The Effect Application of Low Intensity Pulse Ultra Sound on Wounds Healing and Angiogenesis of Diabetes Mellitus Type 2 Rat Model

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ABSTRACT
Wound healing disorder are often found in people with diabetes mellitus. Low Intensity Pulse Ultrasound (LIPUS) therapy was developed to accelerate the wound healing process. The purpose of this study was to determine the effect of LIPUS on wound closure and angiogenesis after skin excision in rats model diabetes mellitus type 2. Twenty-four Sprague dawley rats that match the inclusion criteria were made into type 2 diabetes mellitus by injecting Nicotinamide and Streptozotocin, then devided into six group: DM3, DM7, DM14, DML3, DML7, DML14, with 4 rats each. All rats were made excision wound with punch biopsy in the back area. DML group receive LIPUS therapy in the wound area (frequency 3 MHz, intensity 0.5 W/cm², duty cycle 20%, duration 3 minutes every day) for three days (DML3), seven days (DML7) and fourteen days (DML14). Wound area was measured and the tissue was staining with hematoksciline eiosin to observe the density of angiogenesis. Two Way ANOVA used to analyze the differences in the variable wound area and angiogenesis density. Post Hoc LSD to analyze the differences in variables between groups and the day of observation, showed the area of the wound in the DML was lower than in the DM group significant on day 3 and 7, whereas the density of the angiogenesis in DML group was higher than DM group significant on day 7 and 14. The Pearson test showed a correlation between the wound closure and the angiogenesis density (p = 0,000; r = -0,785). Conclusion: LIPUS therapy accelerate wound closure on day 3 and 7 and increase the density of angiogenesis on day 7 and 14.

1. Introduction
Wounds are damage or disruption of normal anatomical structures and functions in the body. Wounds can vary from simple, that is, to the epithelium of the skin and deeper wounds extending to the subcutaneous tissue. Injuries can occur due to surgery, mechanical or thermal trauma which can cause damage to other structures such as tendons, muscles, blood vessels and bones. Diabetes is a chronic metabolic disorder resulting in hyperglycemia due to defects in insulin secretion, insulin action, or both, leading to a physiological predisposition to myrovascular, macrovascular, and neuropathic complications. Diabetes mellitus type 2 occurs due to decreased ability of insulin to work in peripheral tissues (insulin resistance) and β cell dysfunction so that it is unable to produce sufficient insulin to compensate.

Diabetic neuropathy causes a decrease in the angiogenesis process in wound healing and will reduce vascularity and the number of capillaries. DM will inhibit the narrowing of the wound, and chronic wound conditions are difficult to experience healing. Therapeutic methods to accelerate the healing process of wounds on the skin have been developed. The use of mechanical stimulation therapy to accelerate wound healing has begun to be developed, one of the tools
being developed recently is extra corporeal shock wave treatment, electrical stimulation and Low Intensity Pulsed Ultrasound (LIPUS). Mechanical energy is delivered by LIPUS into living tissue as acoustic pressure waves which will produce micromechanical tensile strength on living tissue which will stimulate the formation of angiogenesis and produce the effect of accelerating wound healing. The purpose of this study was to determine the effect of LIPUS therapy on the speed of wound constriction of type 2 diabetes mellitus models, to determine the effect of LIPUS therapy on increasing the amount of angiogenesis in the healing of punch biopsy wounds in type 2 DM model mice and to see the correlation between the amount of angiogenesis and the narrowing of punch biopsy wounds in rat models. DM type 2 in LIPUS treatment.

2. Methods

The research ethical clearance number 0012 / EC-FKH / Ex / 2020 issued by the Faculty of Veterinary Medicine UGM Yogyakarta. Twenty-four Sprague dawley rats aged 3-4 months with a body weight of 200-250 grams were adapted for 2 days before being made into type 2 diabetes by injecting Nicotinamide and Streptozotocin. All rats were randomly selected and then divided into 2 groups, namely the LIPUS therapy group (DML) and without LIPUS therapy (DM), then each group was further divided into 3 groups according to the day of decapitation (days 3, 7, and 14), namely DM3, DM7, DM14, DML3, DML7 and DML14. Surgery and maintenance are carried out at the PSPG UGM Nutrition Laboratory.

