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RESEARCH ARTICLE

Effect of low-intensity pulsed ultrasound on mast cell degranulation and fibroblast expression on type 2 diabetes mellitus rats wound healing process

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ABSTRACT

Impaired wound healing is one of the Diabetes mellitus complications. Low-intensity Pulsed Ultrasound (LIPUS) therapy may accelerate the impaired wound healing. The use of LIPUS therapy in the early inflammatory phase can induce mast cell degranulation, and in the proliferative phase it can increase collagen synthesis by fibroblasts. The purpose of this study was to determine the effects of LIPUS therapy on mast cell degranulation and fibroblast expression in the healing process of punch biopsy wound in rats with type 2 diabetes mellitus. Twenty-four *Sprague dawley* (n=24) were designed into type 2 diabetes mellitus by injecting Nicotinamide and Streptozotocin, then divided into six groups: diabetes mellitus without LIPUS (DM3, DM7, DM14) and diabetes mellitus with LIPUS (DML3, DML7, DML14), 4 each, and punch biopsy wounds were made on the dorsal skin. The DML group received LIPUS therapy in the wound area (frequency 3 MHz, intensity 0.5 W/cm^2, duty cycle 20%, duration 3 minutes every day for 3 days (DML3), 7 days (DML7), and 14 days (DML14). The wounded tissue area was stained with toluidine blue to observe mast cell degranulation and immunohistochemical type HSP-47 to observe fibroblast expression. Two-Way ANOVA and Post Hoc LSD tests were used to determine the differences in mast cell degranulation and fibroblast expression. The results showed that mast cell degranulation and fibroblast expression in the DML group were higher than in the DM group (table 1). Pearson test showed a correlation between mast cell degranulation and fibroblast expression (p= 0.00; r= 0.839). LIPUS therapy increases mast cell degranulation and fibroblast expression in type 2 diabetes mellitus rat model. The higher the mast cell degranulation, the higher fibroblast expressions.

Keywords: fibroblast expression; Low-intensity Pulsed Ultrasound (LIPUS); mast cell degranulation; type 2 diabetes mellitus

INTRODUCTION

Wounds are defined as damage or impairment of normal anatomical structure and function.[1] Wound healing is the body's physiological process of replacing and restoring the functions of damaged tissue. Having a supportive environment on the wound surface is essential to maximize wound healing potential.[2] Factors which can interfere wound healing are infections, necrotic tissues, and vascular conditions. In addition, there are also physical and psychological factors such as nutritional status, disease state (diabetes, cancer, arthritis), and mental health problems, which can impact wound healing.[3]

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 microvascular, macrovascular, and neuropathic complications.[4] Type 2 diabetes mellitus occurs due to the decreased ability of insulin function in peripheral tissues (insulin resistance) and β cell dysfunction so that it is unable to produce enough insulin to compensate.[5]

Diabetes causes decreased mast cell degranulation, decreased microvascular response to mast cell mediators (histamine, cytokines and arachidonic acid) in the inflammatory phase, which is a sign of the body's inability to respond to acute inflammatory conditions in wounds.[6,7] Fibroblasts will experience dysfunction in collagen secretion in people with diabetes mellitus, Hence the collagen

produced is easily degraded. The decrease in the number of fibroblasts occurs due to the increased apoptosis and the decreased proliferation.[8]

Ultrasonic therapy has been used for decades as a physical therapy to accelerate wound healing.[9] Various biological effects associated with ultrasonic therapy have been inve stigated *in vitro* and *in vivo*. Fyfe and Chahl (1984)[10] stated that ultrasonic therapy induces degranulation of mast cells and increases vascular permeability in rat leg joints, thereby releasing histamine which plays a role in wound healing. Zhou (2004)[11] stated that ultrasonic on normal skin can promote fibroblast proliferation by activating the integrin receptor and the Rho-associated coiled-coil-containing protein kinase (Rho/ROCK), Src, and ERK signaling pathway.

This study aims to determine the effect of LIPUS therapy on mast cell degranulation and fibroblast expression in the excision wound healing process on the dorsal of type 2 diabetes mellitus model rats, and to determine the correlation between mast cell degranulation and fibroblast expression in the excision wound healing process of type 2 diabetes mellitus model rats.

