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Effectivity of Nano herbal Andaliman (Zanthoxylum acanthopodium) to the Vascular Endothelial Growth Factor (VEGF) expression in burn wound in diabetic rats

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***Abstract*— Diabetes mellitus (DM), commonly known as a metabolic disease, is characterized by hyperglycemia caused by the inability of the pancreas to secrete the hormone insulin, impaired insulin action, or both. Hyperglycemia will usually increase Reactive Oxidative Stress and trigger oxidative stress so that it can interfere with every phase of wound healing. Vascular Endothelial Growth Factor (VEGF) plays a role in the angiogenesis process in providing nutrition to wound tissue. One of the natural herbal plants that has the ability to heal burns in DM patients is Andaliman (Zanthoxylum acanthopodium). This is because Andaliman contains terpenoids, flavonoids, tannins, and saponins. This study aims to determine the effectiveness of Andaliman nano herbal against VEGF protein during the healing process of burns in diabetic rats. In this study, Experiments were designed into two groups of burn rats: untreated and treated with Andaliman nano herbal. Treatment and surgery were performed on the days of 0, 4, 8, 12, and 16, respectively. Furthermore, the VEGF protein expression using immunohistochemical staining was analyzed. The results showed (Kruskal-Wallis test) that administration with the Andaliman nano herbal was highly effective in increasing VEGF expression from the day of 4 to day of 16 ($p<0.05$, $p=0.000$). It can be concluded that the nano herbal Andaliman is highly effective in increasing the expression of VEGF during the healing process of burns in diabetic rats.**

Keywords*— *Nano herbal Andaliman, Burn Wound, Diabetes Mellitus, VEGF

I. INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia due to increased levels of glucose in the blood that occurs due to the inability of the pancreas to produce insulin, impaired insulin action, or even both. Lack of insulin or the inability of the body's cells to respond to the hormone insulin causes hyperglycemia. Fasting blood glucose level which higher than 126 mg/dL or random blood glucose level up to 200 mg/dL is considered Diabetes Mellitus [1].

Diabetes mellitus can inhibit the wound healing process. Hyperglycemia conditions can increase Reactive Oxidative Stress (ROS) and trigger oxidative stress that interferes with every phase of wound healing, namely the inflammatory phase, the proliferative phase, and the maturation/remodeling phase which have long-term effects on morbidity, mortality, and quality of life [2].

Vascular Endothelial Growth Factor (VEGF) plays an important role in wound healing and tissue repair. In this case, VEGF plays a role in inducing endothelial cell migration and inflammation in the wound area. It also plays a role in the process of forming collagen which is needed for the wound healing process [3].

Diabetes-related mortality and morbidity are associated with hyperglycemia and impaired wound healing. Many synthetic drugs that are devoted to the treatment of diabetic wounds are expensive and can trigger allergies or resistance, so the search for new drug alternatives is very important and needed [4].

Indonesia has various kinds of plants that are efficacious as medicine, one of which is Andaliman (Zanthoxylum acanthopodium). Several studies have shown that andaliman contains alkaloids, flavonoids, triterpenoids, cardiac glycosides, saponins, and tannins [5-8]. Andaliman has antioxidant, antimicrobial, and immunostimulant properties [9-11]. Flavonoids also have anti-inflammatory effects and help protect blood vessel walls [12].

Any changes that occur in the nanoscale size greatly contribute to changes in physicochemical properties in relation to increasing the efficacy of drug molecules. Based

on this, Andaliman is processed into smaller particle sizes, namely nanoformulation. Nanoformulated herbal medicine (Nano herbal) is expected to be more effective in the absorption process, thus providing greater benefits due to its administration in high concentrations to obtain maximum results [13].

VEGF plays an important role in stimulating the formation of new blood vessels (angiogenesis) and increasing their permeability. The process of angiogenesis also takes part in the process of tissue repair to provide nutritional cofactors involved in tissue regeneration. Increased levels of VEGF during the wound healing process will stimulate neoangiogenesis and will directly increase MMP-1, TIMP, and MMP-2 secretion from endothelial cells and MMP-1, MMP-2, MMP-9 secretion from vascular smooth muscle [14].

The purpose of this study was to identify the effectiveness of the administration of Andaliman nano herbal on the expression of Vascular Endothelial Growth Factor (VEGF) protein in diabetic white rats (Rattus norvegicus L.) exposed to burns. Experimental observations were made on days 0, 4, 8, 12, and 16 during the wound healing process. Research with the proposed method, which is based on nano herbal Andaliman, is still very rarely found.

