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Growth factors of hypoxia Freeze-Dried Adipose Stem Cell-Conditioned Medium (FD ASC-CM) and Fresh Adipose Stem Cell Conditioned-Medium (FR ASC-CM): a comparative study

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Growth factors of hypoxia Freeze-Dried Adipose Stem Cell- Conditioned Medium (FD ASC-CM) and Fresh Adipose Stem Cell Conditioned-Medium (FR ASC-CM): a comparative study

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ABSTRACT

Background: Adipose Stem Cells (ASC) are multipotent stem cells which mechanism of action is mainly secretion of various paracrine molecules known as secretome or conditioned medium (CM). Adipose Stem Cell-Conditioned Medium (ASC-CM) collects several soluble factors, such as cytokines, chemokines, and Growth Factors (GF) secreted by almost all types of living cells in the extracellular space. The freeze-dried (FD) form of ASC-CM can reduce the instability of the fresh (FR) form of ASC-CM. This study aims to compare the GF levels between FD ASC-CM and FR ASC-CM as a comparative study.
Methods: ASC-CM was prepared from human adipose tissue. After we got the FR ASC-CM, some of them were changed into an FD form. We measured the several GF levels using ELISA methods in ng/L. Data were presented in mean and standard deviation as well as analyzed using SPSS version 25 for Windows.
Results: The average levels of EGF in both FD ASC-CM and FR ASC-CM were 256.80±9.70 ng/L and 290.20±53.20 ng/L, followed by TGF-β (564.20±12.70 ng/L and 633.10±11.40 ng/L), PDGF (2.90±0.19 ng/L and 2.90±0.20 ng/L), and VEGF (711.10±21.70 ng/L and 788.70±204.80 ng/L) respectively. There was a statistically significant difference in TGF-β levels between FD ASC-CM and FR ASC-CM ($p=0.002$). However, there was no a significant difference in EGF, PDGF, and VEGF levels between FD ASC-CM and FR ASC-CM ($p>0.05$)
Conclusion: There was a statistically significant difference between FD and FR ASC-CM only in TGF-β levels.

Keywords: ASC-CM, Growth Factors, Freeze-Dried, Fresh
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INTRODUCTION

Stem cells are a population of immature tissue precursor cells capable of self-repair and differentiation into various types of cells in the tissue.[1] Mesenchymal Stem Cells (MSC) is a multipotent stromal cell that can differentiate into various cell types such as adipocytes, osteoblasts, chondrocytes, fibroblasts, and myoblasts. MSC include Bone Marrow Stem Cell (BMSC), Umbilical Cord (UC)-MSC, Placenta (P)-MSC, Umbilical Cord Blood (UCB)-MSC, Amniotic Fluid (AF)-MSC, and Adipose Stem Cell (ASC).[2] The therapeutic effect of SC is mainly on the ability to produce soluble factors, referred to as secretome or Conditioned Medium (CM), which have several biologic activities, including inhibition of fibrosis and apoptosis, proangiogenesis, stimulation of proliferation and/or differentiation of tissue progenitor cells and immunomodulation.[3-7] The composition of CM depends on the SC source.[8] Adipose stem cell-conditioned medium (ASC-CM) is a collection of several soluble factors, such as cytokines, chemokines, and Growth Factors (GF), secreted by almost all types of living cells in the extracellular space.[1,7] The ASC-CM contains various GF such as Vascular Cell Adhesion Molecule (VCAM)-1, Vascular Endothelial Growth Factor (VEGF)-2, Platelet-Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF)-β1, TGF-β2. Epidermal Growth Factor (EGF) and Transforming Growth Factor-β (TGF-β) play a role in wound healing, tissue regeneration, neurogenesis, collagen and elastin synthesis, and extracellular matrix (ECM) remodeling.[9] Transforming Growth Factor-β and PDGF are factors that play a role in maintaining the balance of tissue modification and tissue repair processes that involve many cytokines.[10] Besides SC source, the type and level of GF also were affected by preconditioning modification methods such as hypoxia condition.[11] Hypoxic environment

exposed to ASC produces increased CM containing VEGF and/or HGF, as well as GF and other differentiation factors, such as TNF-α, FGF, and EGF.[12]

A standardized isolation process for CM is still lacking and some isolation methods such as Size-Exclusion Chromatography (SEC) or Ultracentrifugation (UC) are not scalable and also an aggregation or breakup may occur.[13] The freeze-drying technique, also known as lyophilization, aims to remove water content in a product. Freeze drying I is the process of freezing a material after the material is placed in a vacuum, allowing a material to be sublimated from the solid phase into a gas without disturbing the liquid phase. Three freeze-drying processes include freezing, primary drying, and secondary drying to produce the final product with the desired moisture.[14] In a study conducted by Peng and colleagues, it was shown that FD BMSC-CM is a promising biomaterial that may stimulate the formation of chronic wounds.[15] However, there are limited studies that used ASC-CM in FD form and compared it to FR form to compare the GF level between FD ASC-CM and FR ASC-CM.

