The Effect of Topical Toman Fish (Channa micropeltes) Extract on Macrophages and Lymphocytes in Diabetes Mellitus Wound Healing

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The Effect of Topical Toman Fish (*Channa micropeltes*) Extract on Macrophages and Lymphocytes in Diabetes Mellitus Wound Healing

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Abstract. Oral administration of Toman fish extract is proven to accelerate wound healing in rat model of Diabetes Mellitus. Toman fish extract contains albumin which may elevate antioxidant level and reduce inflammation. These changes will later enhance cell proliferation and inflammatory cell activity in wound healing process. This study was aimed to determine the topical application effect of 20% toman fish extract on macrophages and lymphocytes in Diabetes Mellitus wound healing. It was an experimental research using post-test only with control group design. This study was performed on 36 diabetic male wistar rats which was comprised of negative control group without given any treatment, 20% Toman fish extract ointment group and 10% Haruan fish (*Channa striata*) extract ointment group. Rats were sacrificed on day 4, 8, and 14 to investigate wounded area with biopsy technique which later obtained histological preparation using Haematoxylin-Eosin staining. The preparation was then observed using microscope to count macrophages and lymphocytes number. One-Way ANOVA test demonstrated a significant difference in macrophage number on day 4 ($p=0.002$), day 8 ($p=0.000$), day 14 ($p=0.000$). The result of the test also indicated a significant difference in lymphocyte number on day 4 ($p=0.001$), day 8 ($p=0.001$), and day 14 ($p=0.001$). Furthermore, Post Hoc LSD test resulted in a significant difference ($p<0.05$) of macrophage and lymphocyte in day four, eight, and fourteen among all treatment groups. Toman fish extract ointment at 20% concentration for topical application may raise the number of macrophage and lymphocyte than those of negative control and 10% haruan fish extract ointment on day 4 and 8 with gradual decrease on day 14.

Keywords: Albumin, Diabetic wound healing, Lymphocyte, Macrophage, Toman fish extract ointment.

1. Introduction

Diabetes Mellitus is the precipitating factor which induce complication in various organs including oral cavity in the form of xerostomia, gingivitis, periodontitis and candidiasis [1,2]. Hyperglicemic condition promote the decline of cell ability for phagocytosis entailing longer wound healing time [1,2,3]. Indonesia ranks 6th in the world for its high Diabetes Mellitus prevalence and 38.113 South Kalimantan populations were reported by Basic Health Survey to suffer from Diabetes Mellitus in 2013 with gradual increase in 2018 [2,4]. Empirically, Haruan fish (*Channa striata*) extract has been utilized by health practitioner as a basic ingredient in supplement product thus Haruan fish (Channa striata) demand elevates significantly. Alternative compound is mandatory to prevail over the shortage in Haruan fish (*Channa stria*ta) population [5,6].

Haruan fish (*Channa striata*) and Toman fish (*Channa micropeltes*) are categorized in the same genus [6]. Both species contain albumin to accelerate body tissue or cell rehabilitation which undergo destruction [6,7,8]. Albumin is the main protein required for wound healing process [9]. Albumin in inflammation stage acts as an osmotic pressure regulator in circulatory system to prevent the worsening of edema [8]. In diabetic wound inflammation, the number of macrophages increase in day 4 and then reduce in number in day 8 with gradual decrease to day 14 [8,9,11,12]. Notwithstanding, the number of lymphocytes in diabetic wound elevate in day 4 [13,14]. Lymphocytes in diabetic wound healing is essential for antigen binding which activates and produces lymphokine. Phagocytosis process reveals that lymphocyte and macrophage interchangeably stimulate each other in a persistent way to eliminate antigen [13]. Wound healing phase subsequently enters proliferative and maturation phase after phagocytosis until the final achievement of wound closure [15,16].

