The effect of injection of ADSC compared to AAPE on collagen density in aging skin (animal study)

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Abstract

Introduction: Aging is a biological process that induce changes to the structural integrity and physiological function of skin. Structural changes of skin are a result of dermal atrophy, decreased collagen, the loss of subcutaneous fat, the loss of inherent elasticity, and increased melanogenesis. By definition, a stem cell is characterized by its ability to self-renew and to differentiate along multiple lineage pathways. ADSC is a mesenchymal stem cell from human adipose tissue, with similar potential properties as stem cells derived from bone marrow.

Objective: The aim of this study was to compare the effect of adipose-derived stem cells (ADSC) and their secretomes Advanced Adipose-derived Stem Cells Protein Extract (AAPE) in aging skin.

Methods: ADSC were isolated from lipospirates obtained from healthy donors, after obtaining written consent and ethical approval, using liberase enzymatic digestion, washed with steril PBS and centrifuged. After the phase, ADSC were seeded directly after isolation with liberases and cultured, then produced the conditioning media (AAPE) by amplified hypoxia. We analyzed the ADSCs (2,5 x 10⁵ cells) and their secretomes (0,1ml in 0,4ml NaCl 0,9%) by subcutaneous injection on the back of a mice (with range age 48 weeks), and followed every two weeks after injected until six weeks and stained with Van Giesson staining, for measured the density of collagen.

Results: Collagen density increased after ADSC was injected to the skin. Statistical analysis shows a significant increase of collagen density compared to control group and AAPE (p<0.05). We conclude that ADSC had anti-aging or regenerative potential by stimulating collagen synthesis of dermal fibroblast. ADSC will be a new modality treatment for aging skin in future.

Keywords: adipose-derived stem cells, aging skin, secretomes

Background

Aging skin is a progressive biological process which induces the structural and physical changes of the skin. These changes starts in the third decade. All happens by environment destruction, and it will affect the appearance of the skin.¹² Aging skin is also a degenerative multisystem process that involves the skin and all the compartments which support the skin such as bone, cartilage and subcutaneous tissue. Skin changes are the effect of atrophy in dermis, decrease of collagen, loss of subcutaneous fat tissue, loss elasticity and increased melanogenesis.³ The progress of medicine in recent times has focused on two aspects, degenerative prophylaxis and medication.⁴

Stem cells have the ability to produce newer cells and always differentiate as they progress.⁵ In dermatology, much research have been done using stem cells, that can increase the retention of...
fat autologous transplantation, antioxidant and whitening effect. Adults have adipose-derived stem cell (ADSC) as a mesenchymal cell in the human adipose tissue and have the same characteristic like other stem cells derived from bone marrow.\(^4,6\)

ADSC is a kind of mesenchymal stem cell from human adipose tissue, have essentially the same properties as stem cells derived from bone marrow. AAPE is the conditioned media of ADSC that had effects to stimulated collagen synthesis and migration of fibroblast during the wound healing process. And these results may be associated with numerous other factors produced by ADSC.\(^7\)

**Aim of the study**

To compare the effect of adipose-derived stem cells (ADSC) and their secretomes Advanced Adipose-derived Stem Cells Protein Extract (AAPE) in aging skin.

**Material and methods**

**Study design**

This study is an *in vivo* experimental study on rats to see the effect of ADSC and AAPE. The independent variable were ADSC and AAPE, and the dependent variable were the back skin of mice that injected with ADSC and AAPE 2 weeks after injection, and follow up after 6 weeks by histopathology examination. This study was conducted in Dermama Biotechnology Laboratory in Surakarta, Central Java Indonesia, from November 2014 to January 2015.

**Study sample**

The subjects were *sprague-dawley* mice species, taken from Laboratorium Penelitian dan Pengujian Terpadu (LPPT) unit IV from Veterinary of Gajah Mada University. We used 14 healthy female mice, 48 weeks of age. According to Kim *et al.* study, that used 28-week old mice undergoing accelerated intrinsic ageing and these criteria generally accepted that markedly aged species mean \(> 75\) years in humans and \(> 18\) months in mice.\(^6\) These mice fulfilled inclusion criteria, with average of body weight from 250 – 350 grams. The back side was cleaned, then marked with each length and width of 10 cm and 4 cm. The exclusion criteria were mice with abnormal development. The drop out criteria were dead mice.

