

## EFFECT OF HUMAN ADIPOSE-DERIVED STEM CELL IN COLLAGEN GEL ON RELATIVE EXPRESSION LEVEL OF VASCULAR ENDOTHELIAL GROWTH FACTOR-A OF DEEP DERMAL BURN HEALING

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### ABSTRACT

**Objectives:** The objectives of this research was to measure the relative expression levels of vascular endothelial growth Factor-A (VEGF-A) in the deep dermal burns treated with human adipose-derived stem cell (hADSC) in collagen gel in each observational day (days 7, 14, 21, and 28).

**Methods:** This study used 20 male Sprague Dawley rats, divided into four groups of observation days. Each rat received three burn injuries and then given different treatments (hADSC in collagen gel, collagen gel, and control). Deep dermal burn injury on the dorsal was made by placing a metal plate with 250°C for 15 s. The relative expression of mRNA VEGF-A was determined by RT-qPCR technique and calculated using  $2^{-\Delta\Delta Ct}$ .

**Results:** On the 7<sup>th</sup> day, the relative expression level of VEGF-A in the wound treated with hADSC in collagen gel was significantly different from the collagen gel and control group ( $p < 0.05$ ), whereas the control and collagen gel group was not significantly different. The relative expression level of VEGF-A in wound treated with hADSC in collagen gel, collagen gel only, and control was (mean  $\pm$  standard error of the mean)  $17.93 \pm 4.37$ ,  $7.54 \pm 2.63$ , and  $5.44 \pm 1.59$ , respectively. On the next observation days, the result showed that the relative expression level of VEGF-A was not significantly different between the three treatments. The relative expression level of VEGF-A has decreases from day 7 to 28 days. The decrease of VEGF-A relative expression level hADSC in collagen gel group was significantly different on the 7<sup>th</sup> day to the 21<sup>st</sup> and 28<sup>th</sup> days ( $p < 0.05$ ).

**Conclusion:** The provision of hADSC in collagen gel increases the relative expression of VEGF-A early in the wound healing process compared to the without a hADSC group.

**Keywords:** Angiogenesis, Burn injury, Human adipose-derived stem cell, Vascular endothelial growth Factor-A.

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### INTRODUCTION

Burns are a form of damage and or loss of tissue due to contact with sources that have very high temperatures, chemicals, electricity, radiation, or very low temperatures [1]. Burns become a global health problem because it is one of the main causes of morbidity and mortality. Based on the WHO database, in 2016, is estimated about 265,000 people die from burns each year. Approximately 90% of cases of burns occur in developing countries. The highest incidence occurred in Africa and Southeast Asia [2]. Burn wound healing is a complex process consisting of an early phase of abrupt energy depletion and necrosis, followed by inflammatory phase, delayed cell death, formation of granulation tissue, matrix formation, and remodeling [3]. The wound healing process begins immediately after injury by releasing various molecular compounds from the blood vessels [4]. Angiogenesis on burn healing process is indispensable because the blood vessels carry oxygen and nutrients to the damaged tissues, carrying immune cells, as well as preparing wound areas for tissue regeneration and repair [5]. There are three growth factors, which play an important role in the formation of blood vessels. They are fibroblast growth factor, vascular endothelial growth factor (VEGF), and angiopoietin-1. VEGF signals are essential for the development of the endothelial and hematopoietic cell. Proliferation and migration of endothelial cells are a marker of the formation of new blood vessels. VEGF is a growth factor that maintains the survival of endothelial cells, angiogenesis inducers, and preserves vascular permeability [6,7]. VEGF proteins include VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. Among these, VEGF-A is the most important protein and plays an important role in the migration and

proliferation of endothelial cells [6,8,9]. VEGF proteins are secreted by various cell types such as keratinocytes, smooth muscle cells, platelets, neutrophils, macrophages, and fibroblasts, including endothelial cells [10]. Until now, the autograft method is a "gold standard" to replace damaged skin organs in massive burns. The limited source of skin organs from the same individual becomes a problem, especially in massive burns [11]. The application of stem cell therapy is expected to be an efficient solution in the treatment of burns [12,13]. Mesenchymal stem cells can be isolated from adult tissue such as cord blood, bone marrow, and fat tissue [14-16]. Stem cells from fatty tissue commonly abbreviated adipose-derived stem cell (ADSC) can produce from waste liposuction patients in beauty clinics or plastic surgery. Transplantation using mesenchymal stem cells in the treatment of burns is require the material as a scaffold to avoid stem cells flowing and wasting away. This material is used to support ADSC. Collagen is an important component in the formation of granulation tissue during the wound healing phase [17]. Human ADSC (hADSC) administration is expected to increase VEGF-A secretion in the healing process of burns.

