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EFFECT OF *CHANNA MICROPELTES* IN THE GRANULATION, FIBROSIS AND NECROSIS OF DIABETIC WOUND HEALING

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Diabetes mellitus is a metabolic disorder characterized by persistent hyperglycemia due to insulin insufficiency.[1] Based on data from the International Diabetes Federation (IDF), there are around 10,7 million Indonesians who suffer from diabetes mellitus so that Indonesia ranks 7th out of 10 countries with the highest number of diabetes sufferers in the world.[1] One of the symptoms of diabetes mellitus is delayed wound healing. Delayed wound healing occurs when the structure of the skin tissue, nerves, blood vessels and other supporting tissues are damaged, yet blood glucose control is no longer sufficient to repair the wound. Delayed wound healing in diabetes will increase the risk of wound complications that will hinder the healing process, and lead to complications such as fibrosis and necrosis.[2]

Diabetes is characterized by hyperglycemia that increases the production of reactive oxygen species (ROS). Increased ROS will induce oxidative stress on amino acids and proteins that will result in high advanced glycation end products (AGEs). The high production of AGEs that interacts with the receptor of advanced glycation end product (RAGE) causes activation of proinflammatory cytokines and growth factors, resulting in delayed wound healing. The high production of AGEs can also stimulate extracellular matrix production (ECM) that promotes fibrosis.[3] Hyperglycemic condition that can increase ROS can also reduce tissue tolerance to ischemia, causing initial skin lesions that may lead to necrosis.[4]

Decreased regulator for ROS formation such as antioxidants can modulate diabetes complications, namely delayed wound healing, fibrosis and necrosis. One of the antioxidants can be obtained from food intake such as fish. Toman fish or *Channa micropeltes* (CM) is a species that

is often consumed by the South Kalimantan people of Indonesia. CM has a bluish-black body with a band-like pattern from the back to the lateral line. In the juvenile part, there is a black-orange-black line that extends from the mouth to the tail to the middle of the body.[5] CM contains albumin, omega 6 fatty acids (arachidonic acid) and omega 3 fatty acids, vitamin C and zinc.[6] Oral CM at a dose of 16 mL/ kg BW has been shown to have antioxidant properties that can increase SOD and reduce MDA in diabetic wounds.[7] The oral administration of 16 mL/kg BW dose has also been proven to increase the amount of neovascular on day 4 and reduce the amount of neovascular on days 8 and 14 in diabetic wounds.[8] The use of CM extract at a dose of 16 mL/kg BW orally is proven to general optimal wound closure and wound contraction clinically in Wistar rats as diabetic model on day 14.[9] However, the histopathological description of wound healing is yet to be known. This initiates the necessity of study that analyze the effect of 16 mL/kg BW dose of CM extract administration on changes in the histopathological picture of wound healing granulation tissue, fibrosis and necrosis of diabetic Wistar rats on day 14.

MATERIALS AND METHODS

This study has obtained ethical feasibility by the Faculty of Dentistry, University of Lambung Mangkurat with No. 112 / KEPK-FKGULM / EC / IV / 2020. This research was a true experimental design with a posttest-only and control design. The study population was Wistar rats (*Rattus norvegicus*) with inclusion criteria of healthy male Wistar rats aged 2-3 months and weighed 200-300 grams. Exclusion criteria were mice that were dead, abnormal (injured), hematuria, and presenting with weight loss that exceeds 10% body weight after adaptation. The sampling technique used was simple random sampling with two treatment groups, namely the diabetic model of Wistar rat group which was given BR2 feed only for 14 days (the control group) and the diabetic Wistar rat model group which was given BR2 feed and CM extract at 16 mL/kg BW dose orally for 14 days, with 7 replications respectively.

The Making of CM Extract

CM was bought at the traditional market "Pasar Subuh Martapura" located in Martapura, South Kalimantan, Indonesia. The fish was initially cleaned from its scales, blood, head and abdominal contents, then weighed 18 kg for the flesh that was later steamed in a pot for ± 30 minutes at 70-80ºC temperature. The flesh was then wrapped in a flannel cloth and put into a hand press for pressing process. The resulted extract was put into a test tube and centrifuged for 15 minutes at a speed of 6000 rpm. The centrifugation result was then separated from impurities, the oil and water phases of the extract were taken. The separated extract was stored in a dark glass bottle that was covered with aluminum foil and a clean pack. CM extract was then stored in a refrigerator with a temperature of ≤ 4ºC to prevent damage due to oxidation and contamination.

