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Effect of Adipose-derived Mesenchymal Stem Cells in Photoaging Balb/C Mouse Model

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Abstract Cell therapy using stem cells restores organs or tissue damaged by trauma, include of photoaging. Adiposed-derived stem cell (ADSC) have relative advantages in accessibility (less invasif), abundance stem cell compared to other kinds of stem cell and easier to get adipose in large quantities than bone marrow. In this Study, we investigated whether subcutaneous injection of ADSC significantly increase collagen synthesis, epidermis and dermis thickness, colagen density, and microvascular density in Photoaging Balb/C mice. We use 27 mice, divided by 3 group. The first group (A) were given UVB light irradiation and stem cell. The B group were given UVB light irradiation and placebo (NaCl 0.9%). The C group as the control group was untreated (no UVB light irradiation and no stem cells).The results showed that $1x10^4$ ADSC subcutaneuous injection significantly increase collagen synthesis in Balb/C mice and collagen density in photoaging. In conclusions revealed that Adiposed-derived Mesenchymal stem cells stimulate colagen density, and improved photoaging.

Keywords: *Adiposed-derived stem cells, photoaging, Collagen*

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1. Introduction

Stem cells therapy is interesting therapy recently. Many scientist in the world make experiment for stem cells. Stem cells may potentially be useful in the regenerative medicine. There have been many clinical applications for mesenchymal stem cells, including ADSCs for compensation of skin defects and wound healing. In previous study, demonstrated that ADSCs stimulated collagen syntesis during the wound healing process. Adiposed-derived mesenchymal stem cells display multi-lineage developmental plasticity, less invasif, and are similiar to bone marrow-derived mesnchymal stem cells (BM-MSC).(Konno M, et all, 2013; Lee SH, 2011; Kim et all, 2011)

Indonesia has the tropical weather with ample sunlight that highly risks skin for damage or premature aging. Skin premature aging is a skin aging process that fasther than truly. Individual aging was highly affected by various factors either internal or external factors.(Fisher GJ, et all, 2012) The internal factors were mostly genetic factors, disease such as atherosclerosing, diabetes mellitus and osteoarthritis while external factors were exposure to sunlight, pollution, cigarette smoke and unhealty diets (Scharrfetter –kochaneck et all, 1197).

Exposure to UV may result in changes in epidermis and dermis that may cause skin become thin, wrinkled, dry, and rough. (Rabe J, 2006; Ichihashi, 2009)Apart from that, UV exposure may cause skin thickening, uneven pigment distribution and the increase of fluid loss through skin, therefore skin looked dry.(Kligman, 1986; Berneberg et all, 1997; Charffetter-Kochanek et all, 1991) Change in human skin caused by UV exposure were similiar with those of in mice experimentally exposed to UV..

Photoaging process is correlated with epidermis and dermis. The epidermal thickness can be irregular, increased epidermal thickness or pronounced epidermal athrophy. The most pronounced histolgic change is the accumulation of elastin-containing material, known as solar elastosis (Rabe J, 2006, Rijken, 2011). Collagen which composes over 90% of the skin's total protein, becomes disorganized. Collagen is one of the main building blocks of human skin, providing much of the skin's strength. TGF-β promotes collagen formation while AP-1 promotes collagen breakdown by up-regulating enzymes called matrix metalloproteinases (MMPs) (Pierard GE, 2010; Fisher GJ et all, 2002).

Uvb may cause skin inflammation and erythema and also directly and indirectly induces biological effect, including the formation of photoproduct, primarily cyclobutane pyrimidine dimers. Through telomere disruption and telomere-based DNA damage responses, these photoproducts may upregualt DNA repair capacity

and induce mutations in coding DNA that lead to cancer. UVB may significantly reduced antioxidant effect at skin, refuced the ability of skin to protect itself against free radical generated by UV radiation(Kern s, 2006)

Adiposed-derived mesenchymal Stem Cells (ADMSCs) are easily obtained from subcutaneous fat tissue have the relative advantages of accessibility and abundance. (Kern S, et all, 2006; WS Kim et all, 2009). Some studies have repoted on the wound-healing effects of adult stem cells by proliferating fibroblast and secreting cytokines. Re-epitalization and angiogenesis were observed after application of bone marrow-derived mesenchymal stem cells to wound site. Wound healing effects of ADMSCs mediated by stimulating collagen synthesis of dermal fibroblast (Kim et all, 2011)

In previous study, premature aging induced by UV light exposure was caused by the formation of ROS on the skin by the exposure to UVB light (290-320 nm wavelength) and UVA (320-400 nm wavelength). Albino Skh-1 hairless mice were reported to be used in a study as model animals for premature skin aging that utilized UV exposure. In Indonesia this strain of mice are not available therefore in this study Balb/C strain was used as the animal model of premature skin aging in human (Kim et all, 2009).

