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Research Article
Effect of *Channa micropeltes* for Increasing Lymphocyte and Fibroblast Cells in Diabetic Wound Healing

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Abstract

Background and Objective: Diabetes mellitus (DM) has the complications in the form of a wound healing process which take a long time. The DM patients of Indonesia have ranks 4th largest in the world. Empirically the people of South Kalimantan believe that Haruan fish (*Channa striata*) and Toman fish (*Channa micropeltes*) can accelerate wound healing. Both of them have the same genus that contain omega-6, albumin and zinc which can accelerate wound healing. To analyze the effect of toman fish extract orally at a dose of 16 mL kg^{-1} of rat as alternative therapy of diabetic wound healing by increasing the number of lymphocyte and fibroblast cells on the 2nd, 4th and 8th days. **Materials and Methods:** This study was experimental with post test only control design. The sample used 36 male wistar rats, 200-250 mg weight as the model of diabetic wound healing on the back of skin. The study consisted of 3 groups: negative control (only fed), treatment (given toman fish extract 16 mL kg^{-1}) and positive control (given haruan fish extract 13.54 mL kg^{-1}) given for 2 times a day. On day 2, 4 and 8 rats were sacrificed and did the biopsy, then did the histopathology examination by Hematoxylin Eosin to see the number of lymphocytes and fibroblast cells. **Results:** Shapiro Wilk normality test ($p>0.05$) and Variance Levene's Homogeneity Test ($p>0.05$) obtained normal data distribution results and homogeneous data variance, then followed by LSD *post hoc* test. The highest mean value of the number of lymphocytes in the treatment group for the 4th days (15.20 ± 0.84) and the lowest in the negative control group for the 2nd day (5.60 ± 2.30). The highest mean value of the number of fibroblasts in the 4th day treatment group (24.20 ± 1.79) and the lowest in the negative control group 2nd day (10.60 ± 1.14). **Conclusion:** The administration of toman fish extract orally at a dose of 16 mL kg^{-1} BB of rat can be alternative therapy of diabetic wound healing by increasing the highest lymphocyte and fibroblast cells on 4th day.

Key words: *Channa micropeltes*, diabetic wound healing, toman fish extract, fibroblast cells, lymphocyte and haruan fish

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus contributes in several complications and one of them is in the form of delayed wound healing[1]. Patients diagnosed with Diabetes Mellitus in South Kalimantan had reached 1.4% from total citizen with age older[2] than 15. It is estimated that the prevalence of Diabetes Mellitus in Indonesia will reach 21.3 million people in 2030 and will be ranked 4th among the countries with highest prevalence of Diabetes Mellitus worldwide[3].

Empirically, people in South Kalimantan believe that haruan fish (*Channa striata*) and toman fish (*Channa micropeltes*) can accelerate wound healing process[4]. Haruan fish is less available nowadays so it is necessary to provide an alternative resource such as toman fish. Toman fish can be used as an alternative as it is easy to be cultivated because of its fast regenerative property[5].

Currently, there are abundant amount of haruan fish extracts marketed in capsulate formation[6]. It is widely distributed because haruan fish extract contains albumin and fatty acid. Albumin content found in haruan fish extract is estimated around 4.53% which is equal[7] to 13.54 mL kg^{-1} BW. It has an ability to accelerate incised wound contraction on Wistar-strain white rat and also accelerate diabetic rat's wound healing process on day 8.

Haruan fish has the same genus as toman fish which results in similar bioactive contents. The previous study showed that toman fish has anti-inflammatory effect[8]. Administration of toman fish extract can accelerate wound healing process in rat with 16 mL kg^{-1} rat BW dosage[9,10]. However, the administration of toman fish extract 16 mL kg^{-1} rat BW to accelerate diabetic wound healing process has not been yet discovered so far. Wound healing process has several phases which comprised of inflammatory, proliferative and maturation phase[11].

Acute inflammatory phase in diabetic wound healing process occurs from day 2 while chronic inflammatory phase will occur[12] from day 4-8. Inflammatory phase starts right after the occurrence of the wound until day 5 approximately. Polymorphonuclear (PMN) cell is the first inflammatory cell which migrate to wound site and afterward will be replaced by macrophage which infiltration is induced by lymphocytes. Lymphocyte has a role in specific immune response, whether in humoral response which is provided by B cell or cellular response which is provided by T cell. The role of lymphocyte is to release lymphokine which has an impact on inflammation process. Lymphokine will influence the aggregation and chemotaxis of macrophage in wound healing process. End of inflammatory phase will be marked by the reduction of inflammatory cell number and followed by the occurrence of proliferative phase[13]. Proliferative phase includes the formation of fibroblast. It is induced by Fibroblast Growth Factor-2 (FGF-2) signaling pathway. Normally in wound healing process, fibroblast will migrate to wound site and achieve its maximum number[14] on day 7-14.