MATERIALS AND METHODS

The research ethical clearance number 0012/EC-FKH/Ex/2020 was issued by the Faculty of Veterinary Medicine UGM Yogyakarta. Twenty-four male Sprague dawley rats aged 3-4 months with body weight of 200-250 grams were adapted for 2 days before being made into type 2 DM by injecting Nicotinamide (NA) and Streptozotocin (STZ). All rats were randomly selected and then divided into 2 groups, namely A (with LIPUS therapy) and B (without LIPUS therapy), then divided into 3 groups according to the day of decapitation (day 3, 7, and 14). Surgery (punch biopsy excision) and maintenance (foods and drinks) are carried out at the Pusat Studi Pangan dan Gizi (PSPG) UGM Nutrition Laboratory.

A 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 was performed by intramuscular injection of ketamine at a dose of 80 mg/kgBW. 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, using Seamless Premier Uni-Punch (disposable biopsy punch) model 9033525 (Figure 1).

Mechanical restraint method was carried out to fix the rats during the treatment.[12] The application of Low-intensity Pulsed Ultrasound (LIPUS) was performed once a day by attaching the LIPUS transducer which had 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 once a day for 3, 7, and 14 days (Figure 2).

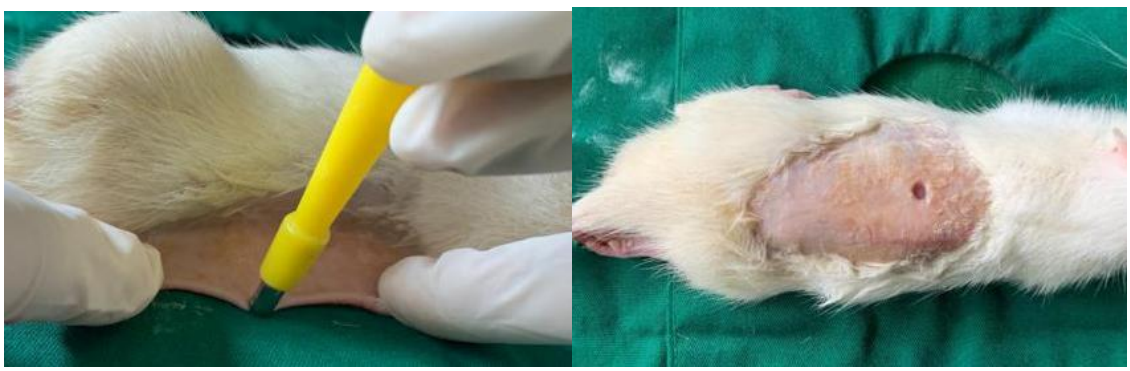

Figure 1. The process of making a punch biopsy excision wound on the dorsal of Sprague dawley rats that have been made into type 2 DM.

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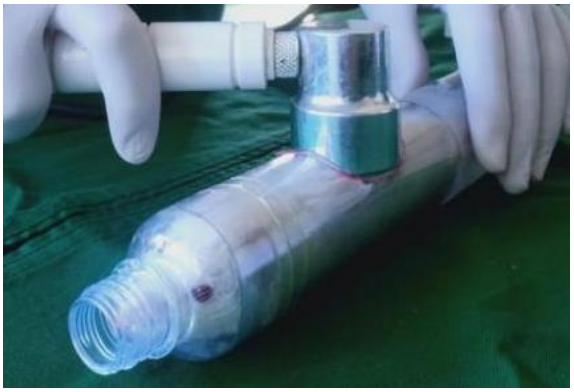

Figure 2. The process of giving LIPUS therapy with mechanical restraint method.

The skin tissue on the dorsal of the rats was taken in the form of a 3x3 cm^2 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 containers containing 10% formalin buffer. The rats were sacrificed after sampling using intramuscular injection of ketamine (150 mg/kgBW) and after that incinerated according to the standard.