Punch biopsy excision wound was made on the 5th day after NA and STZ induction. Rats were given a prophylactic intramuscular injection of cefazolin at a dose of 50 mg / kgBW. Anesthesia is performed by intramuscular injection of ketamine at a dose of 80 mg / kg BW. The dorsal haircut was performed while the rats were anesthetized, disinfected with 10% povidone iodine, then excised a round biopsy with a diameter of 5 mm, sub cutaneous depth (Figure 1).

Mechanical restraint method was carried out to fix the mice during treatment. The application of Low Intensity Pulsed Ultrasound (LIPUS) is carried out once a day by attaching the LIPUS transducer which has been previously smeared with a coupling agent to the wound. The dose of administration and duration of LIPUS therapy for each subject in group A were the same, namely 3 MHz frequency, 0.5 W / cm² intensity, 20% duty cycle and 3 minutes exposure duration per day for 3, 7, and 14 days (Figure 2).

The skin tissue on the back of the mice was taken in the shape of a 3x3 cm square with the post-excision wound in the middle and subcutaneous depth according to the day of observation (3rd, 7th, and 14th day). Tissue samples taken were stored in a container containing a buffer of 10% formaldehyde. The rats were sacrificed after sampling using intramuscular injection of ketamine (150 mg / kgBW) and after that the rats were incinerated according to the standard.

The rats soaked in 10% formalin buffer were processed into histological preparations. The staining process is carried out after all the preparations have been made. The clinical condition was observed that the wound area was measured indirectly using ImageJ application. Hematoxylin Eosin staining was used to see angiogenesis. Wound narrowing is the reduction in wound area when measured on days 3, 7 and 14. Measurements were made by taking clinical photographs with a millimeter block size comparison, and then the actual wound area was measured by the ImageJ application.

The angiogenesis observation was done by counting the number of new blood vessels per 6 field of view with a magnification of 400x, added up, then divided by 6 to obtain the average of each slide. The new blood vessels on histological observation appear to be cavities surrounded by flat-shaped endothelial cells which contain erythrocytes.

The data obtained were tested using Two-way ANOVA to analyze the effect of LIPUS therapy and the length of treatment days on wound narrowing and the amount of angiogenesis in each group. After that, the Post Hoc test was carried out using the LSD (Least Significance Difference) method to determine which variables had differences between groups and days.
Finally, the Pearson test was performed to determine the correlation between wound narrowing and the amount of angiogenesis.

3. Results

The widest wound was found in the DM3 group, while the narrowest was in the DML14 group. The highest amount of angiogenesis was in the DML7 group, while the least was in the DM3 group. The group of DM mice that were given LIPUS therapy had narrower wounds and denser angiogenesis than the group of DM mice that were not given LIPUS therapy.

The Two Ways ANOVA test results showed that there were significant differences in the variable wound area and the amount of angiogenesis both between groups and between observation days with a value of p = 0.000 (p <0.05) (table 2). Comparison of the wound area and the amount of angiogenesis between groups and the day of observation followed by the Post Hoc LSD (Least Significance Difference) test.

Based on the results of the Post Hoc LSD test for the wound area variable, it was found that there was a significant difference (p <0.05) in the variable wound area between groups (Figure 4) on the 3rd and 7th day. The variable wound area between groups on day 14.

Based on the results of the Post Hoc LSD test for the variable amount of angiogenesis, it was found that there was a significant difference (p <0.05) in the variable amount of angiogenesis between groups (Figure 5) on day 7 and day 14. There was no significant difference found p = 0.152 (p <0.05) on the variable amount of angiogenesis between groups on day 3.

Based on the results of the Post Hoc LSD test for the variable amount of angiogenesis between groups and the day of observation (Figure 5), there was a significant difference (p <0.05) in the variable amount of angiogenesis between the observation groups day 3 to day 7 and day 3 to day 14. Insignificant results were obtained in the observation group day 7 to day 14 both in the DM group p = 0.070 (p > 0.05) and DML p = 0.216 (p > 0.05).

Pearson's correlation test was conducted to determine the correlation between wound area and the amount of angiogenesis. Figure 6 shows that there is a significant correlation between the area of the wound and the amount of angiogenesis with a p value of 0.000 (p <0.05), while the correlation coefficient (r) is -0.742 (negative), meaning that the more angiogenesis the narrower the wound.