**Isolation process and culture of adipose-derived stem cells (ADSC)**

ADSC were isolated from liposprates obtained from healthy donors (n=8; age: 30-50 years) after obtaining written consent and ethical approval. Briefly, raw liposprates were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Thereafter, they were treated with 0.18 Wünsch units (WU) of liberase in PBS for 30 minutes at 37°C. The enzyme was inactivated with an equal volume of DMEM (Dulbecco’s-Modified Eagle’s Medium Low Glucose) containing 10% FBS (Foetal Bovine Serum) and cell suspensions were centrifuged at 1200/G for 10 minutes to obtain a cell pellet. The pellet was resuspended in PBS supplemented with 10% FBS and passes through two filters of 250 μm and 100 μm to remove debris. After a second centrifugation at 600/G for 8 minutes, mononuclear cells were counted on a hemocytometer and seeded at a density of 105 on a 25 cm² tissue culture dish and incubated in a humidified cell incubator with 5% CO₂.

**Evaluation of Colony Forming Unit Factors (CFU-F) ability of ADSC**

ADSC were seeded directly after isolation with liberases of collagenase in 25 cm² flasks at a density of \(2 \times 10^4\) cells/cm² containing DMEM low glucose supplemented with 10% FBS for MSC, 2 mM L-glutamine, 100 IU/mL penicillin and 10 μg/mL streptomycyne. After a 10 day culture the colonies consisting of more than 10 cells were counted under an inverted optical microscope IX70 Olympus.

**Flow cytometry**

After obtaining cultured cells, the cells were trypsinized and stained with monoclonal antibodies against CD166, CD105, CD73, CD90, CD106, CD45, CD14, HLA-DR and CXCR4. Briefly, \(1 \times 10^5\) cells suspended in 100 μL of staining buffer (PBS and 2% FBS) were added to a test tube containing an appropriate amount of each antibody. Cells were incubated in the dark for 30 min at 4°C. Stained cells were washed and collected using a FACS Canto cytometer and analyzed with FACS Diva software.

**Measurement of proliferative potential of Mesenchymal Stem Cells (MSC)**

In order to compare the proliferative activity of ADSC and bone marrow-derived mesenchymal stem cells (BM-MSC), \(1 \times 10^4\) cells were seeded on 24-well plates and grown under standard culture conditions (DMEM containing 10% FSC for MSC) or in a serum-free medium, UltraCulture (Cambrex, East Rutherford, NJ, USA) supplemented with 1 μM dexamethasone, 50 μM ascorbic acid and cytokines: 10 ng/mL epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), 1 ng/mL...
platelet-derived growth factor (PDGF) and 100 ng/mL nerve growth factor (NGF). Both culture media contained 2 mM L-glutamine, 100 IU/mL penicillin and 10 μg/mL streptomycin. The medium was changed every fourth day. After 4, 7, 10 and 14 days in culture, the cells were detached from a plate and were counted on a hemo-cyтомeter. Population doubling time was calculated using the formula: \( \log N / \log 2 \) where \( N \) is the number of cells at indicated day of culture divided by the initial cell number.

**Animal Study**

*The subject of this study and the methods on the injected area*

Before injection on back area, these mice underwent general anesthesia using ketamine with average dosage of 44 – 300 mg/kg/weight intramuscular. These mice were divided into 3 groups, first group as control, second group was injected with ADSC and third group was injected with their secretomes (AAPE). After injection, we marked these area with marker, and follow up were started in second, fourth and sixth weeks later. The biopsy was done at the same time, by using the punch technique (5mm) and immersed in 10% formalin solution for histopathology examination.

**Histopathology examination and collagen deposition analysis**

Specimen were stained with Van Giesson (VG), then examined by the pathologist. This examination assessed the collagen deposition of each preparation. Examination of collagen deposition was done manually using standard microscope and captured with optilab camera in 40x magnification until all sections were seen. Every preparation was examined in 100 power field then counted the collagen deposition.

**Ethical clearance**

This ethical clearance was obtained from Veterinary of Medical Faculty Gajah Mada University.

**Data analysis**

Collagen density was measured by computer program which compared control and treated groups, and evaluated at the same time.

The statistical results was examined with one way ANOVA.