Rat skin soaked in 10% formalin buffer was processed into histological preparations (Figure 3). The staining process was performed after all the preparations had been made. Toluidine blue staining was used to observe the degranulation of mast cells, while to observe the expression of fibroblasts IHC, HSP-47 staining was used.

Mast cell degranulation was observed using a 400x magnification microscope. 10 photographs were taken from different skin layers, 4 photos of the papillary stratum, 3 photos of the mid-reticular stratum, and 3 photos of the lower part of the reticular stratum. The number of degranulated mast cells from these 10 photos was then added up in units of MCs/mm^2.

Observation of fibroblast expression was performed by counting the number of cells per 6 field of view with a magnification of 400x, added up, then divided by 6 to obtain the mean of each slide. The fibroblasts stained with Immunohistochemistry (IHC) Heat-Shock Protein (HSP) 47 appeared brownish in color, spindle-shaped and tended to be arranged in a fascicular or cartwheel pattern. Mast cell degranulation and fibroblast expression was observed by trained pathology anatomy residents.

The data of mast cell degranulation and fibroblast expression were tested by using Two-Way ANOVA to analyze the effects of LIPUS therapy and treatment days on mast cell degranulation and fibroblast expression in each group. The Post Hoc test using the LSD (Least Significance Difference) method to determine which variables had differences between groups and days, and the Pearson test was conducted to determine the correlation between mast cell degranulation data and fibroblast expression.

RESULTS

Random Blood Sugar (RBS) levels of type 2 diabetes mellitus rats in this study increased to >200 mg/dL, starting on D+5 after NA and STZ induction. The increase in RBS in rats was also accompanied by other symptoms of diabetes such as a lot of eating, lots of drinking, strong urine odor, and more urine. The increased RBS level was indicated by the value of p= 0.028 (p<0.05) in both the DM and DML groups. This increase in RBS occurred due to the damage to Langerhans beta cells in the rat's pancreas due to STZ induction.[14] NA induction carried out 15 minutes before STZ induction is proven to be able to control the RBS levels of rats so that they do not increase too high. The beneficial effect of NA administration on blood sugar was the protection of pancreatic Langerhans beta cells against STZ, as well as increasing

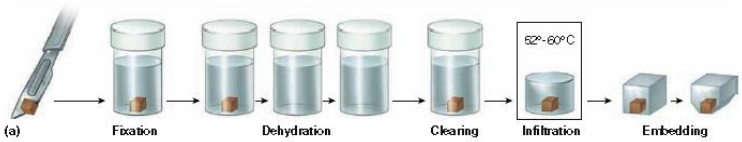

Figure 3. The process of making histological preparations[13]

insulin in the blood. The protective mechanism of NA against STZ-induced Langerhans beta cells included two main effects, namely PARP-1 inhibition and NAD^+ supply.[15]

The highest mast cell degranulation was found in the DML7 group, while the lowest was in the DM14 group. The highest fibroblast expression was observed in the DML14 group, while the lowest was in the DM3 group. The group of DM rats that were given LIPUS therapy had higher mast cell degranulation and fibroblast expression than the group of DM rats that were not given LIPUS therapy. Microscopic features of mast cell degranulation and fibroblast expression between DM and DML groups on the day of decapitation are shown in Figures 4 and 5.

The results of the Two-Way ANOVA test showed that there were significant differences in the variable mast cell degranulation and fibroblast expression in intergroup variables with a p value of <0.05. The results of the Two-Way ANOVA test also showed significant differences in the variable mast cell degranulation and fibroblast expression on the variables between observation days with a value of p <0.05 (Table 2).

Comparison of mast cell degranulation and fibroblast expression between groups and observation days were followed by the Post Hoc LSD (Least Significance Difference) test. Based on the results of the Post Hoc LSD test, it was found that there was a significant difference (p <0.05) in the mast cell degranulation variables both between groups and between observation days (Figure 6). The results of the Post Hoc LSD test on the fibroblast expression variable showed significant differences (p<0.05) in the fibroblast expression variable both between groups and between observation days (Figure 7).

