THE INFLUENCE OF TOMAN FISH (Channa micropeltes) EXTRACT ON THE NUMBER OF NEOVASCULAR IN DIABETES MELLITUS WOUND HEALING

In Vivo Study on the Back of Male Wistar Rat (Rattus norvegicus)

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ABSTRACT

Background: Patients with diabetes mellitus (DM) have delayed wound healing. This happens because of the depletion of neovascular number. Neovascular plays an important role in wound healing by restoring blood flow to the wound area, so the new tissue gets enough oxygen and nutrition supply to proliferate. Toman fish (Channa micropeltes) contains albumin that can increase and decrease the number of neovascular, so it’s potentially become an alternative medicine to accelerates wound healing in DM. Purpose: The aim of this research to prove the effect of toman extract dose 16 ml/Kg BW rat orally on the number of neovascular in wound of diabetic wistar rats on the 4th, 8th and 14th day. Materials and methods: This research used true experimental method with posttest only control group design. This research used 36 wistar rats which were then divided into 9 groups, toman fish extract dose 16 ml/Kg BW of rat group, haruan fish extract dose 13.54 ml/Kg BW of rat group and BR2 feed only group. Research result: The results of One Way-Anova and Post Hoc Least Significant Difference tests showed there was a significant difference between group of toman extract and group of feed only, while on group of toman extract and group of haruan extract showed there was no significant difference on 4th, 8th and 14th day. The average value of toman extract-given group on 4th, 8th and 14th day were 14.75, 7.5 and 6.75 respectively. Conclusion: Toman extract has effects which are increasing the number of neovascular on 4th day and decreasing the number of neovascular on 8th and 14th day in wound healing with DM.

Keywords: Diabetes mellitus, Toman fish extract, Neovascular, Wound healing, Wistar rats.

INTRODUCTION

Diabetes mellitus (DM) is a disease of metabolism characterized by an increase in glucose level in the blood (hyperglycemia) caused by defects in insulin secretion and work.1 DM patients have manifestations in the oral cavity such as xerostomia, oral candidiasis, and periodontitis.2,3,4 According to International Diabetes Federation data in 2013, Indonesia ranked 7th in the world with the highest DM prevalence in the age group of 20-79 years.5 Based on Riskesdas data in 2013, the prevalence of DM diagnosed by doctors in South Kalimantan reached 1.4% while prevalence the undiagnosed DM reaches 2.0%.6 Diabetes mellitus give rise to some complications. One of them is delayed wound healing.7 The people of South Kalimantan believe that eating haruan fish (Channa striata) and toman fish (Channa micropeltes) can accelerate wound healing.8,9 Currently, haruan fish is difficult to be cultivated, so it needs another alternative as haruan substitute, namely a toman fish that is more easily cultivated because it has the fastest growing nature in Channidae family.10 Giving toman extract with dose 16 ml/Kg BW toward rat has been proven to accelerate wound healing process.11 Toman fish contains protein, fat, water, albumin, water soluble vitamins (B and C) and...
Toman fish has the highest albumin in the Channidae family of 5.35% . Albumin as an antioxidant proves to accelerate wound healing.

In the wound with DM, there is an increase of Reactive Oxygen Species (ROS) caused by hyperglycemia, so the wound is delayed to heal. The increase of ROS will cause excessive oxidative stress. Oxidative stress will cause damage or even apoptosis in endothelial cells, resulting in the decreasing of neovascular number. Neovascular is one of components in wound healing which functions by bringing oxygen and nutrients from the blood to the wound area so that new tissue can proliferate. Neovascular decrease in wound with DM caused the delayed in wound healing process.

Toman fish contains albumin that acts as a secondary antioxidant. Albumin has a cysteine and thiol bonding which is binds excessive ROS, resulting in the decreasing of ROS. Reactive Oxygen Species with normal level can initiate angiogenesis process for neovascular formation in the proliferative phase so that it can progress into the maturation phase until the wound heals.

The results of research that support the use of toman fish extract in accelerating the wound healing is still very limited. Research on wound healing has been widely practiced, but the effect of using toman fish extract (Channa micropeltes) on neovascular number in the wounds of wistar rat with diabetes mellitus is not exist yet. Based on the thing above, it is necessary to do research to know the effect of toman fish extract (Channa micropeltes) with dose 16 ml/Kg BW orally on the neovascular number in DM-induced Wistar rats on the 4th, 8th and 14th day. The result of this study is expected that toman fish extract can be an alternative medicine to accelerate wound healing in DM.

