Clinical Nutrition Experimental 24 (2019) 34–44

Contents lists available at ScienceDirect

Clinical Nutrition Experimental

journal homepage: http://www.clinicalnutritionexperimental.com

Growth factors profile in conditioned medium human adipose tissue-derived mesenchymal stem cells (CM-hATMSCs)

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ARTICLE INFO

Article history:
Received 9 March 2018
Accepted 11 January 2019
Available online 21 January 2019

Keywords:
Conditioned medium
Human adipose tissue derived-mesenchymal stem cells
Growth factor

SUMMARY

Background: Stem cell-derived by conditioned medium has potential as regenerative agent of skin cells because it contains many growth factors, cytokines and other regenerative biomolecules. Conditioned medium (CM) from human adipose tissue-derived MSCs (CM-hATMSCs) also has been known enriched growth factors that play an important role in epithelial wound repair, reduce wrinkles, and enhance wound healing.
Objective: This study was performed to evaluate growth factors in CM such as Transforming Growth Factor (TGF)-β1, TGF-β2,

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https://doi.org/10.1016/j.yclnex.2019.01.002

Fresh frozen plasma
Wound healing

Vascular Endothelial Growth Factor (VEGF)-2, Vascular Cell Adhesion Molecule (VCAM) 1, Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) in CM-hATMSCs treated with Fresh Frozen Plasma (FFP) and non-FFP at various passages.
Methods: This study used ELISA method to measure the growth factors in CM-hATMSCs.
Results: FGF was the highest modulator among FFP and non-FFP-treated cells at many passages compared to the others growth factor. Both FFP and non-FFP-treated cells showed significant difference ($P < 0.05$) in some growth factors. However, there was no significant differences in TGF-β1 at passage 3,7,11 and 15 in FFP-treated cells and non-FFP-treated cells.
Conclusion: In summary, the highest concentration level found in CM-hATMSCs is FGF both in FFP and non-FFP-treated cells. FGF as growth factor composition in CM-hATMSCs has potential as wound healing and regeneration of cell and it can be used in anti-aging products.

1. Introduction

Tissue engineering has become the new promising therapies. Human mesenchymal stem cells (hMSCs) are the sources of adult stem cells for cell therapy and tissue engineering [1]. 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-derived stem cells (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 [2], easy access and high cell numbers [3]. ATMSCs also exhibit multilineage 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 [4].

Conditioned medium (CM) contains various growth factors and tissue regenerative agents, which were secreted by the stem cells [5]. CM from MSCs involves collagen synthesis and suggest that CM-MSCs might be a potential candidate for preventing UV-induced skin damage [6]. CM is rich in growth factors and cytokines which will satisfy regulatory requirements and expected to have less stringency compared to stem cell therapy products in the form of cell preparation [7]. CM is 'cell-free' there no risk of Graft Versus Host Disease (GvHD) and no side effects provided cord tissue donor is screened, tested and is free from infectious diseases [7].

The CM from stem cells has growth factors and cytokines such as VEGF Platelet-derived Growth Factor (PDGF), Hepatocyte Growth Factor (HGF), basic Fibroblast Growth Factor (bFGF), Macrophage Stimulating Protein (MSP), Keratinocyte Growth Factor (KGF) and Insulin-like Growth Factor 1 (IGF-1) [8]. These cytokines and growth factors play active in regeneration of cell and angiogenesis [9]. In culture media, fetal bovine serum (FBS) usually used as a standard, but its substitution is required to avoid any disadvantages such as cell contact with animal compounds. The human blood-derived has been widely conducted as it is have several advantages compared to FBS [10]. Therefore, we conducted our research to evaluate the concentration of each modulator in early, moderate, late passage in different medium (FFP and non FFP).

