THE EFFECT OF TOMAN (Channa micropeltes) FISH EXTRACT ON EPITHELIAL THICKNESS IN DIABETES MELLITUS WOUND HEALING

(In Vivo Study on the back of male Wistar rat (Rattus novergicus))

Nina Audina¹, Amy Nindia Carabelly², Didit Aspriyanto³
¹ Dentistry Faculty Lambung Mangkurat University, Banjarmasin
² Oral Pathology Department, Dentistry Faculty Lambung Mangkurat University, Banjarmasin
³ Pedodonsia Department, Dentistry Faculty Lambung Mangkurat University, Banjarmasin, Banjarmasin

ABSTRACT

Background: Re-epithelialization is the process of formation of new epithelium which is important in wound healing process, the faster the process then the sooner the process of wound healing. Toman fish (Channa micropeltes) is trusted as an alternative medicine to accelerate wound healing. Toman fish contains albumin which acts an antioxidant. Antioxidant can reduce Reactive Oxygen Species (ROS) which increased at state of hyperglycemia in people with diabetes mellitus (DM), Thus accelerating the process of Re-epithelialization on diabetes wound. Purpose: The Purpose of this research was to prove that fish extract Toman dose 16 ml/Kg body weight effect on thickness of the epithelium of Wistar Rat Injuries Induced DM are seen on the 2, 4 and 8 day. Material and methods: This research was true experimental design with posttest-only with control design with complete random design. This research used 27 Wistar rats are divided into 9 treatment groups, Toman fish extract dose 16 mL/Kg BW, Haruan fish extract 13,54 mL/Kg BW, and BR2 feed only. The calculation of epithelial thickness was calculates using the ImageJ software application by measuring histopathological results. Research result: The result of Two way Anova test and Post Hoc LSD test showed that there was a significant difference of effect on group Toman fish extract 16 mL/Kg BW with Haruan fish extract 13,54 mL/Kg BW and BR2 feed only to increase the thickness of the wound of epithelium of Wistar rat Induced DM. Toman fish extract showed the highest mean of epithelial thickness with 2nd day (76,69 µm), 4th day (99,21 µm) and 8th day (124,23 µm). Conclusion: There was effect of Toman fish extract dose 16 mL/Kg BW on increased epithelial thickness of Wistar rats wound on induced diabetes mellitus are seen on the days 2, 4 and 8.

Keywords: Diabetes mellitus, Toman fist extract, Epithelial thickness, Wistar Rats.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder, caused by the deficiency of insulin production by pancreas or the ineffective use of insulin in the body. This resulted in an increase in glucose concentration in the blood (hyperglycemia).⁴ From the Riskesdas data on 2013, it was obtained that the number of DM patients aged 15 and above in Indonesia is about 6.9% which consist of 12,191,654 people. In addition, the data for DM patients in South Kalimantan was consisted of 38,113 people.⁵ Diabetes mellitus causes some manifestation in the oral cavity such as gingivitis and periodontitis, candidiasis, xerostomia, tongue burning, alveolar bone resorption and a bad wound healing complications.⁶ In recent years, most people with diabetes mellitus injury prefer to take herbal medicine that is safer and has no adverse side effects compared to conventional medicine.⁷ The people in South Kalimantan believe that consuming Haruan fish (Channa striata) and Toman fish (Channa micropeltes) can accelerate the wound healing process.⁸ Currently Haruan fish is hard to find, so other alternative fish are needed that is...
more easily cultivated and have fast growing properties such as Toman fish.\textsuperscript{5,7}

DM wound encounters increase in Reactive Oxygen Species (ROS), so the wound heal slowly and sometimes fail to heal.\textsuperscript{10,11,12} Toman fish contains albumin which is a non-enzymatic antioxidant that functions as radical scavenger.\textsuperscript{8,9} Albumin functions as radical scavenger which works to capture excessive ROS resulting in ROS depletion so it can help to speed up the wound healing process.\textsuperscript{8,9,13,18}

In the inflammatory phase, the monocyte appears to assist the phagocytic process of microorganisms which will later turn into macrophages that will secrete the required growth factors during wound healing process.\textsuperscript{15,16,17} Epidermal Growth Factor can stimulate the process of migration and keratinocyte proliferation in the process of reepithelization during wound healing.\textsuperscript{18} Reepithelization is one component of the proliferation phase characterized by the tissue proliferation (granulation tissue), angiogenesis, and reepithelization which after proliferation phase will be continued to synthesizing the Extracellular Matrix (ECM) process in the maturation phase until the wound heal.\textsuperscript{19,15} Reepithelization is an important process in wound healing and is used as a parameter for a successful wound healing.\textsuperscript{17}

The research that supports Toman fish extract ability to accelerate wound healing is still very limited. Research on wound healing has been done many times, but there has been no research on the effect of Toman fish extract on epithelial thickness in diabetes mellitus wound healing. The purpose of this study is to know the effect of given Toman fish extract of 16 mL / kg BW dosage given orally on the epithelial thickness of Wistar rat induced with diabetes mellitus on day 2, 4 and 8.