Based on the information above, there are limited numbers of studies which support the use of toman fish extract 16 mL kg^{-1} rat BW in diabetic wound healing process. The aim of this study to analyze the effect of toman fish extract orally at a dose of 16 mL kg^{-1} BB of rat as alternative therapy of diabetic wound healing by increasing the number of lymphocyte and fibroblast cells on the 2nd, 4th and 8th days.

MATERIALS AND METHODS

Ethical approval of this study was obtained in the form of ethical letter no 072/KEPKG-FKGULM/EC/VI/2018 by Research Ethics Committee, Faculty of Dentistry, University of Lambung Mangkurat. It is a true experimental study with posttest-only control group design. The research was done in Biochemistry Laboratorium of Medical Faculty, University of Lambung Mangkurat. The sample of this study was male Wistar-strain rat, age 2-3 months, 200-250 g body weight. Inclusion criteria comprised of healthy rat (active with good appetite) while exclusion criteria comprised of death rat and unhealthy rat (limp, weight loss more than 10%). The sample consisted of 12 male Wistar-strain rat which divided into 3 groups. Each group will be used as negative control group (given BR2 only), treatment group (given toman fish extract of 16 mL kg^{-1} BW) and positive control group (given haruan fish extract of 13.54 mL kg^{-1} BB) which given twice a day (in the range of 8-12 h) using gastric tube for 14 days.

Making of the extract: Flesh of toman fish or haruan fish was weighed for 9.8 kg from total body weight of 11 kg which obtained for the extraction process. The flesh was steamed in a closed pan at low-flame for 30 min until 750 mL pale yellowish liquid obtained from the flesh. The flesh then covered with sterile gauze to be proceeded under hydraulic press. Toman fish and haruan fish extract obtained were then put into reaction tube for 7.5 mL. The extract then centrifuged for 15 min with a speed of 6000 rpm to attain 700 mL of liquid and 50 mL of deposition. The deposition then separated to obtain 700 mL of liquid only. The liquid was then put into dark glass bottle which covered by aluminum foil and clean pack and saved into refrigerator. Toman fish extract was weighed for 16 mL kg^{-1} BW and haruan fish extract for 13.54 mL kg^{-1} BW.

Diabetes mellitus induction: Rats in this study were suffered from Diabetes Mellitus after injected with a dose of 35 mg kg^{-1} Streptozotocin (STZ). Rats' blood glucose level was measured before and 7 days after induction with STZ. Rats were then diagnosed with Diabetes Mellitus when blood glucose level reached $\geq$126 mg dL^{-1}.

Incised wound formation: Rat adaptation was done for a week before wound formation. The fur on the back of the rat then shaved with 3 cm diameter and cleaned by 70% ethanol. Rat was then sedated by the inhalation of 5 mL diethyl ether until being put to sleep. The wound was made at 1 cm length and 2 mm depth using sterile scalpel and blade no 11, the blood resulted from the wound formation was cleaned using aquadest. The wound which had been made then bandaged with sterile gauze. Drug administration for the wound was in the form of toman fish extract and haruan fish extract which given orally twice a day (in the range of 12 h) using gastric tube for 14 days. After the 14th day of the study, rat was given back to research centre to be used for another future study.

Making of preparate: The preparate was made after Wistar rat's back tissue incision. The tissue was fixated in container filled with fixation liquid (buffer formalin) for 24 h. The tissue was then put into embedding cassette and rotated in tissue processor for approximately 14.5 min using 10% BNF solvent. The paraffin block was made by filling the scaffold with melted paraffin wax and attached the tissue inside. The block was then cut using microtome in 5 µm width. The slice of the tissue was put onto object glass. Staining using Hematoxylin Eosin (HE) and mounting was done, respectively.

Observation and cell count: Observation and cell count was done by using light microscope (Olympus, America) which provided with digital camera in 400 times magnification. Determined area was divided into three fields of view. The count result from three different fields of view were then summed and divided to obtain mean value of the data. Data then tabulated and analyzed.

Statistical analysis: Hypothesis test used in this study was One way ANOVA parametric test. The result obtained from Shapiro-wilk normality test ($p>0.05$) and Levene's of Variant Homogeneity test ($p>0.05$) provided normal data distribution and homogeny data variants.