2. Materials and methods

2.1. Isolation and expansion of hATMSCs

Adipose tissue resulted from 3 donors female liposuction with 25–30 years old were put into schott bottle 250 ml or 500 ml fulfilled with transport medium 80% MEM-α (Gibco, A1049001), 1% Antibiotic and antimycotic (Gibco, 15240062) and FFP (Indonesia Red Cross, Bandung) in ice bag with informed consent using the guidelines approved by the Institutional Ethics Committee at Universitas Padjadjaran, West Java, Indonesia (No:1062/UN6.C1.3.2/KEP/PN/2016). After that, the fat was filtered by cell strainer 100 μm (SPL, 93100) and washed with phosphate buffered saline (PBS) (Gibco, 14200075), then transferred into 15 ml tube (SPL, 50015). Briefly 30 ml of 0.075% collagenase type I (Gibco, 17100017) was added into tube and centrifuged (MPW-2000) at 1200 rpm, 10 min at room temperature. Then, the cell pellet was inserted into flask with completed medium consist of 80% MEM-α, 20% FFP, 1% antibiotic and antimicotic and 1% heparin (Inviclot, IH2983) [11–14].

2.2. hATMSCs culture and CM-hATMSC preparation

hATMSCs cells of P2 were seeded at density 10^4 cells/cm^2 on plastic-surfaced culture disks with 80% MEM-α, 20% FFP, 1% antibiotic and antimicotic, 1% heparin incubated in a humidified, 37 °C, 5% CO_2, when cultures reached 80% confluences, cells were detached using 0.25% trypsin EDTA solution (Gibco, 25200072). Detached cells were cultured in another flask until confluence was achieved for P3. Briefly, the medium was collected and centrifuged at 1600 rpm for 5 min at room temperature, and the supernatant was filtered by a 0.22 μm filter unit (TPP, 99722) and used as CM-ATMSCs and stored in −80 °C [15–17].

2.3. Measurement of growth factor level in CM-hATMSCs (TGF-β1, TGF-β2, VCAM1, VEGF2, FGF, EGF)

Some growth factors that presence in CM-hATMSCs were measured using ELISA Assay (Elabscience, H0110 (TGF-β1); H1587 (TGF-β2); H5587 (VCAM1); H0111 (VEGF2); H0483 (FGF); H0059 (EGF)). One hundred microlitres of sample and standard working solution was prepared approximately in each well, and then incubated 90 min at temperature of 37 °C. The solution in each well was removed and then added with biotinylated detection Ab working solution (100 μl). The solution was mixed up gently then incubated at 37 °C, for 60 min. After incubation, the solution was aspirated from each well then added wash buffer (350 μl) and soaked for 1–2 min, this method was replicated three times. HRP conjugate working solution (100 μl) was added, then incubated again for 30 min, at 37 °C. After incubation, the solution was washed again five times with 350 μl of wash buffer. Briefly, 90 μl of substrate reagent was added and then incubated until the color changed into blue. Incubation was done again in 15 min at temperature of 37 °C (protected from light). After that, the solution was added with 50 μl stop solution (the solution changed color into yellow). Absorbance was measured at 450 nm using ELISA reader (Multiskan Go, Thermo Scientific, USA) [16].

2.4. Statistical analysis

Data are presented as mean and standard deviation, the differences among groups were analyzed using one-way analysis of variance (ANOVA) with SPSS 20.0 statistical package, and $p < 0.05$ were considered as statistically significant, along with Tukey post hoc test and 95% confidence interval and independent t-test for comparing between two treatments.

3. Results

Samples used were treated with Fresh Frozen Plasma (FFP) and without FFP at many passage. Growth factors that were observed in this study are TGF-β1, TGF-β2, VEGF, VCAM1, EGF, and FGF. Figs. 1 and 2 describe the result of VCAM1, VEGF, EGF, FGF, TGF-β1, TGF-β2 level of CM-hATMSCs treated FFP