Based on those mentioned above, this study aims to compare the growth factors of hypoxia Freeze-Dried Adipose Stem Cell-Conditioned Medium (FD ASC-CM) and Fresh Adipose Stem Cell Conditioned-Medium (FR ASC-CM).

METHODS

This research is a comparative study. It was conducted at the Stem Cell and Tissue Bank Department of Dr. Soetomo General Hospital, Surabaya. The materials of this research were ASC-CM. The procedures for obtaining adipose tissue from the donor, culture process and separation of secretome were performed legally in accordance with international standards for tissue donor and stem cell culture from humans. In both levels, FR and FD of ASC-CM, growth factors were measured by high sensitivity enzyme-linked immunosorbent assay (ELISA) kits. The reagent kits used for ELISA evaluation of growth factors level is human VEGF ELISA kits bioassay technology laboratory (BT lab), human TGF beta ELISA kits bioassay technology laboratory (BT lab), human PDGF ELISA kits bioassay technology laboratory (BT lab) and human EGF ELISA kits bioassay technology laboratory (BT lab)

Fresh ASC-CM

A piece of adipose tissue was put into a sterile centrifuge tube of collagenase A type I 0.2 mg/ml and incubated for 16-18 hours at 37°C until the tissue is completely digested. Complete growing medium (alpha MEM + penicillin 200 U/ml – streptomycin 200 U/ml + 1% amphotericin B + NEAA 100nM + 10% FBS) was added to the was digested and then centrifuged at 250 g for 10 minutes. The supernatant was discarded, then the cell pellet was resuspended with the complete growth medium, and a cell count was performed. Cell cultures were performed according to the protocol at the Stem Cell and Tissue Bank Department of dr. Soetomo General Hospital, Surabaya, Indonesia. The cell pellet was cultured with complete growth media and incubated at 37°C 5% CO_2 for 24 hours. Growth medium replacement was carried out 24 hours after incubation, and then the growth medium was changed every 3 days until the cells reach 80-90% confluence. Confluent cells were sub-cultured with the warm trypsin method using 0.25% EDTA trypsin. The growing medium was removed, then added 2 ml of 0.25% trypsin EDTA into the petri dish culture and incubated at 37°C for 3 minutes. Complete growth medium as much as 4 ml was added to the petri dish culture for ceasing trypsin act. The cell suspension was put into a centrifuge tube and centrifuged at 250 g for 5 minutes. The supernatant was then removed, then cell pellets were resuspended with a complete growth medium and a cell count was performed. The cell was then cultured again in petri dish culture with a density of 1.2 x 10^6 cell/cm^2 for cell expansion. Growth factor levels were measured using high-sensitivity ELISA kits.

Freeze-dried ASC-CM

After we got the FR form of ASC-CM, some of them were processed to become FD form. A total of 1000 ml FRASC-CM was divided into a petri dish as many as 25, each containing 40 ml. The first stage was freezing with a temperature of -95°C and the product will be frozen first. After freezing, the product will be placed under vacuum, this allows the solvent to freeze in the product to evaporate without going through the liquid state. The hot temperature was applied to speed up the sublimation process. Condensation temperature low removed the solvent that evaporates in a vacuum so that back to solid form. The final product of this process was the pinkish-white powder of ASC-CM. Growth factor levels were measured using high-sensitivity ELISA kits.

Data Analysis

The data were collected and entered in the *Statistical Package for the Social Sciences* (SPSS) version 25.0 (SPSS, Inc., Chicago, Illinois) for Windows. Before the statistical test was carried out, the normality test was carried out using the Shapiro-Wilk test because the number of samples in this study was less than 30. The results of the normality test showed that the data were normally distributed ($p>0.05$). Meanwhile, the statistical comparison analysis test used in this study is the *Independent T-Test*.