MATERIALS AND METHOD

The implementation of the research began with asking the research permit and ethical clearance issued by the Ethics Committee of Faculty of Dentistry, University of Lambung Mangkurat No.022 / KEPKG-FKGULM / EC / IX / 2017. This study used true experimental design with a complete randomized posttest-only design. The population of this study was Wistar rats. The inclusion criteria of this study sample were male Wistar rats, weight 250-300 gram, 2-3 months age and healthy condition (active and had a good appetite). The sampling exclusion criteria were 10% post-body weight loss after adaptation in the laboratory, unhealthy mice (inactivity and lack of appetite), abnormal mice (injury or defect) and dead mice. The researcher used experimental animals divided into 9 groups i.e. 3 groups of negative control with only BR2 feeding, 3 treatment groups with BR2 feeding plus toman fish extract dose 16 ml/Kg BW Wistar rats, and 3 positive control group with BR2 feeding plus haruanfish extract dose 13.54 ml/Kg BW Wistar rat. Each group consisted of 4 rats which is sacrificed on the 4th, 8th and 14th day.

This research procedure began with the sampling of toman fish and haruan fish. The collection of toman fish and haruan fish was obtained from Martapura Traditional Market. Toman and haruan fish that used in this study had a total weight of 11 kg. The portion used is the flesh of the toman fish and haruan fish. Each sample of toman and haruan fish was cleaned by the head and entrails first and continued with the disposal of the scale, and then it weighed till 9.84 kg sample were obtained. The flesh was put into a container and steamed in a pan for ± 30 minutes, then 750 ml the pale-yellow liquid that obtained from the flesh was taken and set aside. Toman or haruan fish flesh then wrapped with flannel cloth and put in a hydraulic press tool for pressing. As much as 7.5 ml of toman or haruan fish extracts were put into the test tube and centrifuged for 15 min at 6000 rpm. Centrifugation resulted in the obtain of 700 ml of fluid and 50 ml of sediment which was then separated. Toman fish extracts or fish were stored in dark glass bottles and covered with aluminum foil and clean pack.

The diabetic mouse model was obtained by injecting STZ to the rat at a dose of 35 mg/kg. The rats were fed and then checked using glucometers after 7 days. Rat declared to have diabetes when blood glucose level ≥ 126 mg/dL. The condition of rat with diabetes was weak, inactive and no appetite.

The treatment toward rat was started by adapting rats for 1 week in the laboratory atmosphere, then divided into 9 treatment groups with 36 samples of rats and numbered according to the group. The rat were taken and given sedatives using diethyl ether. The incision wound was made 1 cm in-length and 2 mm in-depth at the back of the Wistar rats using a scalpel and disposable blade number 11, the blood that came out was cleaned with aquadest. The wound on the back of the rat was then bandaged with gauze and bandages. Each group was given the following treatment:

**The treatment I:** Groups 1, 4 and 7 were fed orally by BR2 toward rat with DM, then sacrificed on the 4th, 8th and 14th day. **Treatment II:** Groups 2, 5 and 8 were fed by BR2 plus toman extract 16 ml/Kg BW orally to the rat with DM, then sacrificed on 4th, 8th and 14th day. **Treatment III:** Groups 3, 6 and 9 were fed by BR2 plus haruan fish extract 13.54 ml/Kg BW orally to the rat with DM, then sacrificed on 4th, 8th and 14th day.

Treatment of each rat was given orally to each treatment group using stomach food pipe and given twice a day for 14 days. The rat in each group were
sacrificed to see the neovascular number of wound healing process with inhalation using 5 ml diethyl ether at 4th, 8th and 14th day. The inhalation process was done by inserting the Wistar rat into the beaker bottle and then closed so that ethyl did not evaporate, wait for a while until the Wistar rat died. The researcher took the tissue on the Wistar backs by biopsy on wounds around the dermis area of 1 cm in-length and 2 mm in-depth. The biopsied tissue was fixed with a 10% (BNF) Neutral Buffer for 24 hours, then the tissue was processed and planted on paraffin blocks. The paraffin block then cut to 5 μm thickness and placed on the object glass and inserted into the waterbath. The pieces were dried and then stained with Hematoxylin-Eosin (HE). All the rats that had been sacrificed were buried. The burial of the animals is done by cleaning the parts of the animal organs that are not used, then wrapped with cloth and buried with a depth of ± 25 - 50 cm.

Histopathologic observations to calculate the neovascular number of Wistar wounded back were observed with a light microscope with 100x magnifications at 5 fields on the connective tissue of the wound edges.