II. Material and Method

A. Materials and tools used

In the experiment, 50 healthy and active adult male Wistar rats (Rattus norvegicus L.) (aged 8-11 weeks) weighing 150-200g (obtained from animal cages at the Faculty of Mathematics and Natural Sciences, North Sumatra University, Medan) were selected as animals test. The number of repetitions test was calculated based on the Federer formula: (t-1) (n-1) ≥15. Standard pellet feed which produced by PT. Charoen Pokphan CP551 Medan is also used as food. Alloxan Monohydrate was used to increase blood glucose levels completely intraperitoneally at a dose of 125 mg/kg BW to the lower abdomen of rats. As for anesthesia, Ketamine-HCl is injected intramuscularly at a dose of 50 mg/kg BW. About 0.9% NaCl physiological fluid was used to clean the wound while Polyclonal Antibody Vascular Endothelial Growth Factor was used as VEGF antibody. For reference support, Catalog No.PAA143Ra01, Brand: Cloudclone. 23603 W. Fernhurst Dr., Unit 2201, Katy, TX 77494, USA and the nano herbal andaliman was selected. Several tools used in this study were digital scales, autocheck GluCare®, iron plate (1 x 1.5 cm), mouse cages were generally made of plastic, lined with woven wire, while the bottom of the cage was lined with rice husks and a light microscope.

B. Research Location

This research was carried out at the Laboratory of Animal Physiology, Department of Biology, FMIPA, USU, and the Laboratory of Anatomical Pathology, Faculty of Medicine, USU, Medan. Any scientific use of animals has received ethical clearance from the Animal Research Ethics Committee, Faculty of Mathematics and Natural Sciences, University of North Sumatra.

C. The Manufacturing process of Nano herbal

Andaliman was obtained from traders coming from Dairi Regency, North Sumatra. This botanical nomenclature was determined based on plant experts' analysis from the The Herbarium, Biology Department laboratory at the University of North Sumatra. The fruit part of Andaliman had been washed, cleaned, and dried at room temperature for 1-2 weeks before the experiment. Nano herbal andaliman was obtained from the High Energy Milling (HEM) at the Indonesian research institute (LIPI, Jakarta). The procedure of nanosizing herbal andaliman was balls function as a crushing medium into a larger diameter jar. HEM was turned on for 2 hours [15]. The particle size of nano herbal Andaliman was analyzed using Particle Size Analyzer/PSA (diameter: 700-800 nm) [6]. Nano herbal andaliman was given at a dose of 100 mg/kg BW [6].

D. Research design

In this study, a completely randomized design (CRD) was used. The experiment is divided into two groups, i.e., the first group (K1), identified as rats' burn wound without treatment, and the group (K2), as the second group identified as rats' burn wound treated with nano herbal andaliman. Both groups were observed on the 0, 4, 8, 12, and 16 days.

E. The Induction of diabetes mellitus in rats

Before administering Alloxan injection, initial measurement of Blood Glucose Level was carried out in experimental animals. Then, the rats were intraperitoneally injected with Alloxan Monohydrate at a dose of 125 mg/kg BW around the abdominal area. In the next day, when another measurement of Blood glucose Level was carried out for the second time, the blood glucose level had reached 200 mg/dL, and the rats were declared Diabetic [1]. Measurement of blood glucose level was carried out from the tail vein, i.e., the lateral vein, using an auto check GluCare® measuring device.

F. The process of creating and treating the burn wound

Before creating a burn wound, the rats' fur around the lateral area of the back (about 3 cm from below the ear) was shaved and disinfected with alcohol. The Rats were anesthetized using Ketamine-HCl at a dose of 50mg/kg Body Weight intramuscularly. Burn wounds were created using a heated iron plate (1.5 cm x 1 cm) placed on the back of the rat for about 1-2 seconds and then removed immediately [15]. The wound is treated according to the treatment of K1 or the "without treatment", then the wound was covered with sterile gauze pads and then plastered. The K2 was smeared with nano herbal andaliman at a dose of 20 mg dissolved with 0.5% CMC Na. Similarly, the wound was covered with sterile gauze pads. Wound treatment was carried out every day based on the agreed observation duration, starting from day 1 until day 16.

The wound was first cleansed with a swab that had been premoistened in 0.9% normal sterile saline. Furthermore, based on each experimental group, the treatment was carried out, the wound was covered with sterile gauze pads, and plastered to prevent dirt or husks from entering the wound area.

G. Surgery

The surgical process on experimental animals was performed on the 0, 4, 8, 12, and 16 days. Experimental animals were first anesthetized with ketamine at a dose of 50 mg/kg BW. Based on the observation days in each treatment group, a cervical dislocation termination was then executed to collect skin tissue from experimental animals. The surgery performed on the 0^{th} day was the wound formation day, surgery performed on the 4, 8 and 12 and 16 days were the homeostasis and inflammatory phase, proliferative phase, and the maturation phase/remodelling during the whole process of wound healing, respectively.