RESULTS

The average levels of EGF in both FD ASC-CM and FR ASC-CM were 256.80±9.70 ng/L and 290.20±53.20 ng/L, followed by TGF-β (564.20±12.70 ng/L and 633.10±11.40 ng/L), PDGF (2.90±0.19 ng/L and 2.90±0.20 ng/L), and VEGF (711.10±21.70 ng/L and 788.70±204.80 ng/L) respectively (Table 1). There was a statistically significant difference in TGF-β levels between FD ASC-CM and FR ASC-CM ($p=0.002$). However, there was no significant difference in EGF, PDGF, and VEGF levels between FD ASC-CM and FR ASC-CM ($p>0.05$) (Table 1). The growth factors level in FD ASC-CM and FR ASC-CM from ELISA measurement were depicted in Figures 1 and 2.

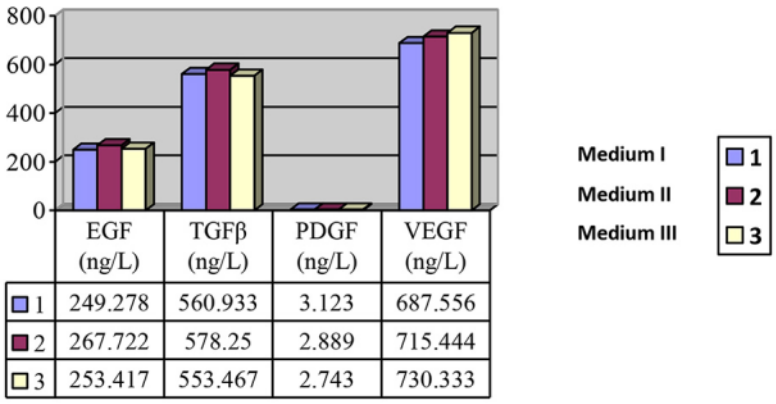
DISCUSSION

Stem cells are a population of immature tissue precursor cells capable of self-repair and differentiation into various types of cells in the tissue.[1] Adipose stem cells produce a series of GF, such as

VEGF, b-FGF, TGF-β1, TGF-β2, HGF, Keratinocyte Growth Factor (KGF), Platelet-Derived Growth Factor AA (PDGF-AA) and Placental Growth Factor (PGF).[16,17] The weakness of ASC is no standard for isolation, culture, expansion, and characterization methods of ASC, as well as proper quality and safety control for cell-based therapy. Each step of preparation, purification and expansion of ASC can affect the quality of ASC.[4,6,7] There are many approaches to making an optimal ASC-CM, such as modifying culture conditions with a hypoxia environment.[11,18] These will cause an increase in cellular function depending on the type of cell and the microenvironment.[6,12,19] It also increased the ability of cell proliferation and repair, thereby increasing the secretion of particular GF such as VEGF and/or HGF, as well as GF and other differentiation factors, such as TNF-, FGF, and EGF.[12,19] Zhou BR et al., examined GF level of ASC-CM by ELISA and reported the concentration of TGF-β1, VEGF, bFGF, KGF, PDGF-A and HGF were 126.76±2.71 pg/ml, 136.04±29.23 pg/ml, 65.09±18.47 pg/ml, 91.43±10.41 pg/ml, 48.12±1.33 pg/ml and 648.43±47.29 pg/ml, respectively.[20] Human ASC-CM cytokines mainly include proinflammatory, angiogenic, and hematopoietic cytokines such as HGF, VEGF, GM-CSF, IL-7, M-CSF, IL-6, IL-8, IL-11, and TNF-α.[21]

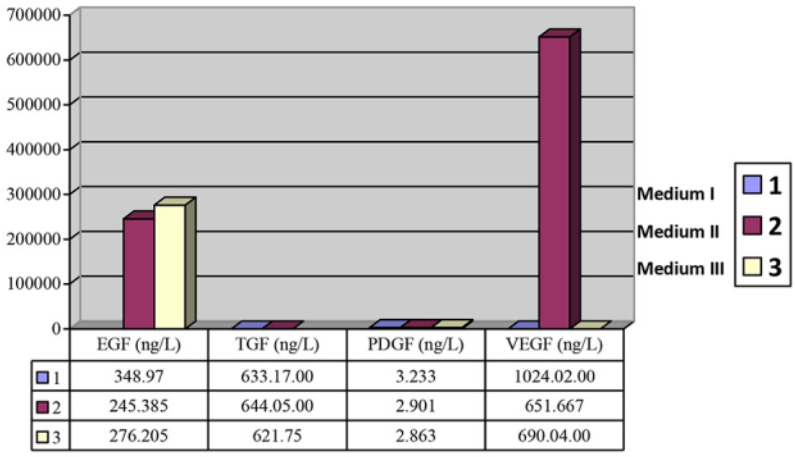
Some of the limitations of CM are that there is no standardization of formulations, characterization of bioactive factors and dosages, delivery optimization techniques and also mechanisms of action remain unclear.[22,23] Storage and transport of ASC-CM should pay attention to the freeze-thaw effect, stability at various temperatures and the effects of freeze-drying components.[6] Freeze drying is one of the most popular drying methods to maintain the quality of drying results, especially for heat-sensitive products. The working principle of a freeze dryer includes freezing the solution, granulating the frozen solution, conditions it under ultra-high vacuum with moderate heating, causing the water in the food to sublimate and produce solid products. The benefits of FD can be stored for a long period, inhibit microorganism activity, reduce oxidative denaturation, and thermal damage.[24] This form of CM has been reported to be stored at -20°C for several months without any change in function.[6]