2. Materials and Methods

2.1. Animal Experiment

Five week old female Balb/C mice at 12-22 gram were provided from animal facility of Bimana Indomedical. All mice were housed in climate controlled quarters (23-25 ℃, at 70-80% humidity) with a 12/12 light/dark cycle. Animals were allowed free access to water and a chow diet and were absorved daily.

The mice were irradiated dorsally using the UVB Philips narrowband, panjang gelombang 311 nm, (Philips, Waldmann F85/100W-01) for sixteen weeks, five times a week. The distance from the lamps to the animals'backs was 20 cm. During exposure, the animals could move around freely in their cage. One cage for 1 mouse. The irradiation dose was 60 mJ/cm, for sixteen weeks.

After photoaging induction, adiposed-derived mesenchymal stem cells (ADMSCs) 1x 10^4 cells were subcutaneously injected into the restricted area of mice. ADMSCs were subcutaneously injected into restricted area of the mice. ADMSCs were suspended in NaCl 0.9% and injected three times in a seven day interval.

2.2. Isolation and Culture of ADSCs

One Balb/C mouse subcutaneous adipose tissue samples were aquired in 2.5 gram. The body weight of mice after treatment(ADMSCs) is 19-24 gram. The obtained samples were enzimatic digested with 0.075% collagenase under gentle agitation for 45 min at 37 ℃ and centrifuged at 2000 rpm for 10 min to obtain the stromal cell fraction. The pellet was filtered with syfon steril filter and resuspended in phosphate buffered saline (PBS) 5-10 ml. The cell suspension was layered onto histopaque and centrifuged at 2000 rpm for 10 min. The supernatant was dicarded. The retrieved cell fraction was cultured overnight at 37 ℃/95% O^2/5% CO2 in control medium DMEM (Dulbecco's modified Eagle Media), 20% fetal bovine medium serum (FBS), penicillin streptomicyn 2%.

After subculture achived until 80% confluency, Adiposed-derived mesenchymal stem cells were cultured and expanded in control medium for two weeks.

ADMSCs expressed CD105 and Stro-1 (stromelycin marker), analyzed by Immunolflouresence (IFA) and Polymerasi Chain Reaction (PCR).

2.3. Skin Replica and Image Analysis

At the time photoaging induction and one week after the final injection of ADMSCs, negative replicas of the dorsal skin surface were taken by using small scissor with pinch the animals's back. To obtain replicas of skin, the skin was marked using and oil-based marker pen. For ease of measurement, all replicas were cut into elipps pieces 1x0.5 cm skin samples were fixed with 10% buffered formalin for hematoxylin and eosin (HE) staining and Masson's Trichrome staining. Staining process referenced from standard procedure of Laboratory of Patology, Animal Primata Center, Bogor Agricultural Institute.

2.4. Hematoxylin and Eosin (HE) Staining

Back skin tissue fixed 10% buffered formalin for 24 hours were cut into 3-5 mm thick slices. Tissues were then embedded in paraffin and cut into 4-5 μm thick slices using microtome. Slices were monted on object glass semared and allowed to dry. Hematoxylin and Eosin staining was performed after deparafiinization. These slices were dip into incresing concentrations of ethanol i.e 95% ethanol in 5 min, in 70% ethanol in 5 min. Slide clean up with running water in 5 min. Harris hematoxylin staining in 5 min, running water for 5 min. Dip into alcohol acid twice. Running water for 5 min. Dip into ammoni water once. Eosin staining for 5 min. Dip into 95% ethanol, twice, 100% ethanol twice, and xylol for 5 min.

2.5. Masson Trichrome staining

The 5 μm slices were deparaffinized by immersion into xylene. Slide hydration with aqua destilation. Fixaxi with Bouin (hematoxylin) for 1 hour, 56 ℃ or save overnight in room temperature. Dish plate was closed, be cold for 5-10 min. Wash with running water until no colour in the slide. Hematosylin iron Weigert staining for 10 min and wash with running water for 10 min. Phosphomolybdic-phosphotungstic staining for 10-15 min. Tranfer aniline blue solution for 10-20 min. Wash with running water. Dehydration was performed using acetic solution 1%.