Figure 1. The process of making a punch biopsy excision wound on the back of Sprague Dawley rats that have been made into type 2 DM.
Figure 2. The process of giving LIPUS therapy with a mechanical restraint method

Table 1. Mean wound area and amount of angiogenesis between observation days

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group of Rats</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(\bar{x} \pm SD)</td>
<td>(\bar{x} \pm SD)</td>
<td>(\bar{x} \pm SD)</td>
</tr>
<tr>
<td>Wound area</td>
<td>DM3, DM7, DM14</td>
<td>23.21 ± 2.16</td>
<td>12.29 ± 1.89</td>
<td>6.65 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>DML3, DML7, DML14</td>
<td>21.01 ± 1.73</td>
<td>10.01 ± 1.97</td>
<td>5.06 ± 1.69</td>
</tr>
<tr>
<td>Total Angiogenesis</td>
<td>DM3, DM7, DM14</td>
<td>0.54 ± 0.08</td>
<td>1.67 ± 0.14</td>
<td>1.41 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>DML3, DML7, DML14</td>
<td>0.83 ± 0.30</td>
<td>2.54 ± 0.42</td>
<td>2.04 ± 0.37</td>
</tr>
</tbody>
</table>

Information:
\(\bar{x}\) = average; SD = Standard Deviation
Figure 3. Microscopic photos of angiogenesis of DM and DML groups on days 3, 7 and 14 of observation.

Table 2. Two Ways ANOVA Test Wound area and amount of angiogenesis between groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>p value of wound area</th>
<th>p value the amount of angiogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (DM and DML)</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
<tr>
<td>Day (3, 7 and 14)</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

Information (*) = significant (p < 0.05)

Figure 4. Comparison of the wound area graph between observation days, (*) = significant (p < 0.05)
4. Discussion

Low Intensity Pulse Ultrasound (LIPUS) therapy is a breakthrough that is currently being developed as a wound healing therapy which has two effects, namely thermal and non-thermal. The non-thermal effect of LIPUS on the skin is in the form of mechanical effects, namely cavitation, acoustic streaming and micromassage. These three things will modulate cell biochemistry, stimulate cell viability and tissue repair, which in turn can affect the structure, function and permeability of the cell membrane which causes the transfer of intra-cellular and extra-cellular ions and stimulates anti-inflammatory molecules, thereby spurring the angiogenesis process and accelerating it. wound healing. Low Intensity Pulse Ultrasound (LIPUS) therapy has the ability to penetrate the deepest tissue of the wound. 

This study was conducted to determine the effect of LIPUS on wound closure and the amount of angiogenesis after skin excision in Sprague dawley rats with type 2 diabetes mellitus model. The observed wound narrowing was the reduction in wound area measured on days 3, 7 and 14 which was compared between DM groups. and DML, while the amount of angiogenesis observed was the amount of angiogenesis per field of view observed on days 3, 7 and 14 which were compared between the DM and DML groups. The strength of this research is that the observation is done clinically and microscopically so that it can be seen the correlation between microscopic cellular factors and clinical conditions in wound healing in DM rats.

Diabetes mellitus will cause an extension of the inflammatory phase, which will affect the decrease of Vascular Endothelial Growth Factor (VEGF) and disruption of the epithelialization process, resulting in a decrease in wound narrowing where the prolonged
inflammatory and infectious process will affect the slow epithelialization process. Wound healing is greatly influenced by the angiogenesis process in the epithelialization process, the faster the epithelialization process, the faster the wound will narrow and heal. Wound narrowing is a parameter of successful wound healing. The process of narrowing the wound can be seen from the narrowing of the wound to closing and functioning normally again.

All study subjects experienced significant weight loss in all groups after induction of NA and STZ, which was due to the effects of hyperglycemia caused by insulin deficiency. Changes in fat tissue and protein metabolism in muscles with an average weight loss of 25% and 14% of the initial body weight after NA and STZ induction. High blood sugar levels in DM sufferers cannot be used optimally to form energy so that a lot of glucose is wasted through the urine so that muscle mass and adipose continue to be used for energy fulfillment.

Blood sugar levels at time (GDS) type 2 diabetes mellitus mice in this study increased to more than 300 mg/dL, starting at H + 5 post induction. NA induction carried out 15 minutes before STZ induction serves to protect pancreatic Beta Langerhans cells from STZ exposure so that blood sugar does not rise too high after STZ induction, NA has been shown to be able to control the GDS levels of mice so they don’t increase too high. The increase in GDS levels is caused by the effect of STZ induction which damages the pancreatic beta langerhans cells and has a direct impact on the disruption of insulin secretion. Insulin is an anabolic hormone that is required in the uptake of glucose and amino acids by peripheral tissues.