Haruan fish and Toman fish may accelerate wound healing process in diabetic Wistar Rat. Topical application of 10% Haruan fish (*Channa striata*) extract depicted an acceleration in incised wound healing of diabetic Wistar Rat [17], while topical Toman fish extract at 20% concentration is proven to enhance incised wound closure on the back of Wistar Rat induced with STZ in day 9 [18]. Topical application of Toman fish extract is conducted in the form of ointment. Toman fish extract ointment contains adeps lanae and vaselin flavum as bases to prolong drug contact on the skin so that drug absorption ability will increase [6,18]. According to that, this study is conducted to analyze the effect of Toman fish (*Channa micropeltes*) ointment application at 20% concentration toward macrophages and lymphocytes in diabetic wound on the back of Wistar Rat (*Rattus novergicus*) on day 4, 8 and 14 in vivo.

2. Material and Methods

This research had obtained Ethical Clearance letter No.079/KEPKG-FKGULM/EC/XII/2018 from Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Indonesia. This study was performed in several steps which were comprised of fish extract manufacturing and animal treatment at Biochemical Laboratory, Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin-Indonesia. Haruan and Toman fish extract oinments were fabricated at Pharmacy Laboratory, Faculty of Mathematics and Science, Universitas Lambung Mangkurat, Banjarbaru-Indonesia. Paraffin blocks were crafted at Anatomical Pathology Laboratory RSUD Ulin, Banjarmasin-Indonesia. Histopathological staining and interpretation were conducted at Histopathology Laboratory, Faculty of Medicine Research Centre, Surabaya-Indonesia. The sample of this study was male Wistar-strain rat, age 2-3 months, 250-300 g body weigh (BW). Inclusion criteria was comprised of healthy rat (active with good appetite) while exclusion criteria was comprised of death or unhealthy rat (limp, weigh loss more than 10%). It was an experimental research using post-test only with control group design. This study was performed on 36 diabetic male Wistar rats comprised of negative control group without given any treatment, 20% Toman fish extract ointment group and 10% Haruan fish (Channa striata) extract ointment group applied topically. Rats were sacrificed on day 4, 8, and 14. Wounded areas were investigated with biopsy technique to produce histological preparation using

Haematoxylin-Eosin staining. The preparation was then observed using microscope to count the number of macrophages and lymphocytes.

2.1. Formulating Haruan and Toman Fish Extract

Toman and Haruan fish were obtained from Ahad Km 7 Market, Banjarmasin, South Kalimantan. Toman and Haruan fish utilized in this research possessed 600-1000g BW [18]. Utilized part was the flesh of the fish. Each sample was scaled and followed by the removal of its digestive organ. Afterwards, the flesh was steamed inside pan for 25-35 minutes under 60oC temperature [19]. Toman fish and Haruan fish flesh was subsequently wrapped with flannel and inserted in hydraulic press instrument for pressing procedure. Derived extract was inserted in reaction tube and was centrifuged for 15 minutes under 6000 rpm. Centrifugation result was separated from contaminating compound. Toman and Haruan fish was kept inside isolated glass bottle and covered using aluminum foil and clean pack [20].

2.2. Manufacturing Haruan and Toman Fish Extract Ointment

The fabrication of Toman and Haruan fish extract ointment was initiated by weighing adeps lanae and vaselin flavum using analytic balance. As much as 16.875 gram adeps lanae and 23.125 gram vaselin flavum were obtained for Toman fish extract while 16.875 gram adeps lanae and 28.125 gram vaselin flavum were prepared for Haruan fish extract. Toman fish extract at 20% concentration and Haruan fish extract at 10% concentration was previously mixed with adeps lanae and gradually poured with the whole volume of Toman fish or Haruan fish extract respectively to the extent that the extract was absorbed by vaselin flavum and digerum homogenously [18].

2.3. Storing Haruan Fish and Toman Fish Extract Ointment

After the mixture of Toman fish and Haruan fish extract to the base homogenously, each extract was poured inside 50 gram ointment pot and stored inside refrigerator.

2.4. Diabetes Mellitus Induction of Wistar Rat

Diabetic rat model was obtained from STZ injection in Wistar rat under 35 mg/kg dosage. Glucose level in rats was examined using glucometer before induced with STZ. Rats were only fed twice a day and glucose level was examined in the respective first three days after STZ induction. Rat was diagnosed with Diabetes Mellitus when glucose level was over 126 mg/dL. Diabetic wistar rat will display limpness and inactivity [8].