Based on statistical results, it was found that the changes of GF level of FD ASC-CM and FR ASC-CM were not significantly different except for TGF-β. Growth factors



	EGF (ng/L)	TGFβ (ng/L)	PDGF (ng/L)	VEGF (ng/L)
1	249.278	560.933	3.123	687.556
2	267.722	578.25	2.889	715.444
3	253.417	553.467	2.743	730.333

Figure 1. Growth factors level in FD ASC-CM from ELISA measurement



	EGF (ng/L)	TGF (ng/L)	PDGF (ng/L)	VEGF (ng/L)
1	348.97	633.17.00	3.233	1024.02.00
2	245.385	644.05.00	2.901	651.667
3	276.205	621.75	2.863	690.04.00

Figure 2. Growth factors level in FR ASC-CM from ELISA measurement

Table 1. Growth factors comparison of FD ASC-CM and FR ASC-CM

Growth Factors	Groups (Mean± SD)		p
	FD form	FR form	
EGF (ng/L)	256.80±9.70	290.20±53.20	0.345
TGF-β (ng/L)	564.20±12.70	633.10±11.40	0.002*
PDGF (ng/L)	2.90±0.19	2.90±0.20	0.644
VEGF (ng/L)	711.10±21.70	788.70±204.80	0.549

EGF: Epidermal Growth Factor; TGF-β: Transforming Growth Factor-β; PDGF: Platelet-Derived Growth Factor; VEGF: Vascular Endothelial Growth Factor; FD: Freeze-Dried; FR: Fresh; *Statistically significant if p-value less than 0.05.

are important biomolecules that instruct cell behavior (for example, cell migration, survival, adhesion, proliferation, and differentiation) and guide regenerative tissue processes.[25] Almost similar in our study, Joseph A et al., reported VEGF level in FD BMSC-CM increased significantly at 72 hours and it did not increase significantly further at 96 hours.[26] From Western blot assay, VEGF, TGF-β1, IGF-1, FGF-2, SCF, and IL-6 were found in FR and FD BMSC-CM.[26] Sun B et al., measured the protein concentration of ASC-CM by Nanodrop 2000 and found that concentrated hypoxia FD ASC-CM was the highest level.[27] They also found no statistical difference between concentrated and unconcentrated hypoxia FD ASC-CM groups in the wound healing process.[27]

In contrast to our study, Peng and colleagues had found that the HGF and VEGF level was significantly lower in FD BMSC-CM than in frozen BMSC-CM.[15] When FR MSC-CM is used on the injured tissue, high levels of GF (bFGF, EGF, TGF-β1, VEGF, HGF, and PDGF) were detected in the Adipose Liquid Extract (ALE).[25] Other FD studies using PRP, such as Huber SC et al., showed that GF levels (VEGF, EGF, TGF, PDGF AA) of FD in PRP were significantly higher than FR PRP.[28] This means that the GF in FD form will increase more than in FR form. The limitation of this study was only used ELISA kit to measure particular GF, so we could not know the whole GF, which contained in both forms of ASC-CM. This study also did not observe both forms of ASC-CM, which become the limitation of its. Further research is crucial so that this FD form may be used as a standard form of ASC-CM.

CONCLUSION

There is no difference between FD ASC-CM and FR ASC-CM in terms of most growth factors. However, there was a statistically significant difference between FD and FR ASC-CM only in TGF-β levels.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

ETHICS CONSIDERATION

Ethics approval has been obtained from the Research Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, with number 2.KE.071.07.2020.

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None.

AUTHOR CONTRIBUTION

All authors are equally responsible for the initial conceptualization, study design, intellectual content, literature search, clinical studies, data acquisition, data analysis, manuscript preparation, and review of the earliest draft of the manuscript.

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