### METHODS

#### Study design

The research protocol has been approved by the Medical Research Ethics Committee FKUI/RSCM, with the protocol number: 17-03-0261. This research was conducted at Animal Laboratory of Research and Development Center of the Indonesian Ministry of Health, Culture Laboratory of Biology of Medicine - Department FKUI, and Molecular Biology Laboratory of Biology of Medicine - Department FKUI.

### Animal models

The samples were randomized male Sprague Dawley rats, 10 weeks old. Based on the calculation of the number of samples, this study used 20 rats with 5 rats for each group. Animal models to be adapted in the laboratory for 1 week, kept in 1 rat in 1 cage with underpad base. Meals and drinks were given on *ad libitum*. The day before the rat treatment was given paracetamol (15 mg/kg BW) dissolved in drinking water, as an analgesic, the administration was continued for 1 week. On the day of rat treatment anesthetized using a combination of ketamine (85 mg/kg BW) and xylazine (10 mg/kg BW) intraperitoneally [18-20] Each rat will get three burns on dorsal area. The wound is made parallel to the line of the mouse vertebra with a distance of 1 cm to the right and left side of the vertebral bone; 0.5 cm below the bone of the scapula, and 2 cm of vertical wound distance. The metal plate with a temperature of 250°C was placed for 15 s to make deep dermal burn injury on the dorsal. Every day wounds were observed. The wound on the skin will appear round-shaped with a diameter of 10 mm. The same step was carried out so that there were three burns for each rat.

### hADSC transplantation

A total of  $1 \times 10^6$  hADSC in collagen gel were administered on the 3<sup>rd</sup> wound. 100  $\mu$ L collagen gel given on 2<sup>nd</sup> wound and 1<sup>st</sup> wound not given any treatment (control).

### Isolation of RNA from skin tissue burns

All wounds were daily observed until the sacrificial day of the rat (days 7, 14, 21, and 28). RNA from the sample tissue was isolated using the total RNA Mini Kit (tissue). The tissue samples were taken 20–25 mg and fed into a 1.5 mL microtube containing 400  $\mu$ L RB Buffer and 4  $\mu$ L  $\beta$ -mercaptoethanol, then homogenized using micropestle until the tissue was destroyed. Transfer the homogenate into a filter column placed on a 2 mL tube, then centrifuged at 1000 rpm for 1 min. The filtrate that passes through the filter column will be accommodated in a 2 mL tube, then transfer the filtrate to a 1.5 mL microtube add 400  $\mu$ L 70% ethanol then homogenized.

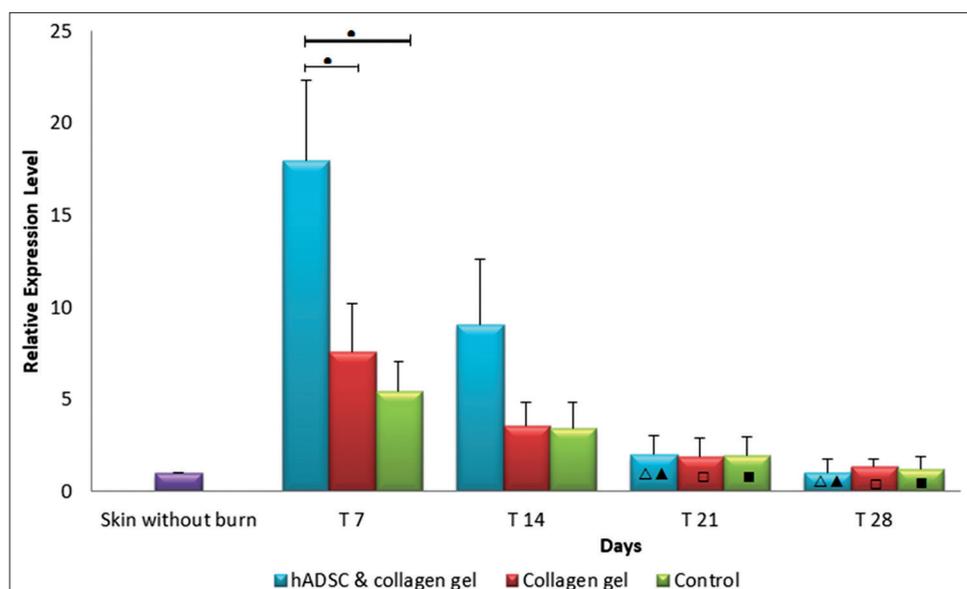
The mixture transferred to an RB column, which has been placed on a 2 mL tube and centrifuged at 12,000 rpm for 3 min. The supernatant passing through the RB column was removed, and the RB column was placed back on the 2 mL tube. Subsequently, 400  $\mu$ L wash buffer was added to RB column and centrifuged at 12,000 rpm for 3 min. The supernatant passing through the RB column was removed, and the RB column was placed back on the 2 mL tube. Then, add 50  $\mu$ L DNase I solution to the middle of RB column and incubate for 15 min at room temperature (20–30°C).