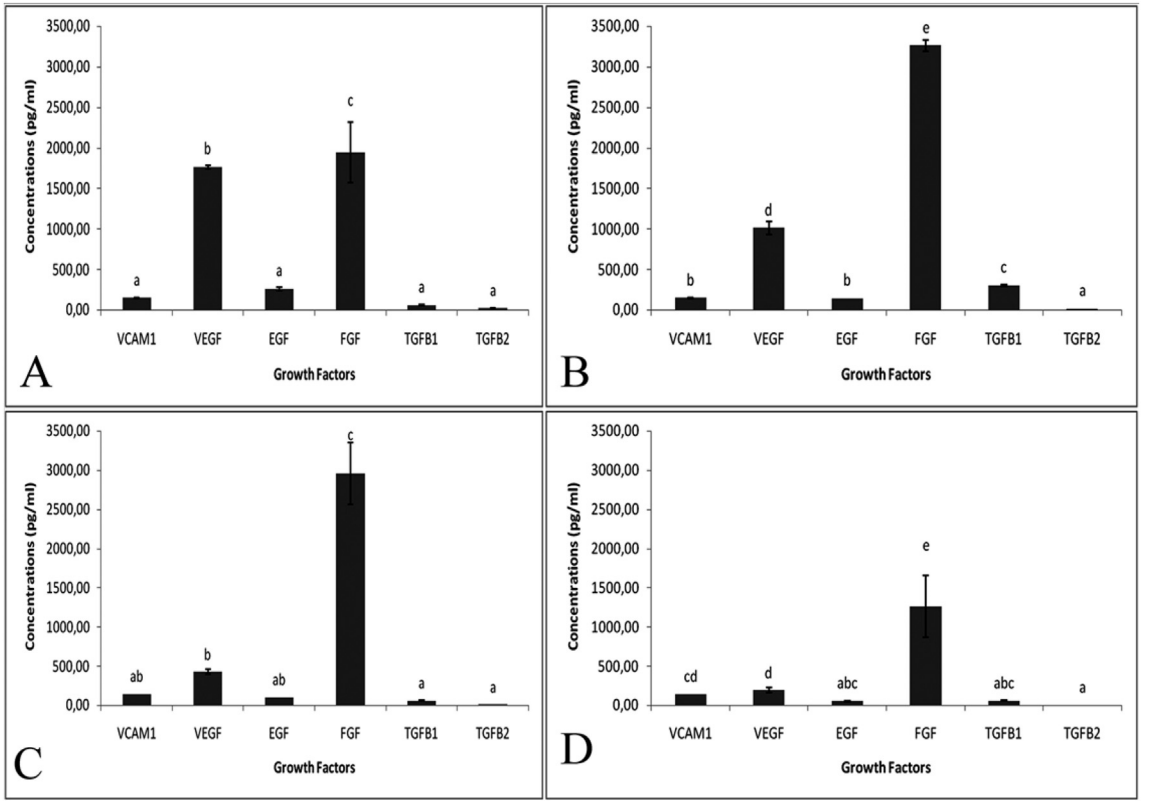

Fig. 1. The concentration of growth factors of CM-hATMSCs (VCAM1, VEGF, EGF, FGF, TGF-β1, TGF-β2) treated with FFP at passage 3 (A), 7 (B), 11 (C) and 15 (D). The growth factors content were measured in triplicate. The histogram are presented as mean ± standard deviation. Different small letters show significant difference among modulators ($P < 0.05$) in P3 (Fig. 1A), in P7 (Fig. 1B), in P11 (Fig. 1C), and in P15 (Fig. 1D), which the data was analyzed using ANOVA and Tukey post hoc test.

and without FFP at P3, P7, P11 and P15, respectively. Fig. 3 describe the comparison between FFP and without FFP at P3, P7, P11 and P15 of VCAM1, VEGF, EGF, FGF, TGF-β1, TGF-β2 level.

Based on Fig. 1, FGF has the highest concentration level of growth factors observed at each passage compared to the others passage. At P3, FGF concentration is 1941.83 pg/ml (Fig. 1A) while TGF-β2 is the lowest concentration in P3 with concentration 21.11 pg/ml. At P7, FGF concentration is 3260.50 pg/ml (Fig. 1B) while TGF-β2 is the lowest concentration (13.24 pg/ml). At P11, FGF concentration is 2960.33 pg/ml (Fig. 1C) while TGF-β2 is the lowest concentration (9.64 pg/ml). At P15, FGF concentration is 1259.17 pg/ml (Fig. 1D) while TGF-β2 is the lowest concentration (5.78 pg/ml). From these results, it also shown that FGF had the highest concentration at P7.