2.5. Wound Construction on the Back of Experimental Animal

Trial animal used was white male rat adapted for a week in laboratory environment. Rat was taken and wound design on rat's back was measured prior to the incision. Procedure was commenced by hand-washing and the application of sterile gloves. Sedative procedure was conducted using 5 ml dosage of diethyl ether inhalation that rat was asleep. Fur around the back was shaved in 3 cm diameter and cleaned using 70% ethanol. Incision wound was achieved in 1 cm length and depth limited to dermal layer on the back of Wistar rat using disposable scalpel and blade no 11. Overflowing blood was cleaned using aquadest [8].

2.6. Animal Treatment

Rats were randomly divided into nine groups with each group was given treatment as follow [4]: negative control groups (1-2) were comprised of 4 diabetic rats given feed twice a day only. Positive control groups (4-6) were comprised of 4 Wistar rats given BR2 feed and 10% concentration of Haruan fish extract applied topically using cotton bud twice a day on the back wound of diabetic Wistar rats. Treatment groups (7-9) were comprised of four rats given BR2 feed and 20% concentration of Toman fish extract topically using

cotton bud twice a day on the back wound of diabetic Wistar rats. Back wound was subsequently covered by sterile gauze which was moistened with NaCl. Additional dry sterile gauze were presented on it.

2.7. Rat Sacrificing Using Diethyl Ether

Rats from each group were sacrificed on day 4, 8 and 14 to observe macrophage and lymphocyte on wound healing process using inhaled anesthesia of 5 ml diethyl ether. Inhalation process was embarked by putting white rats into covered Beaker bottle so that ethyl evaporation would not likely to occur. The process was preceded until white rat was sentenced death. All sacrificed rats were later buried [21].

2.8. Tissue Collection

Tissues for observation were obtained using excisional biopsy technique. Biopsy was conducted by collecting wound tissue and normal tissue around incised back wound of Wistar rat [20].

2.9. Management of Experimental Animal after Tissue Extraction

Unutilized experimental animal organ were buried. Burying of experimental animal organ was performed by cleaning experimental animal organ which was not deployed. Later on, the organ was covered with a fabric and buried in the depth of ± 25 – 50 cm [8].

2.10. Fabricating Preparation

Each biopsy specimen from each treatment group was collected to fabricate histopathological preparation of back wound with the following step [22]: incised area and normal tissue were fixated in 10% Buffer Neutral Formalin (BNF) less than 24 hours. Fixating result was trimmed to ± 1 cm and put in embedding cassette. Organ put in embedding cassette was inserted into a bucket and was rotated using tissue processing machine ± 18 hours with the following steps: 10% formalin (I) for one hour, 10% formalin (II) for one hour, 85% alcohol for one hour, 90% alcohol (I) for 1 hours, 90% alcohol (II) for one hour, absolute alcohol (I) for two hours, absolute alcohol (II) for two hours, xylol (I) for two hours, xylol (II) for two hours, liquid paraffin (I) for two hours, and liquid paraffin (II) for three hours. Tissue was relocated into base mold or a mold containing liquid paraffin and then attached to embedding cassette until it was cold. The preparation was later stored in the refrigerator. Block was placed in microtome and then cut into 5 microns width. Cutting result was selected carefully and put into a water bath with 37oC-47oC temperature. It was kept until no shrinkage observed and obtained using object glass while dried.

2.11. Haematoxyllin Eosin (HE) Staining

Fabricated preparation was stained using Haematoxyllin Eosin (HE) with respective steps [22]: 1) xylol (I) 5 minutes, 2) xylol (II) 5 minutes, 3) xylol (III) 5 minutes, 4) absolute alcohol (I) 3 minutes, 5) absolute alcohol (II) 3 minutes, 6) 80% alcohol 3 minutes, 7) 70% alcohol 3 minutes, 8) Aquabidest/flowing water till clean, 9) Mayer Haematoxyllin 2-5 minutes, 10) Aquabidest/flowing water till clean, 11) Eosin 5-10 minutes, 12) 70% Alcohol 3 minutes, 13) 80% alcohol 3 minutes, 14) absolute alcohol (I) 3 minutes, 15) Absolute alcohol (II) 3 minutes, 16) xylol (I) 5 minutes, 17) xylol (II) 5 minutes, 18) xylol (III) 5 minutes, 19) xylol (IV) 5 minutes, 20) mounting.