**Results**

The comparison of collagen density from each groups i.e the injected groups with ADSC (2.5 x 10^5 cells) and other injected group with 0.1 ml AAPE in 0,9% sodium chloride. In this study, there were significant differences of collagen density between control and ADSC injected groups \((p<0.05)\) in the fourth until the last week of evaluation (sixth weeks). (Figure 1). Collagen density in the ADSC injected group denser compared to the control group. (Diagram 1).

![Figure 1. Van Giesson staining for collagen. A. Control group (skin mice, age 48 weeks); B-C-D. Injected groups with ADSC, evaluated post injection, B. 2 weeks after; C. 4 weeks after; D. 6 weeks after.](image)

![Diagram 1. The differences of collagen density between control group and injected group with ADSC. Evaluated from 2 weeks after until 6 weeks after injected. Collagen density in the ADSC (2.5 x 10^5 cells) injected group were 65,7%, 68,6% and 77,2% during the follow up in 2nd, 4th, 6th week respectively; which denser compared to the control group (57,5%).](image)
In this study, we evaluated the control group and 2 groups with ADSC’s secretomes or AAPE i.e 0.1 ml in 0.9% sodium chloride, injected in the back skin of mice with age 48 weeks.

In ADSC group, the statistical results of collagen density after injection was 65.7% (2 weeks), 68.6% (4 weeks), and 77.2% (6 weeks). In AAPE group the results were 61.5% (2 weeks), 65% (4 weeks), and 71.8% (6 weeks). From statistical analysis showed there was improvement of collagen density in aging skin, p value < 0.05. We can conclude that ADSC is better than AAPE in treating aging skin (diagram 3), however if we compared with control group, both of them can used as new modality treatment for aging skin (diagram 4).

**Discussions**

Human adipose tissue contains differentiated adipose stem cells (Adipose-derived stem cells), as pluripotent mesenchymal cells which have ability to differentiation others cells. There has not been many clinical applications of ADSC was reported about their ability to repair around the damage cells. ADSC produce many growth factors that can repair and renew the damaged cells. The growth factors are good raw materials for skin regenerations, reepilization, wound healing, and ADSC can also repair wrinkles on face.

Conditioning media of ADSC (Adipose-derived stem cells conditioning media) contains many growth factors and has many advantages to cure many skin disease e.c wound healing, renewal and regeneration. Conditioning media of ADSC (ADSC-CM) recently use for biotechnology products such as cosmetics and other drug industries. AAPE (Advanced Adipose-derived stem cells protein extract) are the conditioning media which cultured from ADSC.

**Diagram 3. The collagen density in every group which injected with ADSC and AAPE, that evaluated from 2 weeks, 4 weeks and 6 weeks after injection.** Where the ADSC group was showed good result for increased the collagen than AAPE group.
In this study, we evaluated ADSC and their secretomes effect on the aging skin. There were two factors which can differentiate the aging skin process, the intrinsic factors which are the natural process of aging and as a continuing process that occur in second decade. Where the collagen production in skin decrease or occurs slowly, and where the elastin fiber slowly reduced. ADSC is easily developed and found in many parts of human body, and there aren’t any ethical problem to develop these cells. From Zhang et al. study investigated the anti-aging effect of ADSC, particularly the suppression effect of glycation reaction and functional capacity repair in animal study, which where rapidly aged using d-galactose enzyme. After being injected to the skin, ADSC can reduce the advanced glycation end product (AGE) level while significantly increase the dermal thickness and collagen density, increase the expression of VEGF (vascular endothelial growth factors) and the blood vessel walls in skin. 

In our study, collagen density improved after injection ADSC as well as AAPE injection. Byung-Soon Park et al. investigated the effect of ADSC and their secretomes on aging skin by using animal study and had resulted ADSC produced many growth factors which characterized by increasing of collagen production in injected group, and in this study was done to a woman with many wrinkles in her face, and got improved after 2 weeks of ADSC injection.

Kim et al. study, ADSC and their conditioning media (ADSC-CM) had ability to increase collagen synthesis and migrasion of dermal fibroblast cells during the wound healing process. Where ADSC-CM also can inhibit melanogenesis by reducing the regulation of tyrosinase and expression of tyrosine-related protein-1 (TRP-1) in oxidative cells that induced by chemical agents and ultraviolet B exposure.

In some clinical studies using the ADSC to treat the defect in human skin tissue by radiation, showed progressive tissue restoration and induced new formations of blood vessels. Some prior studies showed ADSC had focused on skin cells trans-differentiation where the ADSC to express their ability to showed epithelial cells, cytokeratin 19, which was evaluated in 4 weeks post wound healing.