The growth factors level of each sample treated without FFP is shown in Fig. 2. Based on Fig. 2, FGF is the highest concentration level compared to other modulators. At P3 (Fig. 2A), FGF concentration is 1404.17 pg/ml, while TGF-β2 is the lowest concentration (9.11 pg/ml). At P7 (Fig. 2B), FGF concentration is 1698.17 pg/ml, while TGF-β2 is the lowest concentration (5.13 pg/ml). At P11 (Fig. 2C), FGF concentration is 1271.33 pg/ml, while TGF-β2 is the lowest concentration (5.04 pg/ml). At P15 (Fig. 2D), FGF (682.50 pg/ml), while TGF-β2 is the lowest concentration (2.49 pg/ml). From the result, it also shown that FGF had the highest concentration at P7.

Fig. 3 showed that the concentration level of each modulator and comparison between FFP and non-FFP-treated cells. FGF concentration at P7, 11, and 15 in FFP-treated ATMSCs are significantly different compared to non-FFP. Concentration level of FGF is higher in FFP-treated ATMSCs than without non-FFP.

4. Discussion

FFP is blood product made from the liquid portion of whole blood, it used as part of plasma exchange, a complex mixture of water, proteins, carbohydrates, fats, and vitamins, and has similar characteristic as Platelet Rich Plasma (PRP) [18]. The WJ-MSCs cultured in medium supplemented with human platelet from donor with blood type O and AB (huPL-ABO) had lower PDT compared to FBS medium in passage 1 to 8 [19]. Growth factors released by platelets in huPL-ABO are effective to stimulate proliferation of WJ-MSCs in vitro [19], this result was in line with previous study that hATMSCs cultured in FFP resulted higher proliferation or lower PDT compared to non FFP medium [20]. This result data shows that higher FFP medium increased cells proliferation, this result was consistent with previous study that growth factors influence cell proliferation, motility, survival and morphogenesis [21].

In comparison, previous study using Platelet Rich Plasma (PRP) supplementation, several growth factors has been observed such as FGF, TGFβ, and VEGF and were at their highest concentration when the proliferation was most enhanced [22]. The cell culture media supplemented with human platelet lysate with AB and O blood type (huPL-ABO) has a higher proliferation cell rate compared with FBS supplemented, high secretion of growth factor in huPL such as platelet-derived growth factor-AB (PDGF-AB), Insulin-like growth factor-1 (IGF-1), TGF-β1 and VEGF [19].

ATMSCs contained various growth factors such as (VCAM1, VEGF2, EGF, FGF, TGF-β1, TGF-β2 (Figs. 1–3), this result was consistent with previous study that MSCs produce MSCs produce various factors, like Ang-1, VEGF, HGF, EGF, PDGF, FGF, KGF and TGF-b [23].

In this study, FGF was the highest concentration compared to other growth factors FFP-treated media and non FFP in P7. FFP and non FFP treated media contained FGF which is in line with previous study that stimulation of FGF is correlated with the upregulation of the expression of anti-angiogenic miR-223 and that the effect of FGF from adipose mesenchymal stem cell-derived extracellular vesicles (AMSC-EVs) are antagonized by the inhibition of miR-223 [24]. FGFs are a family of growth factors involved in wound healing and angiogenesis [21]. Meanwhile, FGF acts to stimulate fibroblast and keratinocyte proliferation [25]. FGF-2 (bFGF) induces differentiation of MSCs to adipogenic, chondrogenic, osteogenic [26,27]. Moreover, bFGF is a factor necessary for MSCs expansion and viability [27,28]. FGF2 is a more potent as angiogenic factor compared to VEGF [29].

TGFβ exists as three isoforms: TGFβ-1, TGFβ-2 and TGFβ-3 which induce proliferation of MSCs and chondrocyte formation [21,30]. TGF-β2 in FFP and non FFP at the P15 showed low concentration. TGF-β1 expression increases in various tissues with damage, especially when accompanied by inflammation [31]. TGF-β1 and another isoform TGF-β2 (at low concentration) do not promote