2.12. Observation of Histopathological Preparation

Histopathological preparation was observed using light microscope (IMERCO IM-910B, made in Germany). Macrophage and lymphocyte was calculated under 400 magnifications in 5 section fields observed with inflammatory cell. Identified macrophage and lymphocyte was counted and compared in each negative control given feed only, treatment groups given 20% concentration of Toman Fish extract topically, positive control given 10% Haruan fish extract topically in diabetic rats [21].

2.13. Statistical analysis

Statistic data in this study was analyzed using Saphiro-wilk normality test ($p>0.05$) and variance homogeneity of Levene's test. Since data was normally distributed and homogenous, data was then analyzed using One Way ANOVA and Post Hoc LSD through SPSS program.

3. Results and Discussion

Table 1. Mean value and standart deviation.

Groups	Day-4		Day-8		Day-14	
	Macrophages	Lymphocytes	Macrophages	Lymphocytes	Macrophages	Lymphocytes
Toman	12.0 ± 0.81	11.7 ± 0.9	16.0 ± 8.1	15.2 ± 1.2	6.0 ± 0.8	6.2 ± 0.9
Haruan	10.25 ± 0.95	9.7 ± 0.9	13.7 ± 0.95	13.0 ± 0.8	8.75 ± 0.95	8.2 ± 0.9
Control	8.75 ± 0.95	8.0 ± 0.8	11.5 ± 0.81	11.0 ± 0.8	10.25 ± 0.95	10.0 ± 0.8

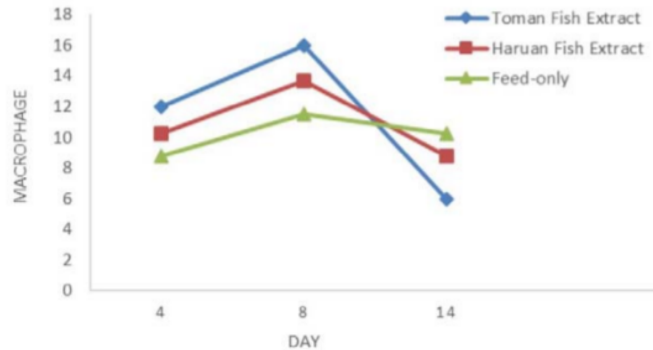

Figure 1. Average number of macrophage cell on back wound of Wistar rats for fourteen days in each treatment.

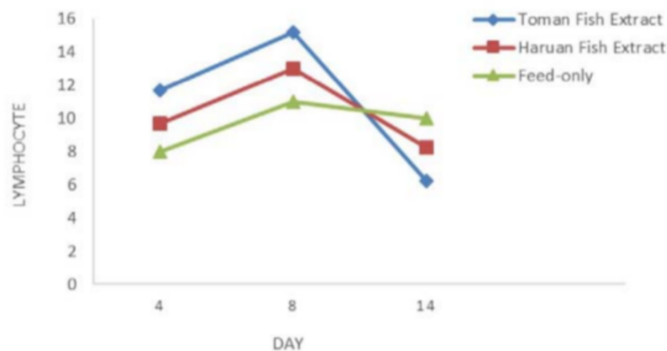

Figure 2. Average number of lymphocyte on back wound of Wistar rats for fourteen days in each treatment.

One Way ANOVA test demonstrated a significant difference in macrophage number on day 4 ($p=0.002$), day 8 ($p=0.000$), day 14 ($p=0.000$). The result of the test also indicated a significant difference in lymphocyte number on day 4 ($p=0.001$), day 8 ($p=0.001$), and day 14 ($p=0.001$). Furthermore, Post Hoc LSD test resulted in a significant difference ($p<0.05$) of macrophage and lymphocyte in day four, eight, and fourteen among all treatment groups.