As for the preparations, skin tissue with a size of 1.5 cm x 1.5 cm was vertically incised until reaching the hypodermic skin layer. The skin tissue preparations were then analyzed using immunohistochemical methods to analyze the VEGF protein expression in the rats' burn wound tissue.

H. Immunohistochemistry

Immunohistochemistry was carried out at the Anatomical Pathology Laboratory, Faculty of Medicine, University of North Sumatra. Histological preparations in the form of paraffin-embedded tissue blocks were cut into 4µm sections using a microtome. The object-glass was first coated with poly lysine then the tissue was deparaffinized in xylene for 2 x 15 minutes. Rehydration was carried out using Alcohol in decreasing concentrations, once for every 2 x 2.5 minutes, and then washed with Tris-buffered (TBS-buffer) saline for 3 hours and 5 minutes. Antibody solutions were prepared with optimized dilution. For around 10-20 minutes, the Antigen retrieval protocol was performed in a decloaking chamber at a high temperature (105°C). Antigen retrieval was performed by having them rehydrated and heated in citrate buffer for 5 minutes (in the microwave) and then washed for 5-minutes using phosphate-buffered saline tween 20 (PBST) three times. A secondary antibody (biotinylated link universal) was exuded into the tissue for 30 minutes after washing. After that, for 5 minutes, it was washed with PBST three times before streptavidin HRP was exuded for 30 minutes, and finally washed again for 5 minutes using PBST three times. Chromogen 3,3'-diaminobenzidine (DAB) was exuded onto the tissue, after that, it was left for 15 seconds and counterstained using Mayer's haematoxylin for 25 seconds. The tissue was then washed with distilled water, dehydrated using graded alcohols and, cleared in xylene before finally getting covered with a cover glass.

All experimental results obtained from the immunohistochemical technique such as the number of positive cells were counted by agreeing with the first and the second observers which were considered positively stained using the Interobserver agreement method. Observations were performed at each preparation using ± 10 field-of-view and cells were also calculated in skin tissue that is antibody-positive [16]

I. Data analysis

The research data were tested statistically using the Kruskal-Wallis test with SPSS 22 program, if the p-value < 0.05 then the test is followed with the Mann-Whitney test to see whether there are any differences between observation days.

III. Result and Discussion

Based on the statistical results obtained from the previous Kruskal-Wallis test, there was a significant difference in VEGF expression in the skin tissue of diabetic rats between each day of observation with p-value = 0.000 ($p < 0.05$). Furthermore, the Mann-Whitney test was carried out to see if there was a difference between each day of observation in each treatment group. The mean value of VEGF expression is shown in Table I.

Based on the statistical results shown in Table I, there was a significant difference in the mean value of VEGF expression between the days of 0^{th} to 16^{th} for each treatment group ($p < 0.05$, $p = 0.000$). The increase in the mean value of VEGF expression was found on the days of 0^{th} to 12^{th}, while the increase was shown to be higher in K2 (4.2 ± 0.98) than in K1 (3.4 ± 0.80). Higher mean value in K2 can stimulate angiogenesis, and increase vascular permeability, resulting in better nutrition distribution, thus promoting faster wound healing.

The 0^{th} day was the first for the wound formation. Hence, both groups produced almost the same (VEGF) expression values and histologic features. It was also shown in both groups that the cells looked irregular, loose, and damaged at the epidermis and dermis as shown in Fig. 1. Meanwhile, the overview histology of the VEGF expression from the 4^{th} to the 12^{th} days have shown that initially damaged cells were started proliferating and migrating from the basement membrane into the wound surface and finally differentiating to form new and regularly shaped cells so that the epithelialization process could occur properly to promote faster wound healing process (Fig 2). Active substances in the nano herbal andaliman were assumed in promoting regular cell shape formation and good epithelialization [5-9].

The administration of nano herbal andaliman can increase the VEGF expression because of the flavonoids, alkaloids, terpenoids, tannins, and saponins. Those substances are proven potential to accelerate the wound healing process [5-6]. Similarly, flavonoids can also protect blood vessel walls and act as anti-inflammatory agents [12,14]. VEGF also takes part in increasing the fibroblast formation, proliferation, and collagen deposition in wound gaps [4].