The next step, after incubation 15 min, added 400  $\mu$ L W1 Buffer to RB column and centrifuged at 12,000 for 2 min. The supernatant passing through the RB column was removed, and the RB column was placed back on the 2 mL tube. Then, add 600  $\mu$ L wash buffer to RB column and centrifuged at 12,000 rpm for 2 min. The supernatant passing through the RB column was removed, and the RB column was placed back on the 2 mL tube. Perform once again giving 600  $\mu$ L wash buffer to RB column and centrifuged at 12,000 rpm for 2 min. The supernatant passing through the RB column was removed, and the RB column was placed back on the 2 mL tube. Then to dry the column matrix, the RB column was centrifuged again at 12,000 rpm for 4 min.

Next step, remove RB column from 2 mL tube and place RB column on sterile 1.5 mL microtube. Add 50  $\mu$ L RNase-free water to the center of the matrix column and incubate for 3 min to ensure that RNase-free water was absorbed by the matrix then centrifuged at 12,000 rpm for 3 min to elute the purified RNA. The concentration and purity of RNA were calculated using nanodrop, taken as much as 2  $\mu$ L. Total RNA was stored at -80°C for a long time.

### Synthesis of cDNA

Furthermore, RNA was synthesized into cDNA. All materials for this procedure should be thawed and spin down before working on ice (0–4°C). The first step was to make the Mixture I (DNase I reaction solution), the RNA template used as 6  $\mu$ L mixed with 2  $\mu$ L 4x DN Master Mix, then incubated at 37°C for 5 min using thermal block. After that,



**Fig. 1:** Average relative expression of vascular endothelial growth Factor-A (VEGF-A). On the 7<sup>th</sup> day of observation, the relative expression of VEGF-A in skin lesions administered by human adipose-derived stem cell (hADSC) in collagen gel was higher than the collagen gel and control group ( $p < 0.05$ ). The 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> days of observation resulted in a relatively different level of VEGF-A expression.  $\Delta$  Indicates statistically significant difference compared with burn treated hADSC in collagen gel at the 7<sup>th</sup> day ( $p < 0.05$ );  $\blacktriangle$  Indicates statistically significant difference compared with burn treated hADSC in collagen gel at the 14<sup>th</sup> day ( $p < 0.05$ );  $\square$  indicates statistically significant difference compared with burn treated collagen gel at the 7<sup>th</sup> day ( $p < 0.05$ ); and  $\blacksquare$  Indicates statistically significant difference compared with control at the 7<sup>th</sup> day ( $p < 0.05$ )

add  $\times 5$  RT master Mixture II and incubate at  $37^{\circ}\text{C}$  for 15 min,  $50^{\circ}\text{C}$  for 5 min, and  $98^{\circ}\text{C}$  for 5 min. There was found a solution of cDNA with a final volume of  $10\ \mu\text{L}$ . The cDNA solution was stored at  $-20^{\circ}\text{C}$  for storage in the long term.