RESULTS

Based on Table 1, the lymphocyte cells count with the highest mean value was found in Toman Group on day 4 (15.20±0.84), followed by haruan group in day 4 (12.40±1.14) and Toman Group on day 8. The lowest mean value was obtained in negative control group on day 2 (5.60±2.30). Then fibroblast cells count showed the highest mean value in Toman Group on day 4 (24.20±1.79), followed by Toman Group in day 8 (21.00±1.00) and Haruan group on day 4 (20.00±1.58). The lowest mean value was obtained in negative control on day 2 (10.60±1.14).

Based on Table 2, there was a significant difference on lymphocyte cells number calculation on day 2 between negative control and toman group ($p = 0.03$). Then it was a significant difference on day 2 between toman and haruan group ($p = 0.001$). There were the significant differences on day 4 between negative control and toman group ($p = 0.000$) and also between negative control and haruan group ($p = 0.000$). Then it was a significant difference on day 4 between toman and haruan group ($p = 0.001$).

Table 1: Mean value and standard deviation

Groups	Lymphocytes	Fibroblasts	p-value
Negative control 2	5.60±2.30	10.60±1.14	0.000
Toman 2	9.00±1.00	21.40±2.88	0.000
Haruan 2	7.20±0.84	18.60±2.07	0.000
Negative control 4	8.20±0.84	15.60±1.82	0.000
Toman 4	15.20±0.84	24.20±1.79	0.000
Haruan 4	12.40±1.14	20.00±1.58	0.000
Negative control 8	9.00±1.41	17.00±2.12	0.000
Toman 8	10.60±1.14	21.00±1.00	0.000
Haruan 8	10.40±1.14	16.20±1.79	0.000

One way ANOVA test, *Significant ($p<0.05$)

Table 2: Significant value of lymphocytes number in all treatment groups

Groups	Negative 2	Toman 2	Haruan 2	Negative 4	Toman 4	Haruan 4	Negative 8	Toman 8	Haruan 8
Negative 2	-	0.000*	0.052	0.002*	0.000*	0.000*	0.000*	0.000*	0.000*
Toman 2	-	-	0.030*	0.322	0.000*	0.000*	1.000	0.052	0.088
Haruan 2	-	-	-	0.218	0.000*	0.000*	0.030*	0.000*	0.000*
Negative 4	-	-	-	-	0.000*	0.000*	0.322	0.005*	0.009*
Toman 4	-	-	-	-	-	0.001*	0.000*	0.000*	0.000*
Haruan 4	-	-	-	-	-	-	0.000*	0.030*	0.017*
Negative 8	-	-	-	-	-	-	-	0.052	0.088
Toman 8	-	-	-	-	-	-	-	-	0.803
Haruan 8	-	-	-	-	-	-	-	-	-

LSD *post-hoc* test, *Significant ($p<0.05$)

Table 3: Significant value of fibroblast number in all treatment groups

Groups	Negative 2	Toman 2	Haruan 2	Negative 4	Toman 4	Haruan 4	Negative 8	Toman 8	Haruan 8
Negative 2	-	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
Toman 2	-	-	0.024*	0.000*	0.024*	0.245	0.001*	0.738	0.000*
Haruan 2	-	-	-	0.016*	0.000*	0.245	0.185	0.05	0.05
Negative 4	-	-	-	-	0.000*	0.001*	0.245	0.000*	0.616
Toman 4	-	-	-	-	-	0.001*	0.000*	0.010*	0.000*
Haruan 4	-	-	-	-	-	-	0.016*	0.404	0.003*
Negative 8	-	-	-	-	-	-	-	0.002*	0.504
Toman 8	-	-	-	-	-	-	-	-	0.000*
Haruan 8	-	-	-	-	-	-	-	-	-

LSD *Post-hoc* test, *Significant ($p<0.05$)

Based on Table 3, the count of fibroblast cells number showed significant difference on day 2 between negative control and toman group ($p = 0.000$), then also between negative control and haruan group ($p = 0.000$). There was also a significant difference on day 2 between toman and haruan group ($p = 0.024$). There was a significant difference on day 4 between negative control and toman group ($p = 0.000$), then also between negative control and haruan group ($p = 0.001$). There was also a significant difference on day 4 between toman and haruan group ($p = 0.001$). There was a significant difference on day 8 between negative control and toman group ($p = 0.002$). There was also a significant difference on day 8 between toman and haruan group ($p = 0.000$).

DISCUSSION

Toman fish contains high-level of albumin approximately 5.35 g dL^{-1} and fatty acid of 7.2 mg. This amount of omega-6 and albumin found in toman fish are higher than the content provided by haruan fish which is only 4.53 g dL^{-1} for albumin and 3.7 mg for omega-6. This two contents can accelerate diabetic wound healing process[15,16].