Induction of Diabetes Mellitus in Wistar rats

Wistar rats were induced to develop type 1 diabetes mellitus by injecting streptozotocin (STZ) at a dose of 40 mg/kg BW. Blood sugar levels were checked with a glucometer before and after STZ induction. Rats were said to have diabetes mellitus if the blood sugar level ≥ 126 mg / dL, checked using a glucometer that was confirmed at the third day after STZ induction.

Experimental Animal Treatment

The incision wound on the back of the rat was made 1 cm long and 2 mm deep. Prior to the injury, the rats were anesthetized for inhalation with 0.75 mL of diethyl ether and kept for 5-10 minutes until the rats fell asleep. The hair of the rats was shaved in 3 cm diameter and cleaned with 70% ethanol. The wound was fabricated using a scalpel and blade no. 11 that was further bandaged using sterile gauze. Diabetic Wistar rats were administered with CM extract at 16 mL/kg BW twice a day (morning and evening) orally using gastric tube for 14 days. After the 15th day, the rats were sacrificed using ketamine-xylazine in a 1: 1 ratio of 0.1 mL for each rat.

Handling of Wistar Rat Carcasses

Wistar rats that had been sacrificed, which skin tissue had been collected, were then buried. Wistar rats were cleaned with water first and wrapped in white cloth which then buried in a depth of ± 25-50 cm.

Skin Tissue Collection and The Making of Histopathological Preparations

Retrieval of skin tissue on the back of Wistar rats was performed using tweezers, scalpel and blade no.11. The skin tissue was cleaned with NaCl and stored in a container containing 70% formalin buffer. Skin tissue samples were made into histopathological preparations through the following steps that were tissue fixation, tissue trimming, processing, tissue embedding, slide making and hematoxylin eosin (HE) staining. Further, the histopathological preparations were observed using a light microscope from Leica Dm 1000 brand with a magnification of 40x10 in 6 fields of view.

Analysis of histopathological preparations

The histopathological preparations were analyzed using three criteria, namely granulation tissue, necrosis and fibrosis. The calculations used are:

a. Calculation of granulation tissue (based on the distribution of necrotic tissue and inflammatory cells) was carried out with the following criteria:[10]
   Score 0: Normal

Score 1: Occasional evidence (25%)
Score 2: Light scattering (30% -50%)
Score 3: Abundant evidence (55-75%)
Score 4: Confluent cell (80-100%)

b. Fibrosis calculations were carried out with the following criteria:[11]
Score 0: Normal
Score 1: 1-25% fibrosis
Score 2: 26-50% fibrosis
Score 3: 51-75% fibrosis
Score 4: 76-100% fibrosis

c. The calculation of necrosis was carried out with the following criteria:[12]
Score 0: Normal
Score 1: Minimal necrosis
Score 2: Mild necrosis
Score 3: Severe necrosis

Data Analysis and Statistical Evaluation

Data obtained from all groups were processed using SPSS software. The histological scores for all groups were presented in the mean rank. The Mann-Whitney U test was used to examine the differences between groups with a level of significance of less than 0.05 ($p<0.05$).

RESULTS

Clinical Examination

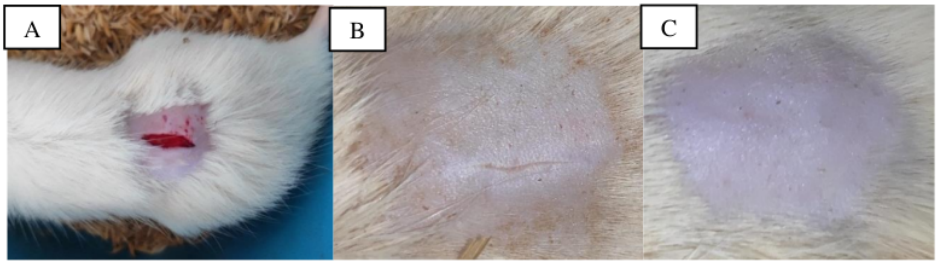

Figure 1: Clinical picture of incision wound in diabetic Wistar rat model on day 14. (A). Wounds in a diabetic Wistar model before being treated with CM extract at 16 mL/kg BW dosage. (B). Wounds in the control group were still presented with redness and yet fully covered. (C). Wounds in t he CM treatment group portrayed complete wound healing.