#### VEGF gene expression test

The material used for the measurement of the relative expression of VEGF-A gene mRNA is THUNDERBIRD<sup>®</sup> SYBR<sup>®</sup> quantitative PCR (qPCR) mixture. The primer used is specially designed to obtain the primary target gene sequence as shown in Tables 1 and 2:

The volume of each reactant is  $20\ \mu\text{L}$  with components of  $4.4\ \mu\text{L}\ \text{H}_2\text{O}$ ,  $0.6\ \mu\text{L}$  primary forward, reverse primer,  $0.4\ \mu\text{L}\ \times 50$  ROX reference dye,  $10\ \mu\text{L}$  THUNDERBIRD<sup>®</sup> SYBR<sup>®</sup> qPCR master, and  $4\ \mu\text{L}$  cDNA template. The solution fed into the Lightcycler capillary. Next, centrifuge at 3000 rpm for 15 s. The solution fed into the polymerase chain reaction PCR machine by following the cycle protocol.

#### Statistical analysis

Statistics analysis used SPSS computer software for Windows (Version 17.0, SPSS Inc., Chicago, IL). The relative expression of VEGF-A in the wound that given hADSC in collagen gel, collagen gel, and control at 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> day was analyzed by one-way ANOVA. The decrease in VEGF-A relativity expression from 7<sup>th</sup> to 28<sup>th</sup> days in various treatments was analyzed by one-way ANOVA. Statistically significant values were defined as  $*p < 0.05$ .

### RESULTS

#### Comparison of the relative expression level of VEGF-A

At the 7<sup>th</sup> day observation, there are significant differences between hADSC in collagen gel group with control and collagen gel group ( $p < 0.05$ ), while between control group with collagen gel group was not significant different ( $p > 0.05$ ). The average relative expression level of VEGF-A in the hADSC group in collagen gel was higher than the collagen gel and control group. On 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> days observation, there were no significant differences between the three treatments. On the day 14 of observation, the relative expression level of VEGF-A of the hADSC in collagen gel group was higher than control and collagen gel group. The average relative expression level of VEGF-A on all treatments at days 21 and 28 is almost as same (Fig. 1).

#### Decrease in relative expression level of VEGF-A by observation days

The relative expression level of VEGF-A was analyzed on 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> days. In all treatments group, there was a decrease in the relative expression level of VEGF-A from day 7 to 28. Calculation of relative expression was done using Livak method. Hence, the average relative expression (mean  $\pm$  standard error of the mean [SEM]) of VEGF-A mRNA on the 7<sup>th</sup> day by giving hADSC in collagen gel  $17.93 \pm 4.37$ ; collagen gel  $7.54 \pm 2.63$  and controls  $5.44 \pm 1.59$ . There was a decrease in VEGF-A mRNA expression the following day. On the day 14, an average expression of VEGF-A mRNA was obtained on wound treated with hADSC in collagen gel of  $9.08 \pm 3.53$ ; collagen gel  $3.57 \pm 1.27$ ; and controls

$3.54 \pm 1.36$ . Furthermore, at the day 21, the expression of VEGF-A mRNA in all three treatments was seen to be almost as same. Expression of VEGF-A mRNA in wound given hADSC in collagen gel  $2.00 \pm 1.02$ ; collagen gel  $1.88 \pm 1.00$ ; and control  $1.94 \pm 1.00$ . On the day 28, there was also a decrease in VEGFA mRNA expression from the previous day in all three treatments. Expression of VEGF-A mRNA in hADSC in collagen gel  $1.03 \pm 0.71$ ; gel collagen  $1.38 \pm 0.37$ ; and control  $1.19 \pm 0.68$ .

### DISCUSSION

In this study, VEGF-A mRNA expression examination started on the 7<sup>th</sup> day after burn. The results showed an increase in VEGF-A mRNA expression on the 7<sup>th</sup> day compared to calibrator (VEGF-A mRNA expression in normal skin-no burns). This can be caused of the effect of hADSC in the collagen gel on the burn tissues by paracrine mechanisms. In addition, the increased expression of sharp VEGF-A mRNA on the 7<sup>th</sup> day was also influenced by the activity of inflammatory cells producing VEGF-A. VEGF is produced by many types of cells that participate in the healing of burns such as keratinocytes, mast cells, macrophages, fibroblasts [10], endothelial cells, smooth muscle cells, platelets, and neutrophils. VEGF secretion by these cells is induced by the hypoxic conditions that occur due to burns [10,21].