Pearson correlation test was conducted to determine the correlation between mast cell degranulation and fibroblast expression. There was a correlation between mast cell

Table 1. Average number of mast cell degranulation and fibroblast expression between observation days

Variable	Rat groups	3rd day (x±SD)	7th day (x±SD)	14th day (x±SD)
Degranulation mast cells	DM	0.57 ± 0.06	0.64 ± 0.05	0.42 ± 0.03
	DML	0.66 ± 0.02	0.72 ± 0.03	0.52 ± 0.03
Fibroblasts expression	DM	17.41 ± 0.54	158.65 ± 7.59	448.25 ± 9.97
	DML	54.90 ± 6.60	196.21 ± 27.33	484.21 ± 11.40

Notes:
x = mean; SD = Standard Deviation

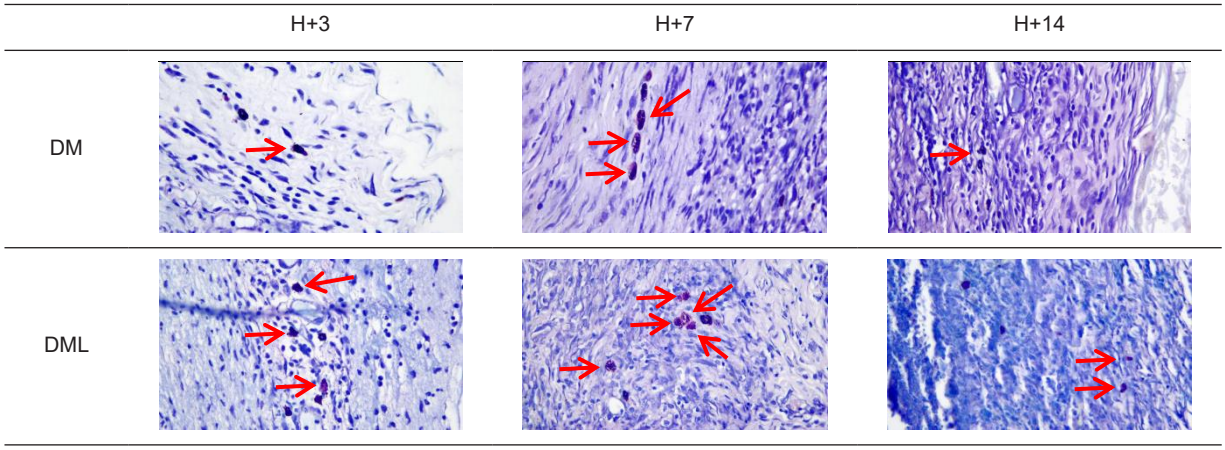

Figure 4. Microscopic picture of mast cell degranulation (shown with red arrow) in DM and DML groups on D+3, D+7, and D+14

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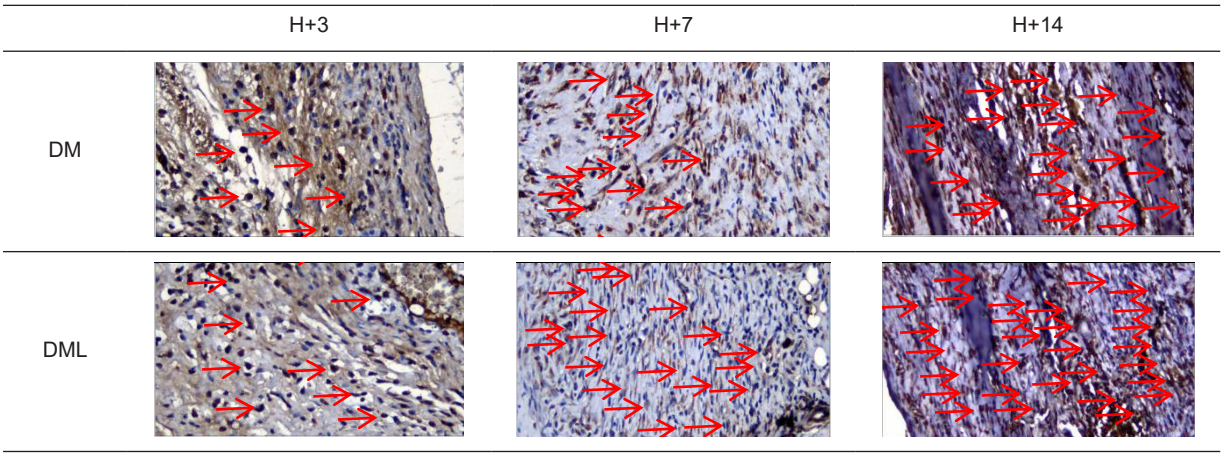