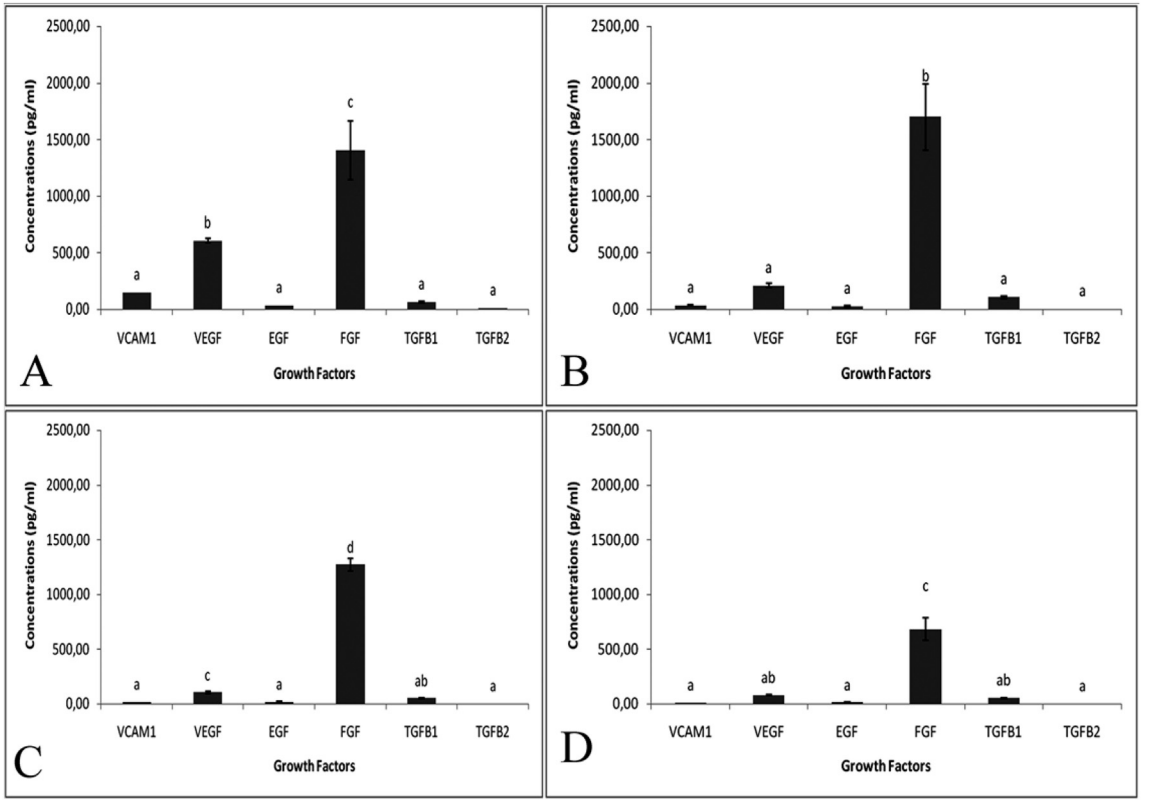

Fig. 2. The concentration of growth factors of CM-hATMSCs (VCAM1, VEGF2, EGF, FGF, TGF-β1, TGF-β2) treated with non FFP at passage 3 (A), 7 (B), 11 (C) and 15 (D). The growth factors concentration were measured in triplicate. The histogram are presented as mean ± standard deviation. Different small letters show significant difference among modulators ($P < 0.05$) in P3 (Fig. 2A), in P7 (Fig. 2B), in P11 (Fig. 2C), and in P15 (Fig. 2D), which the data was analyzed using ANOVA and followed by Tukey post hoc test.