2.6. Density of Collagen

Masson's trichrome staining slide were taken picture with Nikon Eclips 80i. Density of collagen measure with histogram in Image J program**.**

2.7. Analysis Design

Skin histoopatohological changes visualized by HE, and Masson's Trichrome staining were analyzed.

3. Results

The observation of changes on this study based on skin thickness and biopsy analyzed. This study used 27 Balb/C mice, which divided by 3 group. Grup A (@9 Balb/C mice) were given UV irradiated 60 mJ/cm^2, five times a week, in sixteen weeks and given ADMSCs 1x 104 at week 17th, 18th, and 19th. One week after final injection of ADMSCs, replicas of the dorsal skin surface were taken. Grup B (@9 Balb/C mice) were given UV irradiated 60 mJ/cm^2, five times a week, in sixteen week and placebo (NaCl 0.9%) at week 17th, 18th, and 19th. Grup C (@9 Balb/C mice) were given no UV irradiated and no treatment. All datawas analyzed with SPSS 17 program.

Table 1 and Table 2 showed no significant difference in skin thickness (mm) and dermis thickness(m) before and after given ADMSCs. There was significant difference epidermis thickness and density of Collagen.

Table 1. Respon Change before anda after given ADMSCs, Grup A.

		Mean	N	Std. Deviation	Std. Error Mean
Skin thickness (Caliper digital)	Skin Thickness pre-treatment (mm)	2.416	9	.218	.073
	Skin thickness post-treatment(mm)	2.627	9	.293	.098
Dermis	Dermis thickness pre-treatment (µm)	185.967	9	39.552	13.184
	Dermis thickness post-treatment(µm)	192.464	9	49.854	16.618
Epidermis	Epidermis thickness pre-treatment(µm)	41.364	9	18.775	6.258
	Epidermis thickness post-treatment(µm)	24.568	9	7.853	2.618
Kolagen	Density Colagen pre-treatment (pixel)	133.578	9	5.746	1.915
	Density Colagen post-treatment (pixel)	163.008	9	4.900	1.633

Table 2. There was significant or no significant at table 1, Grup A.

		Paired Differences					t	df	Sig. (2-tailed)
		Mean pretreat-Mean post	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Ketebalan kulit	Ketebalan kulit pre-treatment - Ketebalan kulit post-treatment	-.211	.472	.157	-.574	.152	-1.341	8	.217
Dermis	Dermis pre-treatment - Dermis post-treatment	-6.498	46.768	15.589	-42.447	29.451	-.417	8	.688
Epidermis	Epidermis pre-treatment - Epidermis post-treatment	16.797	17.404	5.801	3.419	30.175	2.895	8	.020
Densitas Kolagen	Kolagen pre-treatment - Kolagen post-treatment	-29.430	7.385	2.462	-35.107	-23.753	-11.955	8	.000

Pretreatment and postreatment said significant if p<0.05 (yellow)

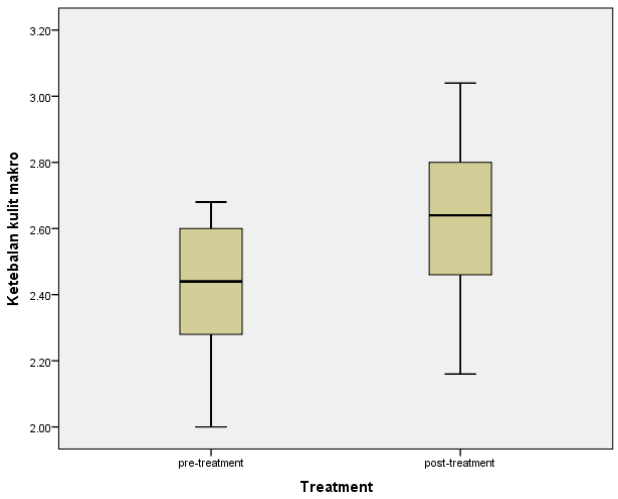

Figure 1. Evaluation of Skin thickness before and after given ADMSCs (mm) with Calliper digital