Figure 5. Microscopic picture of fibroblasts expression (shown with red arrow) in DM and DML groups on D+3, D+7, and D+14

Table 2. Two Ways ANOVA test (comparison of mast cell degranulation variables and fibroblast expression)

Variable	p value degranulation of mast cells	p value fibroblast expression
Groups (DM and DML)	0.000*	0.002*
Day (3rd, 7th, and 14th)	0.000*	0.000*

Notes:
p = Two-Way ANOVA test; * = significant (p<0.05)

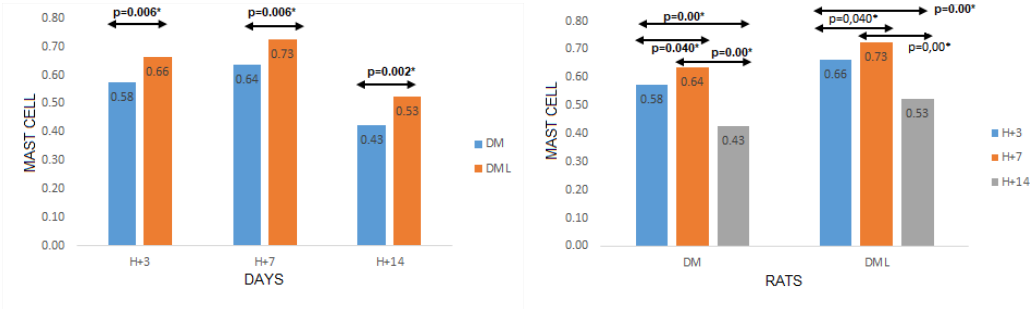

Figure 6. Comparison of mast cell degranulation between groups and between observation days (p <0.05)

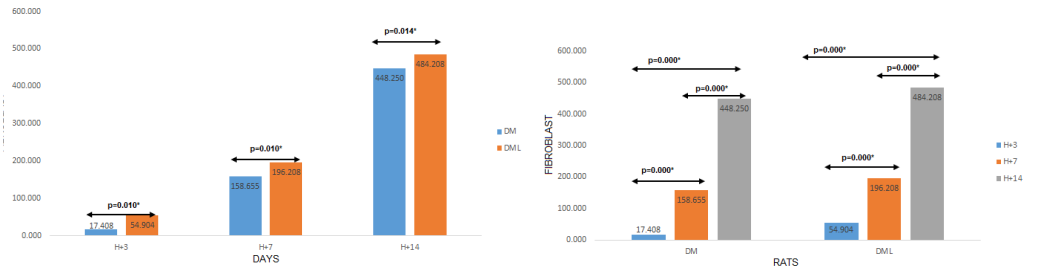

Figure 7. Comparison of fibroblast expression between groups and between observation days (p <0.05)

degranulation and fibroblast expression with a significant p value of 0.00 (p <0.05), while the correlation coefficient (r) was 0.839 (positive) meaning that the higher the degranulation of mast cells, the more the expression of fibroblasts. Conversely, the lower the mast cell degranulation, the lower the fibroblast expression (Figure 8).

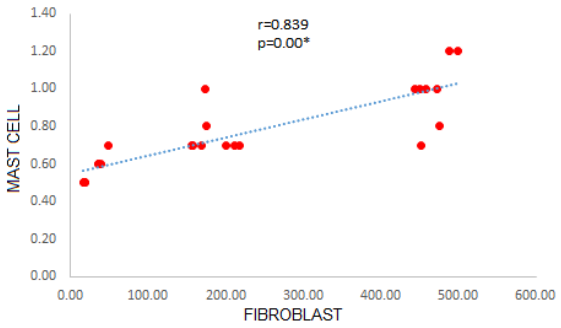

Figure 8. Pearson correlation test between mast cell egranulation and fibroblast expression in the DM and DML groups.