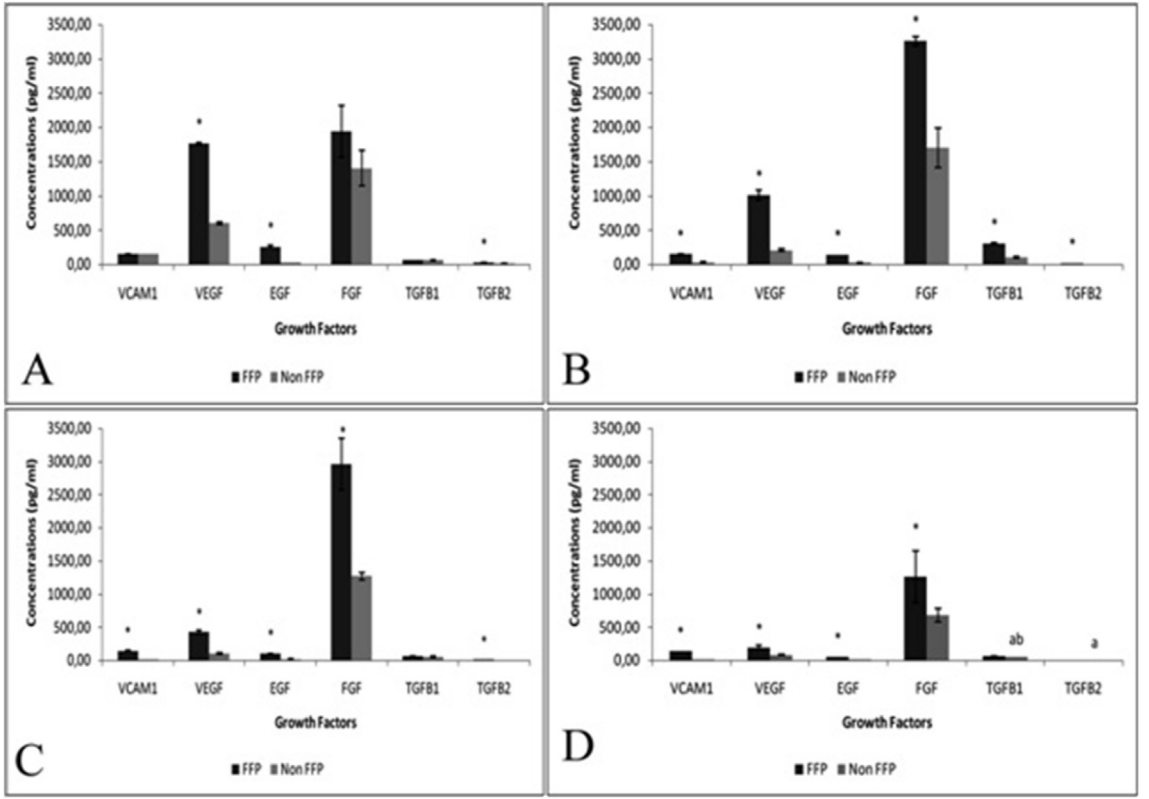

Fig. 3. The concentration of growth factors of CM-ATMSCs (VCAM1, VEGF2, EGF, FGF, TGF-β1, TGF-β2) treated with FFP and non FFP at passage 3 (A), 7 (B), 11 (C) and 15 (D). The growth factors concentration were measured in triplicate. The histogram are presented as mean ± standard deviation. P3 (Fig. 3A), in P7 (Fig. 3B), in P11 (Fig. 3C), and in P15 (Fig. 3D). Asterisk symbol shows significant difference in modulators at difference treatment between FFP and non FFP ($P < 0.05$) which was analyzed with independent sample t-test.

immunosuppression, it reversed the immunosuppressive effect on exerted by activated MSCs, and this effect is through inhibiting inflammatory cytokine-induced iNOS expression in an SMAD3 [32]. The stem cell-derived secreted factors showed that the secreted factor alone without the stem cell itself may cause tissue repair in various conditions that involved tissue/organ damage [33]. Dedifferentiated adipocytes CM which contain TGF-β1 may be useful for aged skin, which stimulate collagen synthesis and accelerate collagen degradation in human dermal fibroblast [34]. TGF-β is involved in a number of processes in wound healing: inflammation, stimulating angiogenesis, fibroblast proliferation, collagen synthesis and deposition and remodelling of the new extracellular matrix [35,36], play a pivotal role in skin aging [37].

ATMSCs secreted EGF both in FFP and non FFP supplementation, this result was consistent with previous study [9,23]. EGF has ability such as wound healing, tissue regeneration, neurogenesis [23]. EGF has played role in skin generation through the epidermal stem cells from mitogenic effects and differentiation of skin stem cells [38]. EGF promote *ex vivo* expansion of MSCs without triggering differentiation into any specific lineage [21,39]. EGF increased NIH3T3 cell proliferation in a bell-shaped dose response, and the maximum cell proliferation was achieved at a concentration of 25 ng/ml [40]. EGF has ability such as skin rejuvenation, production of collagen, elastin, ECM remodeling [41].