Macrophages are the primary producers of pro-inflammatory cytokines in wound. Recent wound healing studies have focused on studying macrophage dysfunction in chronic wounds of diabetic humans and mice. Classically activated or M1-like macrophages are known for killing microorganisms and producing pro-inflammatory cytokines. In contrast, the alternatively activated or M2-like macrophages produce anti-inflammatory factors [23]. Lymphocyte in the form of T lymphocyte and B lymphocyte act for antigen paghocytosis in wound healing process [24].

Diabetes mellitus is a condition produced from the elevation of blood glucose level or hyperglycemia. Hyperglycemia may induce disturbance in wound healing process thus wound healing will be delayed [23]. Acceleration of diabetic wound healing process requires an exogenous material, such as albumin. Albumin is proven to accelerate wound healing process and is presented in Haruan and Toman fish ointment. The level of albumin in Toman fish is higher than Haruan where 5.53% unit of albumin were detected in 20% Toman fish extract ointment while 4.53% unit in 10% Haruan fish extract [12]. This induces an increase in macrophage and lymphocytes with the highest number to be found in 20% Toman fish extract ointment group in day 4 and 8 of this study. Albumin is attested to elevate Nuclear Factor Kappa Beta (NF-kβ) thus increase the number and the activity of macrophage [26]. NF-kβ is a transcription factor to regulate pro-inflammatory process. NF-kβ binds inhibitory protein in cytoplasm to prevent its binding with deoxyribonucleic acid (DNA) which is essential in optimizing NF-kβ function through extracellular stimulation. Albumin is an immunomodulator contained in Toman and Haruan fish extract ointment. This compound shall be trapped by macrophage protein receptor and activate NF-kβ which may elevate the number of macrophage [4,27,28].

Albumin may also increase the level of Prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2). PGE2 is increased during initial inflammatory cascade for insult clearance and PGD2 is increased during the active event of inflammatory resolution and healing [29]. Prostaglandin plays a role in activating M1-like macrophages cell [30]. Activated M1-like macrophages will produce interleukin-1 (IL-1) mediator which subsequently stimulates interleukin-6 (IL-6) to increase lymphocyte differentiation and proliferation [31,32]. Albumin has been proven to increase IL-1 and IL-6 in open heart surgery [32]. IL-6 has important regulatory function in the immune system as a mediator of acute-phase response in wound healing process.

It was early recognized as an important cytokine for T and B lymphocyte differentiation. It is involved in early steps of T cell activation via the promotion of T cells proliferation and/or survival, in B cells terminal differentiation via the secretion of immunoglobulins and down-regulation of MHC class II expression, and it is a differentiation factor for cytotoxic T lymphocytes (CTL) [34].

Diabetic wound is confined in a persistent inflammatory state with elevated levels of pro-inflammatory cytokines and proteases, thus resulting in prolonged inflammation. The transition of macrophages from a pro-inflammatory M1-like phenotype to an alternative M2-like phenotype has been suggested as a pre-requisite for the switch from inflammatory to proliferative phase in the healing of the wound [23]. Albumin elevates the number of Transforming Growth Factor-beta (TGF-β) which acts as M1-like macrophages, T lymphocytes, and B lymphocyte growth inhibitor decreasing the number of macrophage and lymphocyte in 20% Toman fish extract ointment group compared to 10% Haruan fish extract application in day 14 [24,34]. TGF-β may also stimulate fibroblast migration, stimulate and inhibit fibroblast proliferation, stimulate fibroblast collagen synthesis and inhibit endothelial cell proliferation which may accelerate proliferative phase on diabetic wound healing process [24]. Supplementation of Channa striatus (Haruan Fish) capsule can also significantly decreased Tumor Necrosis Factor alpha (TNF-α) in the twelfth weeks [35]. TNF-α is a product of innate cells or CD4 and CD8 T cells effector [36]. Bearing a role as a transporter in proliferative stage, albumin carries not only nutrient but also oxygen required for new tissue formation in wound healing so that diabetes mellitus wound healing acceleration can be achieved [37].

4. Conclusion

Toman fish extract ointment at 20% concentration for topical application may raise the number of macrophage and lymphocyte than those of negative control and 10% haruan fish extract ointment on day 4 and 8 with gradual decrease on day 14.

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