DISCUSSION

Ultrasonic therapy is widely used for examination of clinical features, and it is also a type of physiotherapy for the treatment of hard and soft tissues. Low-intensity Pulsed Ultrasound (LIPUS) is a non-invasive therapy which transmits energy transcutaneously to biological tissue. LIPUS therapy has two kinds of effects, namely thermal and non-thermal. Ultrasonic waves can interact with one or more inflammatory components and initial resolution of inflammation by increasing the immune response, modulating vasoconstriction, lymphocyte adhesion to the endothelium, degranulation of mast cells, macrophage phagocytes, production of growth factors for macrophages, calcium changes in fibroblasts, angiogenesis, cell proliferation T, and osteoblasts. The interaction of these factors will accelerate the tissue healing process.[16]

This quasi-experimental study was conducted to determine the effects of Low-intensity Pulsed Ultrasound (LIPUS) on mast cell degranulation and fibroblast expression in the excision wound healing process on the dorsal of type 2 diabetes mellitus model rats. Mast cell degranulation observed was the number of mast cells that had degranulation seen on the 3rd, 7th, and 14th day. Fibroblast expression was the number of fibroblasts on the day of observation, namely day 3rd, 7th, and 14th.

The body weight of the rats decreased significantly, starting from D+5 after NA and STZ induction until the day of decapitation, indicated by a p value of <0.05 in all groups, both control and treatment groups. According to Nakhaee et al., characteristics of diabetics are weight loss, polyphagia, and polydipsia. Weight loss that occurs in diabetics can be caused by waste of protein in the absence of carbohydrates which are used as an energy source.[17] Nugroho added that the symptoms of thirst and hunger are the result of fluid loss and the body's inability to use nutrients. All of these result in a significant reduction in muscle mass and adipose tissue.[16]

Administration of STZ and NA in adult rats caused partial damage to B cells which led to a decrease in blood insulin and an increase in glucose in these animals. The severity of STZ and NA-induced diabetes was much lower than that of STZ-induced diabetes alone; rats exhibit moderate hyperglycemia and do not require exogenous insulin to survive. Diabetes caused by STZ and NA remains stable for a long time, thus this diabetes model is suitable not only for the short term, but also for long-term animal studies.[17]

The groups that were treated with LIPUS therapy showed more degranulation of mast cells than the groups that were not treated with LIPUS. Ultrasound interacts with several components of inflammation, resulting in an earlier resolution of inflammation. Ultrasonic therapy performed in the inflammatory phase stimulates mast cells, platelets, white blood cells, phagocytes, and macrophages. The sonication process will induce degranulation of mast cells and release arachidonic acid which is a precursor to the synthesis of inflammatory mediators.[18] One of the chemical mediators that plays a role in the wound area is histamine. Mast cells are the main source of histamine which is released through the degranulation process of mast cells. Ultrasonic therapy given immediately after injury can stimulate mast cell degranulation and release histamine. Ultrasonic stimulates mast cell degranulation by increasing cell permeability to calcium, which acts as an intracellular messenger for the appropriate metabolic response.[19] The benefits of ultrasonic therapy with an intensity of 0.5 W/cm^2, which is a 20% broken wave related to non-thermal effects, contribute to the stimulation

of substance transport and modification of cell permeability.[20] Mast cell degranulation increased on the 7th day after injury, along with vascularity and fibroblast count.[21]

A wide variety of biological effects associated with ultrasonic therapy have been identified *in vitro* and *in vivo*. 1-3 MHz frequency and intensity between 0.1-1.5 W/cm^2 are the parameters used. Ultrasonic therapy can affect changes in cell membranes, including cellular adhesion, membrane permeability, calcium ion flux, biological protein levels, and proliferative processes. Non-thermal ultrasonic therapy can increase intracellular calcium levels, fibroblast proliferation, collagen production, and fibroblast migration patterns. These biological effects are in accordance with the theory that non-thermal ultrasonic therapy improves fibroblast function which will increase collagen synthesis and matrix repair.[22]