VCAM-1 was lower at older passage, higher significant difference FFP compared to non FFP (Fig. 3). This result was consistent with previous study that the expression of VCAM-1 was most markedly decreased among the tested markers in the senescent MSCs [42], VCAM-1 affect aging in MSCs [43]. Activation of lymphocyte-associated MMPs is delayed by hours after binding to VCAM-1, and this activation is blocked by inhibition of endothelial cell ROS generation [44]. VCAM-1 mediates leukocyte-endothelium adhesion, and elevated levels of circulating soluble form has been related to systemic inflammation disease [45]. Older passage of MSCs has lower angiogenesis activity compared to early passage, whereas early-passage of MSCs (P2-3) exhibited a significantly higher DNA damage response (DDR) capacity than late-passage cells [46], the cellular senescence can be regarded as a permanent DNA damage response activation [45]. Shortening telomeres produces a persistent DDR, which activates and sustains the senescence growth arrest [45,47,48], in the early passage, MSCs secreted higher VCAM-1 than late passage. The MSCs reduced migratory, and homing ability [45].

CM-hMSCs treatments have ability to improve cutaneous wound healing. CM-hMSCs can stimulated the migration of dermal fibroblasts and increased their expression level of genes to wound healing process [49]. Other study showed, ATMSCs and their secretory factors can stimulate collagen synthesis and migration of fibroblasts during the wound healing process via activation of dermal fibroblasts or growth factors [50]. Dermal of fibroblasts cell (NBL-6) can migrated more rapidly in cell culture in CM than controls, that may increase injury more rapidly [49]. WJ-MSCs release the various cytokines of wound healing such as TGF-βs, CTGF, HIF-1α, VEGF, FGF-2, which can promote the synthesis of collagen and also sirtuin-1 (SIRT-1) as antiaging genes [51]. Passage affect cytokines (IL1-α, IL-6, IL-8) and growth factor (VEGF) level secretion in human Wharton's Jelly MSCs (WJMSCs) [16]. The VEGF level of ATMSCs (Figs. 1 and 2) are higher than VEGF of CM-WJMSCs 24.96–51.86 pg/ml [17]. CM-ATMSCs which secrete TGF-β1 is reported to stimulate collagen synthesis and hyaluronic acid synthase (HAS) expression [42]. In other study, CM from bone marrow MSCs (CM-BMMSCs) which contain growth factors may help in skin rejuvenation and has effect on anti-aging. The CM is also able to protect skin fibroblast from UV-B radiation and ter-buthylhydro peroxide (tbOH) induced oxidative stress [52].

CM from adipose tissue-derived MSCs (CM-hATMSCs) may increase migration, proliferation of fibroblasts in vitro and accelerate of healing process. CM-MSCs has produce some angiogenic factor that induce proliferation and differentiation of endothelial cell [49,53]. In regeneration of cell, CM-ATMSCs also showed significantly increased the number of cells in G1 phase while reducing the number of cells in the S and G2/M phases in cell cycle analysis [4]. CM-ATMSCs can induce G0/G1 growth arrest in U251 cells [54]. CM-ATMSC also inhibited melanoma (skin cancer) growth by altering cell-cycle distribution and inducing apoptosis in vitro [55].

5. Conclusion

The highest concentration level found in CM-hATMSCs is FGF both in FFP and non-FFP treated medium compared to the other mediators. CM-hATMSCs is promising wound healing and able to

regenerate the cells and it can be used for anti-aging products because it contains many growth factors such as TGF-β1, TGF-β2, VEGF2, FGF, VCAM1, and EGF.

Conflicts of interest

None.

Acknowledgements

This study was supported by the Grants-in-Aid from Insinas Riset Pratama, the Ministry of Research, Technology and Higher Education of the Republic of Indonesia No. 43/INS/PPK/E/E4/2017. The authors like to thank to Rizal Rizal, Annisa Amalia, Hanna Sari W. Kusuma, Yukko Arinta, Rismawati Laila Qodariah, and Rahmawati Rahmawati from Aretha Medika Utama Biomolecular and Biomedical Research Center, Bandung, Indonesia for their technical assistants.

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