**MATERIAL AND METHODE**

This study used true experimental method with complete randomized posttest-only control group design. This research began by taking care of the research permit and ethical clearance issued by the Committee of Medical Research Ethics Faculty of Dentistry Lambung Mangkurat University No.020 / KEPKG-FKULM /EC/VIII/2017. The population of this study was Wistar rats. Inclusion criteria were rat with 200 - 300 grams weight, aged 2 - 3 months and healthy conditions (active and have a good appetite). Exclusion criteria in this study were weight loss of more than 10% after laboratory adaptation time, unhealthy rat, abnormal rat (injury or defect) and dead rats. Researcher used 27 Wistar rats divided into 9 groups, which are 3 positive control groups, 3 treatment groups and 3 negative control groups. Each group consisted of 3 Wistar rats seen on days 2, 4 and 8.

This research procedure began with the sampling of Toman fish or Haruan fish. Fish sampling is done in Martapura Traditional market. Toman fish or Haruan fish used in this study weighed 11 kg. The part used was the fish flesh only. Each sample of Toman fish or Haruan fish that were going to be used started by cleaning the head and entrails and continued with the removing of the scales. The flesh then weighed until it reached 9.84 kg in weight. The flesh was put into a container and steamed in a pan for ± 30 minutes, then 7.5 ml pale yellow liquid that comes out of the flesh was taken and set aside. Toman fish or Haruan fish then wrapped with flannel cloth and put into the hydraulic press tool for pressing. 7.5 ml of Toman fish and Haruan fish extract were put into the reaction tube separately and centrifuged for 15 minutes at a speed of 6000 rpm. Centrifugation process resulted in the obtain of 700 ml of fluid and 50 ml of sediment which was then separated. Toman fish extract and Haruan fish were stored in dark glass bottles and covered with aluminum foil and clean pack.

The diabetic rat model was obtained by inducing STZ to rats at a dose of 35 mg / Kg BW. The rat were fed BR2 and then checked using a glucometer after 7 days. The rat were stated has diabetes mellitus when their blood glucose level exceeded 126 mg / dL.\textsuperscript{1,2,1} The physical condition of rat with diabetes appeared to be weak, inactive and lack of appetite.

Treatment in rat was started by adapting rat for 1 week in laboratory atmosphere, then divided into 9 treatment groups with 27 samples of Wistar rats and numbered according to their group. Rats were taken and sedated using diethyl ether. The incision wound was made 1cm long and 2mm in depth at the back of Wistar rats using a scalpel and disposable blade number 11, the blood that came out was cleaned with aquades.

Groups 1, 4 and 7 as negative controls which consisted of diabetic rats given with BR2 feed-only were then sacrificed on day 2,4, and 8. Groups 2, 5, and 8 as treatment group were given Toman fish extract 16 mL / Kg BW orally by using gastric sonde and given BR2 feed, then the rats were sacrificed on days 2, 4 and 8. Groups 3, 6 and 9 as positive control group were given Haruan fish extract 13.54 mL / Kg BB orally by using gastric sonde and given BR2 feed, then rats were sacrificed on the 2nd, 4th and 8th day.

Treatment for each rat was given orally in each group using gastric sonde twice daily for 8 days. The rat in each group were sacrificed to see the epithelial thickness on the wound healing process seen on day 2, 4 and 8. The researchers took the tissue on the Wistar rats back injury by biopsy around the 1 cm length and 2 mm in depth wound. The tissue from doing biopsy was fixated with a
10% Buffer Neutral Formalin (BNF) for 24 hours, followed by tissue processing and implantation on paraffin blocks. The paraffin block was then cut to 5 μm thick and placed on the object glass and inserted into the waterbath. The cutting results were then dried and given Hematoxylin Eosin (HE). All the rats that had been sacrificed were buried.

Histopathologic observations to measure epithelial thickness in Wistar rat wounds were observed with an Olympus BX41 light microscope to be captured with Olympus DP21 camera. Histopathology with 100x magnification with 1 field of view by dividing into 10 calculating lines and measuring the average of its epithelial thickness.

RESULT

Based on the result of the research, the researcher obtained the mean value of epithelial thickness on the 2nd, 4th and 8th day in all groups, as follows.

