCs-CM.

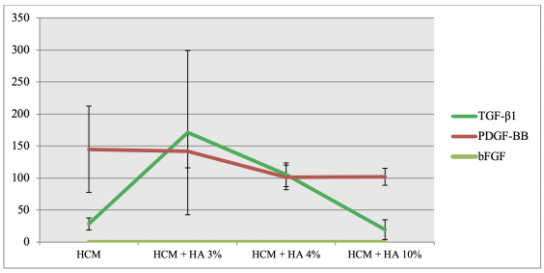

Figure 1: Growth factor levels in crosslinked HA and HCM; HCM: hypoxic conditioned medium; HCM+HA 3%: conditioned medium + hyaluronic acid crosslinking grade 3%; HCM+HA 4%: conditioned medium + hyaluronic acid crosslinking grade 4%; HCM+HA 10%: conditioned medium + hyaluronic acid crosslinking grade 10%

Discussion

In this study TGF-β1, PDGF-BB and bFGF were selected in the analysis due to being the most important GF related to senescent fibroblasts in the ageing skin. Fibroblasts are the cells most responsible for the onset of ageing skin [3]. Fibroblasts are the main cellular elements in the human dermis because these cells are responsible for the synthesis of the extracellular matrix, both collagen, elastin synthesis, and the synthesis of other basal dermis substances.

Ultraviolet A (UVA) exposure in the long term attenuates dermal structures causing premature photoaging. Reactive oxygen species (ROS) yielded from UV radiation leads to oxidation at the cellular level, clinically presented by skin inflammation, erythema, tanning, immunosuppression, photoaging, and skin cancer. Antioxidant molecules (i.e. glutathione, carotenoids, ascorbate, and tocopherol) and proteins (i.e. ferritin, heme oxygenase, glutathione peroxidase, superoxide dismutase, catalase, etc.) ruled as the defences against UVA. UVA nevertheless can transgress to the dermis, altering the dendritic cells, matrix metalloproteinase (MMP), T-lymphocytes, mast cells, endothelial cells, and fibroblasts [12].

Human skin fibroblasts activity is very dependent on the race of various cytokines and GF. Most responsible GF for human skin fibroblast activity is transforming growth factor-β or TGF-β [13]. Exposure to UVA was shown to inhibit the proliferation of fibroblasts, inhibiting the synthesis of collagen by fibroblasts and producing collagen damage due to increased activity of MMP enzyme that yielded collagen fibres breakage [14]. Collagen fibres breakage would lead to a decrease in the mechanical power that yielded wrinkled fibroblasts, and TGF-βII receptors on cell membranes would become sealed against their ligands [14], [15]. Thus, the TGF-β signalling pathway would be disrupted, whereas the TGF-β-Smad signalling pathway is the most important signalling in fibroblast proliferation and collagen synthesis [15].

In this study, low degree HA crosslinking elevated TGF-β1 release in WJSCs-CM. Low degree HA crosslinking provoked the release of GF greater than the higher HA crosslinking degree. HA crosslinking did not provoke increased levels of PDGF-BB and bFGF in WJSCs-CM, both at low and higher degrees. There was almost no difference in other levels of GF, namely PDGF-BB and bFGF between groups of HCM mixtures with various HA crosslinking degrees. This was likely due to HA crosslinking in the HCM mixture causing binding of proteins including GF in HCM so that GF levels would be reduced.

It had been reported that the addition of monomeric exogenous HA to fibroblast culture triggered TGF-β signalling and collagen production. HA which was involved in wound healing and its biological properties depended on its molecular size [16]. Inhibition of HA synthesis in dermal fibroblasts had been shown to cancel the proliferation induction of TGF-β1 [17]. In the study of Quan et al., it was known that HA filler locally injected into the skin would fill the space and push the area around the extracellular matrix (ECM) so that fibroblasts underwent morphological extension [14]. This elongation of fibroblasts was associated with upregulation of the TGF-β signalling pathway.

Quan et al., and Fisher et al., showed that the decrease in collagen content in the dermis would result in a decrease in mechanical power which caused fibroblasts (which were in a statically bound state by collagen fibres) to morphologically became shrinking, so that the TGF-β receptor became closed and did not respond to TGF-β. This was evidenced by a dermal filler of HA injection on ageing skin which increased mechanical power, would cause an increase in TGF-β receptor expression, a change in the morphology of fibroblasts to be longer, and an increase of fibroblast proliferation with the content of procollagen-1. Reduced fibroblast size or decreased mechanical power of the fibroblasts caused the failure of TGF-β/Smad signalling due to decreased expression of TGF-βII receptors [14], [15].

In the processes of wound repair, expression dynamics of growth factor and cytokines, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), exhibited characteristics of temporal and spatial regulation. Impaired wound healing was associated with alteration in growth factors expression pattern [18]. WJMSCs-CM had been shown to increase the regulation of re-epithelialization gene expression, TGF-β, neovascularisation (hypoxia-inducible factor-la) and fibro-proliferation (plasminogen activator inhibitor-1), with an increase of normal human skin fibroblasts proliferation and to aid wound healing on injured mice skin [19]. In a study by Wirohadidjojo *et al*., the benefits of WJSCs-CM was discovered in the recovery of human ageing skin fibroblasts activity due to UVA exposure by increasing proliferation, migration of aging skin fibroblasts and increasing consumption of TGF-β [7]. Conditioned medium derived from fat cells was also proven to stimulate the production of TGF-β1, immunoglobulin binding protein-7 (IGBP-7), collagen type 1 and fibronectin, as well as to restore collagen synthesis through increased procollagen-1 gene expression, suppress MMP-1 release, and restore human skin fibroblast proliferation [20], [21].

In conclusion, low degree HA crosslinking induced the increase of TGF-β1 release in WJSCs-CM. HA crosslinking did not provoke increased levels of PDGF and bFGF in WJSCs-CM, both at low and higher degrees.

Authors Contribution

Authors equally contributed to design, data compiling and analysis, and the composing of the manuscript.

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