All groups that were given LIPUS therapy showed faster narrowing of the wound than the groups that were not given LIPUS therapy. The results showed that the wound in the DML3 group was significantly narrower than the DM3 group (p: 0.006), the wound in the DML7 group was significantly narrower than the DM7 group (p: 0.016), the wound in the DML14 group was significantly narrower than the DM14 group (p: 0.239). There was no significant difference in wound closure on the study day 14. Complication of diabetes is damage to multiple organs and one of them is the pancreas itself due to insufficient nutrition. The result is a decrease in the ability of the pancreas itself to meet the body’s need for insulin. The skin is the tissue that is affected due to the worsening condition of the pancreas due to uncontrolled type 2 diabetes. The longer the ability of the pancreas will decrease which in turn will affect the wound healing process. The results of this study indicate that on day 14 there is no significant difference in wound narrowing, possibly due to a decrease in wound narrowing speed due to disruption of the healing process due to uncontrolled diabetes and an increase in blood sugar in mice every day.

All groups that were given LIPUS therapy showed more angiogenesis when compared to the groups that were not given LIPUS therapy. The results showed that angiogenesis in the DML3 group was not significant more than in the DM3 group (p: 0.152), angiogenesis in the DML7 group was significantly more than in the DM7 group (p = 0.000), angiogenesis in the DML14 group was significantly more than in the DM14 group (p = 0.005). The highest amount of angiogenesis occurred on day 7, at the peak of inflammation during wound healing. Microbubble cavitation causes mechanical injury to the cells and surrounding tissue which will give the effect of "ultrasonic drilling" and cause injury to the endothelial cells of blood vessels, causing temporary inflammation of endothelial cells and triggering the secretion of endogenous VEGF which then triggers the angiogenesis process. The wound healing process of DM is disrupted in the initial phase of inflammation and experiences obstacles to a further stage. Patients with DM will experience a decreased ability to infiltrate immune cells, neutrophils and macrophages which will hinder the wound healing process. The initial phase of the 3rd day of healing in this study is likely to experience interference so that it reduces the effectiveness of LIPUS application to the wound up to day 3, so that on day 3 the amount of angiogenesis between DM and DML shows insignificant results in this study. The decrease in the ability of
immune cell infiltration will inhibit the wound healing process by increasing the inflammation time so that diabetes does not show a significant decrease between day 7 and day 14 of observation. The use of ultrasound can accelerate wound narrowing by significantly increasing angiogenesis in chronic wounds. Angiogenesis in the DML group was more than in the DM group, and the wound in the DML group was narrower on all groups on the day of observation, this proved that LIPUS therapy was effective in increasing the amount of angiogenesis on days 7 and 14 which had an impact on increasing tissue growth rate and wound narrowing, especially on days 3 and 7. The increase in the amount of angiogenesis in LIPUS therapy is caused by mechanical energy delivered by LIPUS into living tissue as acoustic pressure waves which produce micromechanical tensile strength in living tissue which will stimulate the formation of angiogenesis, and produce the effect of accelerating wound healing. Research by Hanawa et al., (2014) in their research states that the use of LIPUS has a significant effect on the formation of angiogenesis in chronic wounds. Wound healing is very much influenced by the angiogenesis process which continues to the epithelialization process which continues with tissue growth and experiences healing. Research by Haus et al., (2014) states that the application of LIPUS will significantly increase the formation of angiogenesis and accelerate wound healing on day 7 and day 15, this is consistent with this study which shows that angiogenesis reaches a significant value on day 7 and 14 between the DM and DML groups. This study proves that the Low Intensity Pulse Ultrasound therapy with parameters of 3 MHz, an intensity of 0.5 W / cm², a duty cycle of 20%, with a duration of 3 minutes of exposure given every day can significantly increase the speed of wound narrowing after skin excision on day 3 (p = 0.006) and day 7 (p = 0.016) rat type 2 diabetes mellitus model. Low Intensity Pulse Ultrasound therapy can significantly increase the amount of angiogenesis on day 7 (p = 0.000) and day 14 (p = 0.005) post-excision wound healing mouse skin type 2 diabetes mellitus model. There is a correlation between wound narrowing and the amount of angiogenesis after skin excision in type 2 diabetes mellitus model mice (p value = 0,000; r = -0.742), meaning that the more angiogenesis the wound is narrower.

6. References