The results of this study indicated that the fibroblasts expression in the group treated with LIPUS therapy was more than the group without LIPUS treatment. Research conducted by De Oliveira, et al using LIPUS with an intensity of 0.3 W/cm^2 (10-20%) and 0.5 W/cm^2 (20%) can increase the percentage of cell viability in fibroblast culture, where significant results are obtained at an intensity of 0.5 W/cm^2 (10%).[20] Maan, et al. stated that low-intensity ultrasonic therapy significantly accelerate wound healing in diabetic rats. The increase in collagen deposition and skin thickness that occurs due to increased fibroblast proliferation, suggests that this therapy improves the integrity of diabetic wounds. Low-intensity ultrasonic therapy can affect the physiology of fibroblasts, stimulating their proliferation *in vitro*.[23]

This study used the LIPUS brand Ultrason 101 produced by Pioneer with a frequency specification of 3 MHz, an intensity of 0.5 W/cm^2, a duty cycle of 20%, and an exposure duration of 3 minutes per day for 3 days, 7 days, and 14 days. Daily low-intensity ultrasonic stimulation with intermittent energy can increase cell proliferation, which is a prerequisite for wound healing.[11] Weinheimer-Haus, et al. conducted their study on diabetic rats with 8 mm diameter dorsal excision wounds, and started the treatment on the day of the injury, stated that there was an increase in granulation tissue and angiogenesis, as well as faster wound closure and re-epithelialization.[24]

The study of Carrer, et al used ultrasonic waves with a frequency of 3 MHz and two different intensities, namely 0.5 W/cm^2 and 2 W/cm^2 in a continuous mode every day for 3 days (inflammatory phase), 7 days (proliferative phase), and 21 days (remodeling phase). Ultrasonic therapy performed during the inflammatory phase (3 days) stimulates mast cells, platelets, leukocytes, and macrophages. Ultrasonic application induces degranulation of mast cells, leading to increased quality of the inflammatory phase, thickening of the epidermis, and initiating the formation of granulation tissue in the wound area. The stimulatory effect in the proliferation phase (7 days) can be observed especially on fibroblasts, myofibroblasts, and endothelial cells. Increasing quantity of collagen types I and III indicates the success of the healing process in the remodeling phase (21 days).[25]

Alkahtani, et al stated that low-intensity ultrasonic therapy doses between 0.5-3 W/cm^2 provide the best results and minimal side effects, and are most effective given in the first and second phases of the wound healing process. Low-intensity ultrasonic therapy can promote healing of open wounds as well as deep tissue wounds. The effectiveness of low-intensity ultrasonic therapy in chronic wounds is not only for the healing process but also for reducing pain, pigmentation, and aroma.[26]

This study uses a gel-based coupling agent as a carrier of LIPUS energy. This is in accordance with Watson which states that the coupling agent used can be water or gel-based, which can fill the space, has sufficient viscosity, provides ultrasonic transmission with minimal effect on the tissue.[19]

The results proved that Low-intensity Pulsed Ultrasound (LIPUS) therapy with a frequency of 3 MHz, an intensity of 0.5W/cm^2, a duty cycle of 20%, and an exposure duration of 3 minutes per day could significantly increase mast cell degranulation and fibroblast expression in the

process of wound healing, punch biopsy, type 2 diabetes mellitus model rats. The observation day of this study was limited to the 14th day, thus the changes in each variable after the 14th day are still unknown. In addition, this study was only conducted on skin wounds, not on other soft tissues, so that further research is needed, with different variables, longer observation days, and wounds on other soft tissues.

CONCLUSION

Low-intensity Pulsed Ultrasound (LIPUS) therapy with a frequency of 3 MHz, an intensity of 0.5 W/cm^2, a duty cycle of 20%, and an exposure duration of 3 minutes daily for 3, 7, and 14 days can increase mast cell degranulation and fibroblast expression in the punch biopsy wound healing process type 2 diabetes mellitus model rats, and there are correlations between the two variables (mast cell degranulation and fibroblast expression).

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