Moon et al. study defined the benefits of AAPE to aging skin, where AAPE showed their effect to increase the proliferation of skin keratinocytes, also their ability to increase keratinocytes regeneration. ADSC-CM can stimulate the growth factors of many cells by autocrine and paracrine mechanisms. How AAPE can activate the keratinocytes is still unclear, but AAPE contains many growth factor as analyzed with proteome analysis i.e 2-D gel assay, MALDI-TOF analysis and antibody array, and can be used as new modality treatment for skin regeneration.

Conclusions

In aging skin, injection with ADSCs and AAPE subcutaneously which were evaluated in 2 weeks, 4 weeks and 6 weeks after the injection showed that ADSCs were more efficacious to increase collagen density than AAPE. However compared to the control group, either ADSCs or AAPE had the same effect to repair aging. It proved that ADSCs and their secretomes can be used as new modality to treat aging skin.

The limitations

In this study in vivo could not be done by using three dimension microscopy.
References

1. Masnec IS, Šitum M. Skin aging. ActaClin Croat 2010;49:515-9
2. Yarak S, Okamoto OK. Human adipose-derived stem cells: current challenges and clinical perspectives. An Bras dermatol. 2010;85(5):647-56.
3. Zhang S, Dong Z, Peng Z, Lu F. Anti-Aging Effect of Adipose-derived stem cells in mouse model of skin aging induced by D-galactose. PLoS One. 2014;9(5):1-7.
4. Ogliari KS, Brum DE, Marinowic D, Loth F. Stem cells in dermatology. An Bras Dermatol. 2014;89(2):286-91.
5. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res. 2007;100:1249-60.
6. Kim JH, Jung M, Kim HS, Kim YM, Choi EH. Adipose-derived stem cells as new therapeutic modality for ageing skin. Experimental dermatology. 2011;20:383-7.
7. Park BS, Jang KA, Sung JH, Park JS, Kwon YH, Kim KJ, et al. Adipose-derived stem cells and their secretory factors as a promising therapy for skin aging. Dermatol Surg. 2008;34:1323-6.
8. Wozowicz SB, Ward SM, Zawisz A, Adamus T, Konieczny P, et al. An improved protocol for adipose tissue-derived stem cell isolation: implications for treatments of bone disorders. European Journal of Medical Technologies. 2014;1(2):6-21.
9. Kim WS, Park BS, Sung JH. Protective role of adipose-derived stem cells and their soluble factors in photoaging. Arch Dermatol Res. 2009;301:329-36.
10. Moon KM, Park YH, Lee JS, Chae YB, Kim MM, et al. The effect of secretory factors of adipose-derived stem cells on human keratinocytes. International Journal of Molecular Science. 2012;13:1239-57.
11. Jafferany M, Huynh TV, Silverman MA, Zaidi Z. Geriatric dermatoses: a clinical review of skin diseases in an aging population. International Journal of Dermatology. 2012;51:509-22.
12. Park BS, Jang KA, Sung JH, Park JS, Kwon HY, et al. Adipose-derived stem cells and their secretory factors as a promising therapy for skin aging. Dermatology Surgery. 2008;34:1323-6.
13. Enggalhardjo M, Wahid S, Sajuthi D, Yusuf I. Effect of Adipose-derived Mesenchymal stem cells in photoagingbalb/c mouse model. American Journal of Medical and Biological Research. 2015;3(1):48-52.
14. Konno M, Hamabe A, Hasegawa S, Ogawa H, Fukusumi T, et al. Adipose-derived mesenchymal stem cells and regenerative medicine. Develop Growth Differ. 2013;55:309-18.
15. Mizuno H, Tobita M, Uysal C. Concise Review: Adipose-Derived Stem Cells as a Novel Tool for Future Regenerative Medicine. Stem Cells Express. 2012;30:804–10.
16. Derby BM, Dai H, Reichensperger J, Cox L, Harrison C, et al. Adipose-derived Stem cell to epithelial stem cell transdifferentiation: a mechanism to potentially improve understanding of fat grafting’s impact on skin rejuvenation. Aesthetic Surgery Journal. 2014;34(1):142-53.
17. Zuk P. Adipose-derived stem cells in tissue regeneration: A review. Hindawi Publishing Corporation. 2013:1-36.