Microscopic Examination
Granulation tissue

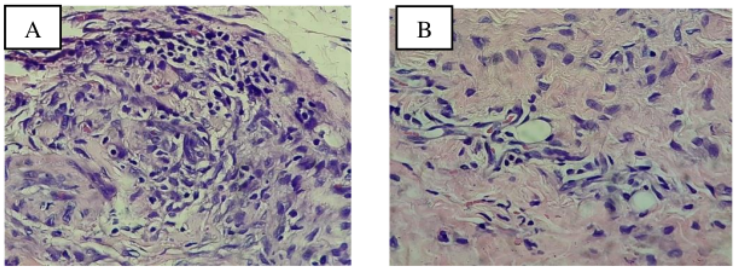

Figure 2: Granulation tissue. (A) The control group shows abundant evidence of granulation tissue with extracellular matrix, inflammatory cells and blood vessels (score 3), magnification 400X, hematoxylin-eosin staining. (B) The CM treatment group showed granulation tissue with extracellular matrix, fibroblasts, inflammatory cells and light scattering blood vessels (Score 2), magnification 400X, hematoxylin-eosin staining.

Fibrosis

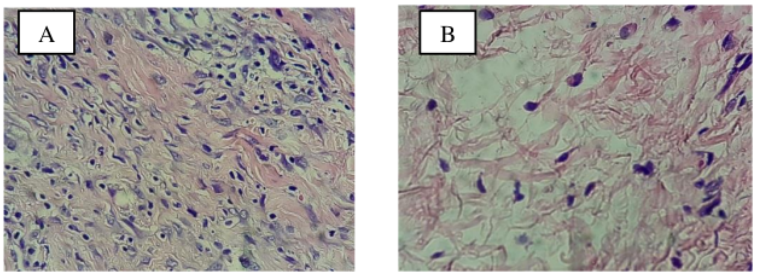

Figure 3: Fibrosis. (A) The control group showed the initial formation of extracellular matrix and collagen fibers, but tissue and inflammatory cells were still persisted, magnification 400X, hematoxylin-eosin staining. (B) The CM treatment group showed fibrosis with an initially-solidified extracellular matrix and collagen fibers formation, magnification 400X, hematoxylin-eosin staining.

Necrosis

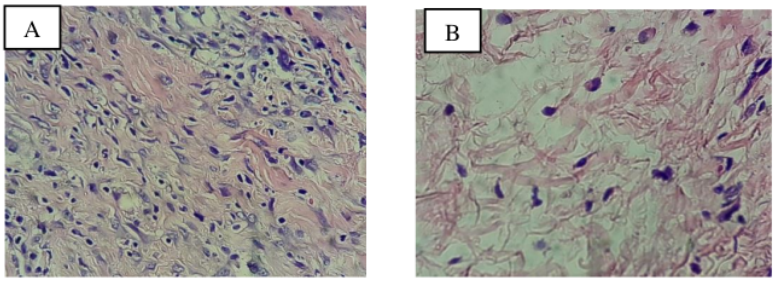

Figure 4: Necrosis. (A). Control group did not show any necrosis, magnification 400X, hematoxylin-eosin staining. (B). The CM treatment group did not show any necrosis, magnification 400X, hematoxylin-eosin staining.

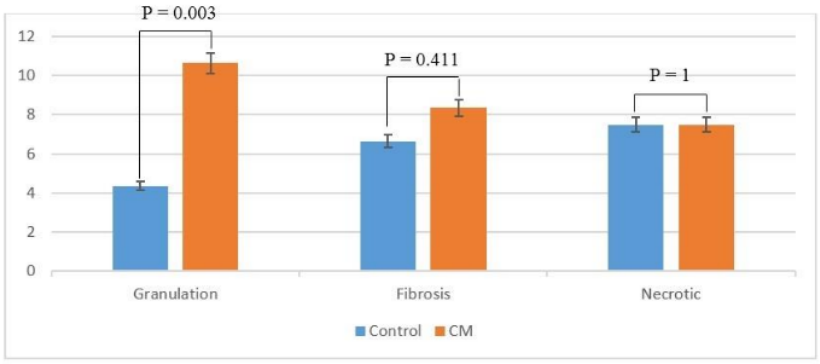

Figure 5: The results of the Mann-Whitney U test on the histopathological features of granulation tissue, fibrosis and necrosis after the administration of 16 mL/kg BW extract of CM orally on a diabetic rat wound, with a level of significance of less than 0.05 ($p<0.05$).