There is an increase of advanced glycated end products (AGEs) in inflammatory phase of diabetic wound healing process. The elevation of AGEs results in higher numbers of Reactive Oxygen Species (ROS) through Nicotinamide adenine dinucleotide phosphate (NADPH) pathway[17,18]. The elevation of ROS in Diabetes Mellitus patient results in the reduction of superoxide dismutase (SOD) activity so that it is necessary to provide alternative antioxidant such as albumin[19]. The administration of toman fish extract which contain albumin can function as antioxidant to bind Reactive Oxygen Species in diabetic wound healing process. Elevation in SOD activity can suppress excessive amount of ROS and will be followed by the reduction of MDA so it can accelerate wound healing process in diabetes mellitus patients[18, 20].

Toman fish extract contain omega-6 which has chemical mediators such as prostaglandin and lipoxin to play their role in inflammatory phase of wound healing process[9]. Diabetic wound healing process has several phases comprised of acute inflammatory phase from day 2-4, chronic inflammatory phase from day 4-8 and proliferative phase[12] from day 8-14.

Toman fish extract also contains higher level of Zinc than haruan fish. Zinc contains in haruan fish has approximate amount of 2.43% and the one contains in toman fish is 2.59%. Zinc is a structural element of superoxide dismutase enzyme which provided in cytoplasm. Superoxide dismutase has an active centre for copper and zinc ion. This enzyme accelerate radical superoxide conversion in the form of Reactive Oxygen Species (ROS) into hydrogen peroxide[9, 21].

The result of this study shows that toman fish extract administration has an effect toward the number of lymphocytes in wound healing process. The count of lymphocyte number shows the highest mean value in toman group day 4. This shows that 4 days of toman fish extract administration can increase the number of lymphocyte to reach its peak to induce macrophage number. Lymphocyte will release lymphokine which induces macrophage chemotaxis. In addition of macrophage function in phagocytosis, they also release several mediators such as growth factor and interleukin.

The result of day 2 showed that toman fish extract administration had significant result compared to negative control group and haruan fish extract group. It was caused by the higher production of antioxidant to induce the elevation of lymphocyte number which later affect the number of macrophage.

The result of day 4 showed that the administration of toman fish extract influenced the number of fibroblast in wound healing process. The count of fibroblast number obtained the highest mean value in toman group day 4. This result showed that 4 days of toman fish extract administration will increase the number of fibroblast which induced by the elevation of macrophage on day 4. The elevation of fibroblast number is a good progress for wound healing process.

The result of day 8 shows that the administration of toman fish extract has a significant result compared to negative control group and haruan fish extract group. This illustrated that toman fish extract has a higher ability than haruan fish extract to increase the proliferation of fibroblast in the end of inflammatory phase.

Macrophage had an important role in wound healing process because of its ability in growth factors production, angiogenesis and also fibrogenesis induction. Activated macrophages will phagocyte bacteria and clean the debris on wound surface, then will be presenting antigen and releasing cytokines that can stimulate the growth and function of T lymphocytes. The result was supported by the research showed the lymphocyte cells decreased in chronic inflammation phase[22].

The transition process of inflammatory phase to proliferative phase will allow macrophage to stimulate cell migration, proliferation and tissue matrix formation. Macrophage induces the release of Transformation Growth Factor (TGF)-β and Fibroblast Growth Factor (FGF)-β to increase fibroblast proliferation[22,23]. Fibroblast will produce collagen to promote new fibrous tissue formation. When collagen and extracellular matrix are synthesized, new epithelial tissue in oral mucosa will be formed to promote the closure of wound surface. It is known as fibroblast phase because fibroblast plays an important role in this phase. This phase also promote the granulation tissue formation, wound contraction and epithelialization. Fibroblast proliferation will determine the end result of wound healing process[23,24]. The result was supported by the research before that increasing the fibroblast cells considered as healing parameter that determines as the final outcome of wound healing[24].

CONCLUSION

Based on the arguments above, it has been proven there is an effect of toman fish extract administration per oral with 16 mL kg^{-1} rat BW dosage on the number of lymphocyte and fibroblast cells in diabetic wound on the 2nd, 4th and 8th days. It can be concluded the administration of toman fish extract mL kg^{-1} BB of rat can be alternative therapy of diabetic wound healing. The Toman fish extract can be increasing the highest lymphocyte and fibroblast cells on 4th day so that resolve the problem of delayed healing in diabetic wound.

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