VEGF-A mRNA expression subsequently decreased from the 14<sup>th</sup> to 28<sup>th</sup> day. On the 21<sup>st</sup> and 28<sup>th</sup> days, VEGF-A mRNA expression was almost same to the value of the calibrator. This is because the condition of wound tissue is approaching the homeostatic state. The pattern of decreased expression of VEGF-A mRNA on the 7<sup>th</sup> to 28<sup>th</sup> day in this study is similar to Brown *et al.* study. The results of Brown *et al.* showed increased VEGF on the 3<sup>rd</sup>-7<sup>th</sup> day after wound injury. Then there was a decrease in the 13<sup>th</sup> day and after 3 weeks did not increase compared to normal [21].

The comparison of expression of VEGF-A mRNA in each treatment showed a significant difference between hADSC in collagen gel with collagen gel and control at the 7<sup>th</sup> day. The expression of VEGF-A mRNA in burn tissue treated with hADSC in collagen gel was higher than without hADSC. This suggests that there is an hADSC in the collagen gel roles to the burn tissue. There are several possible mechanisms for the effects of hADSC on these burns. The provision of hADSC has a direct effect on the damaged tissue by differentiating it into vascular endothelial cells, and VEGF transcription becomes more numerous. hADSC may also give paracrine effect to cells surrounding wound thus affecting rat cells to increase VEGF transcription. Then, the presence of collagen gel can also act as a medium conducive for maintaining cells or tissue exposed hot can survive.

This is reinforced by the results of research conducted by indicating that ADSC administration to the wound showed an activated fibroblast phenotype and resulted in the recruitment of endothelial cells and macrophages increased in the wound area [22,23]. It is known that the cells play a role in VEGF secretion. Therefore, it is possible that hADSC has a paracrine effect on rat niche cells thus affecting the amount of VEGF secretion during the wound healing phase. In addition, other studies showed that allogeneically administratin of stromal vascular fraction increases VEGF secretion on the 10 day, VEGF secretion in the treatment group was higher than control [24].

The results of the study of VEGF-A mRNA expression in burns treated with hADSC in collagen gel on the 14<sup>th</sup> day have shown no significant difference compared with collagen gel and control. However, it still has a higher expression value in treatment with hADSC in collagen gel (mean  $\pm$  SEM), hADSC in collagen gel =  $9.08 \pm 3.53$ ; collagen gel =  $3.57 \pm 1.27$ ; and control treatment =  $3.54 \pm 1.36$ . This suggests that until the 14<sup>th</sup> day,

Table 1: Primer of mRNA VEGF A

Primer	Sequences (5'→3')
Forward primer VEGF A	TGGACCCTGGCTTTACTGCT
Reverse primer VEGF A	CTTCACCACTTCATGGGCTTTC
Forward primer actin beta	GATCTGGCACCCACACCTTCT
Reverse primer actin beta	GGGGTGTGAAGGTCTCAA

VEGF: Vascular endothelial growth factor

Table 2: Cycling protocol

Program	Pre-incubation	Amplification			Cooling
Target ( $^{\circ}\text{C}$ )	95	95	57	72	12
Hold (hh:mm:ss)	00:03:00	00:00:30	00:00:30	00:01:00	00:05:00

hADSC still give the influence to burn tissue, but it has less effect. On the 14<sup>th</sup> day, it is estimated to have entered the proliferation phase. The inflammatory cells that secrete much VEGF early in the healing period may have decreased. On the 21<sup>st</sup> and 28<sup>th</sup> days, VEGF-A mRNA expression in all three treatments was no significant difference. The condition of the burned tissue has reached homeostatic conditions, so there is no induction of VEGF secretion as in hypoxic conditions like in the beginning wound healing process.

Then, collagen gel provides a three-dimensional (3D) structure for hADSC to remain in the wound area and help the inflammatory cells to get to the wound area early in the injury time. In this study, there is no examination by an electron microscope to see the collagen 3D gel structure. Even in the process of wound healing, there is also an extracellular matrix that will provide an adhesion framework for the cell to organize the growth, movement, and differentiation of cells in it [25]. A study by Karimi *et al.* showed no significant differences in wound healing parameters observed among groups given ADSC by injection with untreated groups [26]. studies that use biomaterials to created 3D structures showed better wound healing result [27,28]. This means that the use of collagen gels as the scaffold is required in administration hADSC to the wound tissue.

## CONCLUSION

The provision of hADSC in collagen gel increases the relative expression of VEGF-A in the early of wound healing process compared to the without hADSC. There was a decrease in the relative expression level of VEGF-A on the 7<sup>th</sup> day until the 28<sup>th</sup> day.

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## CONFLICTS OF INTEREST

All authors have none to declared.

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