RESULTS

Based on the research data, the average value of neovascular number on the 4th, 8th and 14th day in all groups were as follows:

Table 1. Mean (Mean ± SD) Neovascular Number in Wistar Rat Back Wound with Diabetes Mellitus (DM).

<table>
<thead>
<tr>
<th></th>
<th>Toman Fish Extract</th>
<th>Haruan Fish Extract</th>
<th>Feed Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>4th day</td>
<td>14.75 ± 1.25</td>
<td>13.50 ± 1.29</td>
<td>11.00 ± 0.81</td>
</tr>
<tr>
<td>8th day</td>
<td>7.50 ± 1.29</td>
<td>8.25 ± 0.95</td>
<td>10 ± 0.81</td>
</tr>
<tr>
<td>14th day</td>
<td>6.75 ± 0.95</td>
<td>7.75 ± 0.95</td>
<td>8.75 ± 0.95</td>
</tr>
</tbody>
</table>

Figure 1. Graphic of Average Neovascular Number on Wistar Rats Back Wound with Diabetes Mellitus on 4th, 8th and 14th day in Each Group.

Figure 1 shows the average amount of neovascular back wound of different wistar rats in each group of neovascular number on 4th, 8th and 14th day. On the 4th day, there was neovascular increase in which is the highest number of neovascular rates in all three groups are the toman fish extract, haruan fish extract, and feed only. On 8th and 14th day, there was a neovascular decrease in which the lowest neovascular rates among the three groups are toman fish extract, haruan fish extract and feed only.

Figures 2, 3 and 4 show the HPA images of wistar rats back wound which was given toman fish extract, haruan fish extract and feed only on 4th, 8th and 14th day.
Figure 2. Histopathologic Image of Neovascular Number on Back Wound between Toman Fish Extract Groups, Haruan Fish Extracts and Feed Only on 4th Day at 100x Magnification.

Figure 3. Histopathologic Image of Neovascular Number on Back Wound between Toman Fish Extract Group, Haruan Fish Extract and Feed Only on 8th Day at 100x Magnification.

Figure 4. Histopathologic Image of Neovascular Number on Back Wound between Toman Fish Extract Group, Haruan Fish Extract and Feed Only on 14th Day at 100x Magnification.

The calculation results of the neovascular number data were tabulated, followed by normality test using Shapiro-Wilk and homogeneity test using Levene's test. Based on the normality and homogeneity test, the result data on the 4th, 8th and 14th day had the normal and homogeneous value (p > 0.05), so it can be continued with One-way Anova Test. One-way Anova test showed the values for 4th day was p = 0.004, 8th day was p = 0.021 and 14th day was p = 0.047. There were significant differences (p < 0.05) on day 4, 8 and 14, so a Post-hoc LSD analysis was conducted to find out which treatment group showed the significant differences.

Table 2. LSD Statistic Test Result for Neovascular Number on 4th day.

<table>
<thead>
<tr>
<th></th>
<th>Toman Fish Extract</th>
<th>Haruan Fish Extract</th>
<th>Feed Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toman Fish Extract</td>
<td>0,156</td>
<td>0,001*</td>
<td></td>
</tr>
<tr>
<td>Haruan Fish Extract</td>
<td>0,156</td>
<td>0,013*</td>
<td></td>
</tr>
<tr>
<td>Feed Only</td>
<td>0,001*</td>
<td>0,013*</td>
<td></td>
</tr>
</tbody>
</table>

Note:
* : There is a significant difference (p < 0.05)

Table 3. LSD Statistic Test Result for Neovascular Number on day 8th day.

<table>
<thead>
<tr>
<th></th>
<th>Toman Fish Extract</th>
<th>Haruan Fish Extract</th>
<th>Feed Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toman Fish Extract</td>
<td>0,335</td>
<td>0,008*</td>
<td></td>
</tr>
<tr>
<td>Haruan Fish Extract</td>
<td>0,335</td>
<td>0,041*</td>
<td></td>
</tr>
<tr>
<td>Feed Only</td>
<td>0,008*</td>
<td>0,041*</td>
<td></td>
</tr>
</tbody>
</table>

Note:
* : There is a significant difference (p < 0.05)

Table 4. LSD Statistic Test Result for Neovascular Number on day 14th day.