Table 1. Average (Mean±SD) Epithelial Thickness

<table>
<thead>
<tr>
<th></th>
<th>Given Feed only</th>
<th>Haruan Fish Extract</th>
<th>Toman Fish Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 2</td>
<td>50.38 ± 13.02</td>
<td>51.56 ± 3.25</td>
<td>76.68 ± 1.77</td>
</tr>
<tr>
<td>Days 4</td>
<td>66.48 ± 7.86</td>
<td>84.09 ± 7.59</td>
<td>99.21 ± 2.54</td>
</tr>
<tr>
<td>Days 8</td>
<td>100.51 ± 1.00</td>
<td>115.68 ± 4.36</td>
<td>124.23 ± 2.79</td>
</tr>
</tbody>
</table>

Figure 1. Graphic of Average Epithelium Thickness

Figure 1. shows the average of epithelial thickness of different Wistar rats’ wound in each group. The greater the increase in epithelial thickness, the better reepithelization process of the wound. The widest epithelial thickness between the three treatment groups was Toman fish extract, Haruan fish extract and feed only group respectively.

Figures 2, 3, 4. show the histopathological section of epithelial thickness on the Wistar rats' backs in one field of view. On the second day there has a phase of inflammation so that the thickness of the epithelium is still small and there is still not seen any thickness in some of them. On day 4, there is an early proliferation phase in which keratinocytes have begun proliferating. On day 8, it enters the peak phase of the reepithelization process with the highest epithelial thickness. This is the histopathologic image of only given feed, Haruan fish extract and Toman fish extract on days 2, 4 and 8. The epithelial thickness will be measured using ImageJ software by measuring from the basal membrane perpendicularly to the outermost epithelial layer.
The collected result data of the average of epithelial thickness was tabulated, followed by normality test using Saphiro-Wilk and homogeneity test using Levene’s test. The result of normality test of Saphiro-Wilk for epithelial thickness in group given Toman fish extract is $p = 0.171$, Haruan fish extract group is $p = 0.259$ and group given feed only was $p = 0.263$. The result of normality test Saphiro-Wilk for epithelial thickness in day 2 group was $p = 0.300$, the group on the 4th day was $p = 0.463$ and group on the 8th day was $p = 0.201$. Based on the result of Saphiro-Wilk normality test for treatment group and the observation day showed $p > 0.05$ so the data obtained was normally distributed. The data were tested with homogeneity test using Levene’s test and obtained for wound epithelial thickness is $p = 1.000$ ($p > 0.05$) which means the data distribution is homogeneous.

Normally distributed and homogeneous data were continued with data analysis using Two-way ANOVA parametric analysis with 95% confidence level. Two-way ANOVA test result showed $p = 0.0001$ ($p < 0.05$) which showed significant difference between treatments group and Post-hoc LSD analysis was done to know which group showed any significant difference.

From table 5.3 and table 5.4, it can be seen that each of the Toman fish extract group, Haruan fish extract and given feed only showed significant difference in each group. The results showed a different average of the epithelial thickness of the wound on Wistar rats’ back in each group. Post-hoc LSD test result showed that Toman fish extract has more in influence on the thickness of wound epithelium of diabetic rat than the Haruan fish extract and given only feed group.

<table>
<thead>
<tr>
<th>Tabel 5.2 Result of LSD statistic test on treatment for epithelial thickness.</th>
<th>Toman Fish Extract</th>
<th>Haruan Fish Extract</th>
<th>Only Feed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toman Fish Extract</td>
<td>0.000*</td>
<td>0.000*</td>
<td></td>
</tr>
<tr>
<td>Haruan Fish Extract</td>
<td>0.000*</td>
<td>0.001*</td>
<td></td>
</tr>
<tr>
<td>Only Feed</td>
<td>0.000*</td>
<td>0.001*</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Tabel 5.3 Result of LSD statistic test for observational day of epithelial thickness</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>0.000*</td>
<td>0.000*</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>0.000*</td>
<td>0.000*</td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>0.000*</td>
<td>0.000*</td>
<td></td>
</tr>
</tbody>
</table>

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

From table 5.3 and table 5.4, it can be seen that each of the Toman fish extract group, Haruan fish extract and given feed only showed significant difference in each group. The results showed a
The statistical test results in this study indicated that there were significant differences in the Toman fish extracts on wound healing processes in diabetic rats compared to normal rats. Post-hoc LSD test result showed that Toman fish extract is more influential on the thickness of wound epithelium of diabetic rat than the Haruan fish extract and given only feed.