TABLE I. MEAN VALUE OF VEGF PROTEIN EXPRESSION ACCORDING TO DAYS OF OBSERVATION IN DIABETIC RATS' SKIN TISSUE

VEGF expression Mean Value	***N***	**Observation Day (X±SD)**				
		0	***4***	***8***	***12***	***16***
K1	25	0.2±0.40	1±0.63	1.6±0.49	3.4±0.80	5.8±0.98
K2	25	0.4±0.49	1.4±0.49	3.2±1.17*	4.2±0.98	5.2±0.98
p-value:		p=0.000 (p< 0.05)				

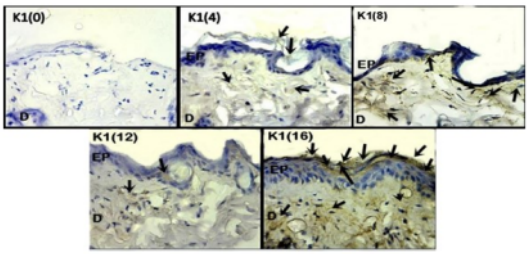

Fig. 1. Expression of VEGF protein in skin tissue of Diabetes Mellitus rats' group (K1) without treatment. Note K1(0): first day of treatment, K1(4): fourth day, K1(8): eighth day, K1(12): twelfth day, K1(16): sixteenth day. EP: Epidermis, D: Dermis, →: (arrow): VEGF expression, Magnification (400x).

On the 8th day, it is clearly shown that for K2 (3.2±1.17*), the mean value of VEGF has increased much more significantly than K1 (1.6±0.49).

On the 16th day, the increasing mean value of the VEGF expression has reached its highest among the previous days (Fig. 1). The basal cell epithelialization process in the epithelium was moved from the edge of the wound into its center before finally covered the whole wound area. At the edges of the wound, the keratinocytes-contained layer would proliferate and migrate from the basement membrane into the wound surface. The keratinocytes that have migrated and differentiated into epithelial cells would migrate towards the center of the wound. When the epithelial cells finally reached the middle of the wound, cell migration would stop and the basement membrane formation started.

The administration of nano herbal andaliman on burns has been shown to have excellent ability to increase VEGF expression on day 16. Andaliman also has antioxidants where the hydroxyl group can bind to carbon in the aromatic ring so that it can capture free radicals generated by lipid peroxidation. Andaliman has flavonoids that have been shown to reduce lipid peroxidation, thereby increasing collagen viability as well as fibroblast proliferation [17]. In addition to that, tannin compound contained in andaliman serves as an astringent thus enabling it to form complex protein that can stimulate nitric oxide. This nitric oxide would help during new tissues formation, thus contributing to an improvement during epithelization process. Andaliman also has saponin in stimulating the synthesis of fibronectin by fibroblasts and altering the expression of the Transforming Growth Factor Beta (TGF-) receptor. TGF-β stimulates the production of Extra Cellular Matrix (ECM) in the proliferative and maturation phases to form collagen and fibronectin. Fibronectin is a large and multi-functional glycoprotein, containing areas that bind to several macromolecules such as collagen, proteoglycans, fibrin and heparin. Fibronectin is found in the early phase of wound healing and induces fibroblast migration. With the stimulation of fibronectin synthesis by fibroblasts, the migration of fibroblasts by fibronectin will be faster as well, where these fibroblasts will be used in the wound healing phase to produce collagen. With the increasing number of fibroblasts migrating to the wound area, the collagen synthesized by fibroblasts will also increase and will accumulate in the extracellular matrix so that the extracellular matrix becomes thicker and the wound heals faster so that it is thought to play a role in the wound contraction process [10,11,13].

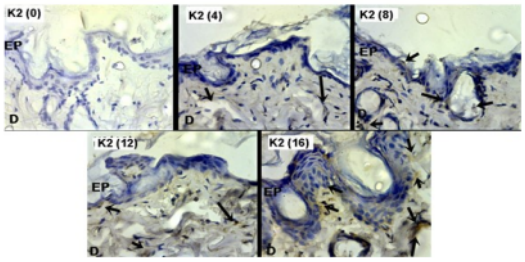

Fig. 2. The expression of VEGF protein in skin tissue of diabetic rats after Nanoherbal Andaliman administration (Group K2). Note: K2 (0): first day of treatment, K2 (4): fourth day, K2 (8): eighth day, K2 (12): twelfth day, K2 (16): sixteenth day. EP: Epidermis, D: Dermis, → (arrow): VEGF expression, Magnification (400x).

IV. Conclusion

The experimental results showed that VEGF expression was significantly found between days of 0th to 16th ($p < 0.05$, $p = 0.000$). There was an increase in the mean value of the VEGF expression, the increase was shown to be higher in K2 (4.2 ± 0.98) than in K1 (3.4 ± 0.80). Andaliman nanoherbal has been shown to be able to increase the histological mean value of VEGF expression effectively in diabetic burns.

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