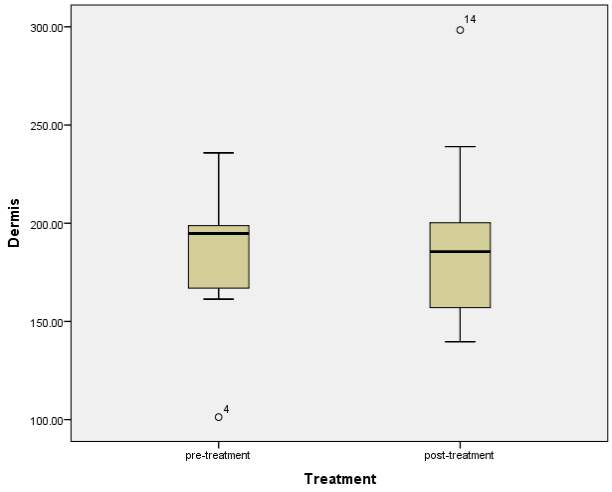

Figure 2. Evaluation of Dermis thickness (µm) before and after given ADMSCs

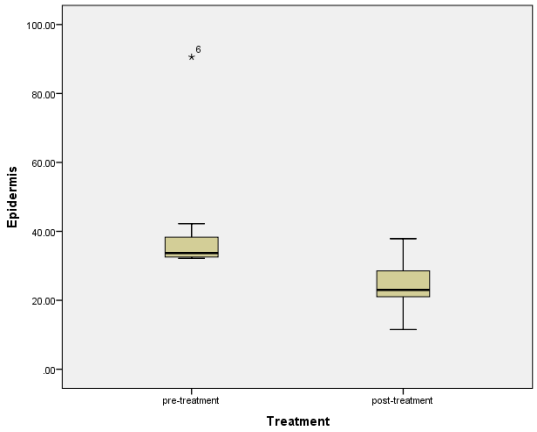

Figure 3. Evaluation of Epidermis thickness (µm) before and after given ADMSCs

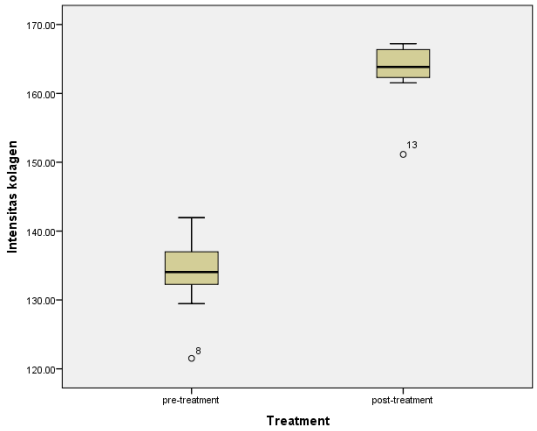

Figure 4. Density collagen before and after ADMSCm injection (pixel)

Figure 5 shows that epidermis thickness decreased after week 20. Epidermis thickness decrease in grup A (stem cells), grup B (placebo) and Grup C (control).

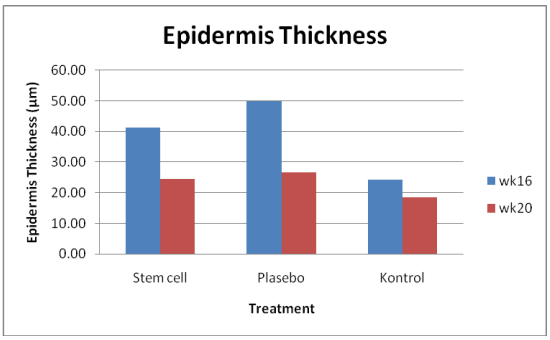

Figure 5. epidermis thickness Grup A(ADMSCs), Grup B (plasebo) and Grup C (control)

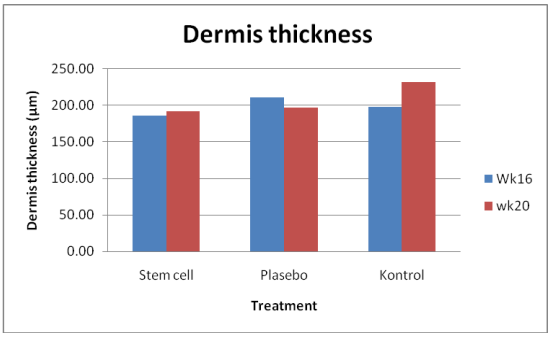

Figure 6. Dermis Thickness in grup A (stem cell), grup B (Placebo) and grup C (control)

Dermis thickness shows at grup A, after injection ADMSCs and control, but dermis thickness decrease in grup B (placebo).