**DISCUSSION**

The normal wound healing process occurs from the inflammation phase, proliferation phase till maturation phase, wherein the inflammatory phase occurs on day 1 to day 3 after lesion was. The inflammatory cells will work for bacterial phagocytosis and provide nutrients to support the wound healing process. In the granulation phase, there are formation granulation tissue, angiogenesis and reepithelization which ended by maturation or remodeling phase. Meanwhile, the diabetes mellitus wound has characteristic of hyperglycemia that can trigger the increase of Reactive Oxygen Species (ROS). In Diabetes mellitus wound, there is poor wound healing process due to persistent inflammation with elevated level of proinflammatory cytokines. The ongoing inflammatory phase will disrupt keratinocyte cells which will lead to error in cell signaling and disrupt cell migration. This occurs due to the occurrence of oxidative stress resulted from excessive increase in ROS. Excessive ROS may inhibit the reepithelization and give rise to the failure of wound closure. 

Excessive Reactive Oxygen Species (ROS) need to be captured by antioxidants. Antioxidant properties can be found in albumin found in Toman fish extracts. Albumin is known as a radical scavenger of antioxidant which in charge of capturing ROS because it has a hydride sulfur bond and a thiol group binds which function to attain the excessive ROS rapidly resulting in a reduced ROS. Low ROS is able to activate MMP-1 which can signal the EGF to help regulate keratinocyte proliferation during wound reepithelization by maintaining normal keratinocyte migration and proliferation.

The healing process in diabetic mellitus wound on epithelial thickness in this study was seen on days 2, 4 and 8. On day 2, there was an inflammation phase where, in normal circumstances the inflammation phase only lasted a few minutes to 25 hours after trauma and the longest time is 3 days. In wound healing in people with diabetes mellitus there is an increase in inflammatory cytokine level that will make prolonged inflammation. This condition can disrupt the cell and reduce cell function to respond to growth factor. Wound in diabetics who have given albumin on day 2 has been an inflammation phase in which macrophages have replaced the role of PMN cells for phagocytosis debris on day 2 - 3 after fibroblasts migration occurred to the wound area and begin proliferate to form granulation tissue before the inflammatory phase ends. The process of tissue proliferation granulation followed by cell migration process are required during the process of reepithelization like a macrophages. Macrophages will secrete growth factors that activate keratinocytes to allow keratinocytes migration into wounds to heal the defects.

The normal wound healing process on day 4 enters the proliferation phase where there has been a formation of granulation tissue to repair damaged tissue. This phase consisted of the granulation tissue formation with the process of new blood vessels formation (neovascularization), infiltration of inflammatory cell especially macrophages and fibroblasts (connective tissue) in the wound area. In the wound healing process in people with diabetes mellitus on day 4 is still undergoing an inflammatory phase where the increasing of inflammatory cytokines continues to occur which will degrade the formation of ECM, interfere the function of keratinocytes and disrupt the fibroblasts which is required in granulation tissue formation. In the healing process of patients with diabetes mellitus that has been given albumin, the granulation tissue has been formed. The process of reepithelization formed after granulation tissue occurs with keratinocyte migration process, keratinocytes begin proliferate to ensure adequate cell for wound wrapping. This proliferation process is aided by various growth factors such as HB-EGF, EGF, TGFα and KGF which helps to create the first layer to cover the wound.

During normal wound healing process on day 8, the proliferation phase occurs where the peak of reepithelization occurs on days 7 to 14. New reepithelization of wounds in people with diabetes mellitus will occur on day 10 to 14. This happens because of The uncontrolled prolong inflammation so reepithelization process is delayed. In the process of wound healing in diabetes mellitus that has been given albumin, albumin play a role in the granulation tissue development process. Albumin acts as a carrier of oxygen and other substances such as bilirubin, fatty acids, ions, hormones and medicines needed in the process of energy formation for the proliferation of epithelial cells and supporting the viability of the granulation tissue so that the wound can be enclosed. The granulation tissue can nourish and can physically support the repair of the upper layers, so keratinocytes can migrate and proliferate to close the wound in epidermal layer. This proliferation and migration occurs on average 17 times higher than normal, since the wound already has a surface so that the epithelial cells can proliferate easily at the wound edges.
fish extract group. Haruan fish extract and only give BR2 feed. Toman fish extract group is the most influential group on the epithelial thickness of wound in diabetes mellitus compared to Haruan fish extract group and only given BR2 feed. Differences in epithelial thickness between groups were due to different albumin levels in both extracts. Based on research Firliyanti et al in 2013, Toman fish contains 5.35% albumin while Haruan fish only contains 4.53% albumin. This is in accordance with research from Murdani et al in 2015, where the Wistar rat with diabetes mellitus given the extract Toman fish containing albumin has a faster healing time. This research is also in accordance with Marjiyanto and Lilis research in 2013, where the higher levels of albumin can accelerate the wound healing process. Based on this research, it can be concluded that there is an effect of Toman fish extract dose 16 ml/Kg BW in the form of epithelial thickness improvements in wounds of Wistar rats induced with diabetes mellitus in day 2, 4 and 8.

REFERENCES