<table>
<thead>
<tr>
<th></th>
<th>Toman Fish Extract</th>
<th>Haruan Fish Extract</th>
<th>Feed Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toman Fish Extract</td>
<td>0,174</td>
<td>0,016*</td>
<td></td>
</tr>
<tr>
<td>Haruan Fish Extract</td>
<td>0,174</td>
<td>0,174</td>
<td></td>
</tr>
<tr>
<td>Feed Only</td>
<td>0,016*</td>
<td>0,174</td>
<td></td>
</tr>
</tbody>
</table>

Note:
* : There is a significant difference (p < 0.05)
Based on the result of Post Hoc LSD test in the table above, it can be seen that there is significant difference between extract fish toman group 16 mL dose / Kg BW Rat with the BR2 feed group only and there is no significant difference of toman fish extract group 16 ml/Kg BW rat with haruan fish extract group 13.54 ml/ Kg BW rat on the 4th, 8th and 14th day. On the 4th and 8th day, there was a significant difference between the toman fish extract group of 13.54 ml/Kg BW rat and the BR2 feed only, while on the 14th day there was no significant difference between the haruan fish extract group of 13.54 ml/Kg BW rat and the BR2 feed only group.

DISCUSSION

The result of this study indicates an elevation in the number of neovascular on the 4th day and the depletion in the number of neovascular on 8th and 14th day where toman fish extract give a better effect than haruan fish extract and feed only to increase and decrease the number of neovascular on regenerating the wound on the back of wistar rat with diabetes mellitus.

Diabetes mellitus (DM) is a disease characterized by the occurrence of hyperglycemia. Hyperglycemia will lead to an increase in excessive ROS. Increased ROS causes oxidative stress. Oxidative stress results in the damage or death to endothelial cell in angiogenesis, resulting in the decreased of neovascular formation and delayed wound healing in patients with DM. Damage to oxidative stress caused by excessive increase in ROS can be treated with the administration of albumin as an antioxidant.

Albumin is a secondary antioxidant that has cysteine and thiol group which acts as radical scavenger by binding copper with high affinity and also binding to metal ions involved in ROS formation, resulting in the decrease in ROS formation. Reactive Oxygen Species with normal level play a role in the wound healing process. Reactive Oxygen Species at normal level can increase the activity of Hypoxia Inducible Factor-1α (HIF-1α) that plays a role in angiogenesis by signaling activation of Vascular Endothelial Growth Factor (VEGF). Vascular Endothelial Growth Factor is a pro-angiogenic factor produced by macrophage cells where macrophages produce various growth factors and cytokines in the proliferative phase. Vascular Endothelial Growth Factor stimulates proliferation, migration and endothelial cell response. Vascular Endothelial Growth Factor with HIF-1α leads to neovascular formation in angiogenesis. This results in an increase in the neovascular number on the 4th day of proliferation after the injury.

The 8th day post-injury showed a decrease in neovascular number. This is due to the 8th day as the beginning of the proliferative phase initiation where the wound begins to close resulting in a decrease of macrophages. The decrease in macrophages causes a decrease in VEGF secretion. Decreased VEGF expression results in the decrease of neovascular number. In addition to VEGF, neovascular decrease is also due to apoptosis of endothelial cells induced by anti-angiogenic factors at the time of granulation tissue begins to form. Albumin in toman fish extract plays a role in granulation tissue formation. Albumin acts as an oxygen carrier and other substances such as bilirubin, fatty acids, metals, hormones and medicine to be used as energy in the proliferation of epithelial cells and support the viability of the granulation tissue.

On the 14th day post-injury, the wound healing process began to enter the maturation phase. The wounds with DM that had been administered fish extract on the 14th day, showed the depletion of neovascular number. This happened because the 14th day of wound healing process, granulation tissue has been formed. The formation of granulation tissue indicates the neovascular formation in the wound area is sufficient to provide oxygen and nutrients from the blood and has supported the viability of the granulation tissue, so that the wound is closed.

The statistic test result in this research showed that there is no significant difference of the toman fish extract group compared to the haruan fish extract group. However it can be seen from the result that there was a difference in the average of neovascular number and the best wound healing rate was found in toman fish extract group. The difference of the average of neovascular number between toman fish extract group and haruan fish extract is caused by difference of albumin content on both extracts. Based on Firlianty research et al, (2014), it is stated that the toman fish contains albumin of 5.35%, while the haruan fish only contain albumin of 4.53%. Albumin on both extracts serve as antioxidants, thus it can accelerate wound healing on diabetes. This is linear with the Murdani study et al, (2016) which is showed that DM-induced wistar rats administered with toman fish extracts containing albumin can accelerate the wound healing compared with the negative control group given only aquadest. This also corresponds to Marijyanto research et al, (2013) which is stated that the higher the albumin level, the higher the rate of wound healing acceleration process occurred.

It can be concluded that there is an influence of toman fish extract which are increasing the number of neovascular on the 4th day and decreasing the number of neovascular on the 8th and 14th day in wound healing with DM.
REFERENCES