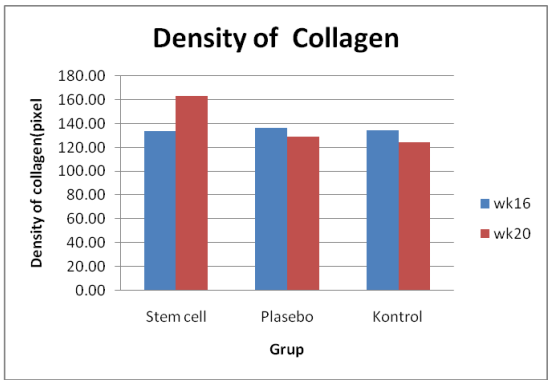

Figure 7. showed dencity of collagen in grup A (stem cell) increased. In Grup B dan C, density of collagen decrease.

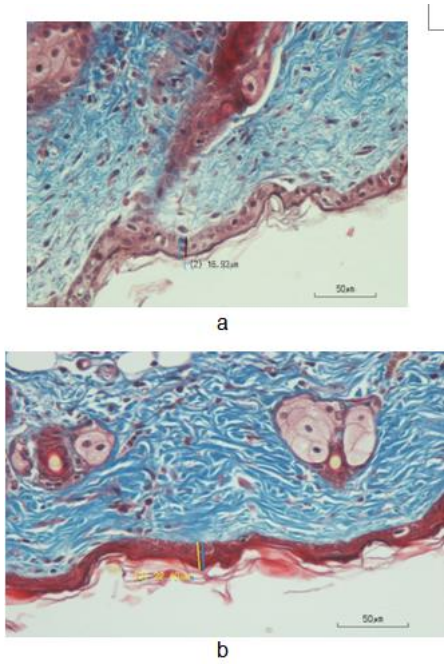

Figure 8. Masson's Trichrome staining, before (a) and after(b) injection ADMSCs showing increasing density of collagen after injection ADMSCs.

4. Discussion

Photoaged skin is characterized by coarse wrinkle. Loss of elasticity, pigmented spots, dryness, verrucous papules and telangectasia. The age at onset and expression of these photoaged characteristics appear to differ between racial phenotypes or pigmentary group. Histopathologically, photoaged skin, there can be a significant increased disorganization of fiber-proteins.

The observation of microscopical changes on UVB irradiated and unirradiated control groups mice skin were performed weekly in pilot study. Based on macroscopic observation of skin UVB irradiated for 16 weeks, compared to those of the nonirradiated control group.

The skin of control mice appeared thin, elastic and smoth meanwhile in mice irradiated showed alterations in their skin which varied from redness, thickening, wrinkles, toughness.

This study, uvb radiation 60 mJ/cm2, five times a week for sixteen weeks make skin photoaging in Balb/C model. Photoaging measure with there was incresed thickness of the skin, thickness of epidermis, and decrease thickness of dermis and density of dermis. Dose and time for UVB irradiation in this study can be model for skin photoaging, which was injection with ADMSCs.

From these results shows that there was decrese of epidermis thickness after injection ADMSCs. Epidermis thickness decrease was significant in statistic (p=0.02). Epidermis thickness decrease in grup B (NaCl 0.9%). The conclusion was ADMSCs was repair the epidermis, but same with NaCl 0.9%. Epidermis will repair itself after we stop irradiation UV.

Furthermore there was increase of dermis thickness after injection ADMSCs but it was no significant in statistic (p=0.688). The dermis thickness decrease in Grup B (NaCl 0.9%a).

Also, there was signicifant increase density of collagen after injection ADMSCs compare grup B (NaCL 0.9%) and grup C (control). Injection of ADMSCs 1x 104 cells increase sinthesis collagen but no significan effect in the thickness of epidermis and dermis.

In conclusion of this study revealed that UV irradiated skin macroskopically underwent histological structure alteration. Epidermis is hiperplasia, hiperkeratosis. Microscopically alteration observed was the reduction of collagen. Uv irradiation in sixteen weeks and given injection ADMSCs make alteration increasing density of collagen. But no significan influence of the thickness of epidermis and dermis. In the skin

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