DISCUSSION

Wound healing is an essential physiological process consisting of various cellular, vascular, and chemical components in an effort to restore wound integrity.[13,14] The results of this study showed that there was a significant difference in the histopathological features of granulation tissue between the control group of diabetic Wistar rat model and the group that were given CM extract at a dose of 16 mL/kg BW for 14 days. Diabetic

conditions in the control group showed a delay in the wound healing process, so that the histopathological granulation tissue in this group presented abundant evidence of extracellular matrix, fibroblasts, inflammatory cells and blood vessels (score 3). Diabetes is characterized by high intracellular glucose levels which have an impact on metabolic processes such as glycation and autoxidation to produce diacilyglicerol (DAG) which is a physiological activator of protein kinase C (PKC). The activation of PKC will phosphorylate nicotamide adenine dinucleotide phosphate (NADPH) oxidase to produce superoxide anion ('O2) which leads to increased production of reactive oxygen species (ROS). Increased ROS induce oxidative stress on amino acids and proteins generating advanced glycation end products (AGEs) production which bind to the advanced glycation end product receptor (RAGE) on the cell surface, thereby activating the transcription factor nuclear factor kappa β (NFκβ). Activation of NFκβ triggers the release of pro-inflammatory cytokines such as TNF-α and IL-1β excessively so that inflammation takes longer and the wound healing process will be delayed.[15,16,17]

In the treatment group of diabetic Wistar rats model, the samples administered with CM showed extracellular matrix, fibroblasts, inflammatory cells and light scattering blood vessels in histopathological features of granulation tissue (score 2). This is because CM contains albumin, omega 6 fatty acids, and omega 3 fatty acids.[6] Albumin functions a media for nutrients and oxygen transportation in the process of granulation tissue development.[18,19] Albumin also functions as an anti-inflammatory agent which can reduce osmotic pressure that will lessen edema in cells.[18] In addition, albumin also functions as an exogenous antioxidant that plays a role in suppressing oxidative stress due to excessive ROS production in cellular injury and hyperglycemic conditions in diabetes through the increased mechanism of SOD enzym[10] Albumin elevates the SOD enzyme by signaling nuclear factor erythroid 2-related factor 2 (Nrf2) in the nucleus that will bind to the antioxidant response element (ARE) sequences to code antioxidants, so that the body's production of antioxidants increases. This rise in antioxidants level will compensate for the amount of ROS so that oxidative stress can be avoided and prolonged inflammation in hyperglycemic conditions can be controlled.[7,20] Omega 6 fatty acids, especially arachidonic acid, function as anti-inflammatory which plays a role in stimulating macrophages in phagocytic neutrophil cells and the residues of the phagocytosis process at the inflammatory stage.[21] Arachidonic [5]cid can also stimulate macrophages to release growth factors such as transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF).[22] Those mediators are the main mediators in the formation of the granulation tissue.[14] Arachidonic acid that enters the body will be metabolized through an enzymatic mechanism, namely the 5-lipoxygenase and cyclo-oxygenation pathways that produce products in the form of leukotrienes (LTB4, LTC4 and LTD4), prostaglandins (PGD2, PGE2, PGF2, and PGI2) and thromboxane A2. These substances can stimulate cell migration, new local vascularization, proliferation and differentiation of fibroblasts such as extracellular matrix synthesis in the formation of granulation tissue.[22,23]

Fibrosis is one of the chronic wound manifestations that can occur in diabetic wounds.[24] The high production of AGEs in DM can stimulate extracellu[22] matrix (ECM) production, causing fibrosis.[3] In this study, there was no significant difference in the histopathological features of fibrosis in the DM group of mice given 16 mL/kg BW dose of CM in comparison with the control group. This happened because the observation on incision wound was performed on day 14 which was presented with no sign of infection. The histopathological features of necrosis were also not presented in the DM group of rats given 16 mL/kg BW dose of CM when compared to the control group of this study. When an injury occurs, the exposed tissue becomes easily contaminated with pathogenic organisms that may cause infection. Diabetes sufferers may experience neuropathy, vascular insufficiency, and decreased neutrophil function.[25] This condition will disrupt the defense mechanisms against infectious agents that enter the body which can lead to complications such as tissue necrosis.[4] In this study, the incisional wounds on the back of the Wistar rats were wrapped using a bandage to prevent infection, therefore no presentation of necrosis was obs[24]ed in diabetic Wistar rat model both in the CM group and the control group. It can be concluded that the CM extract at 16 mL/kg BW dose affects the histopathological description of granulation tissue which presents a better result than those in the control group but did not affect the features of fibrosis and necrosis in diabetic Wistar rats on day 14.

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