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Oral L-Arginine lower neovascularization and the number of fibroblasts but failed to increase nitric oxide and epithelialization in the healing process of male white diabetic wistar rats (*Rattus norvegicus)*

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ABSTRACT

Introduction: Oral L-Arginine is a conditional essential amino acid that plays a role in wound healing in DM. The role of arginine in diabetic wounds is by enhancing blood circulation in the injured area and increasing oxygen supply to the wound tissue. The purpose of this study to prove the administration of oral L-Arginine toward vascularization status in wound healing of male white rats wistar diabetes mellitus.

Methods: A randomized posttest only control group study using with 36 diabetic induced wistar rats (*Rattus Norvegicus*) aged 2-3 months and weighing 180-200gram which then divided randomly into two groups. Nitric oxide level was measured on the third day and each group was then further divided into two groups for examination of neovascularization, fibroblasts and epithelialization on the seventh day and on the tenth day.

Results: Administration oral L-Arginine failed to induce any significant change in Nitric Oxide level and wound gap closure. On the other hand, the results showed that the mean neovascularization was significantly different between the two groups on the 10th day (Control group vs intervention group: 4.22±1922 vs1.89±1364; p=0.009). In addition, the mean number of fibroblast at the 10th day was also significantly different (Control group vs intervention group: 74.11±28.57 vs 38.11±20.90; p=0.008).

Conclusion: In conclusion, oral L-Arginine did not significantly affect nitric oxide and epithelialization while decreased neovascularization and the number of fibroblasts on day tenth in the healing process of male white rats diabetes mellitus

Keywords: L-Arginine, Nitric Oxide, Wound, Diabetes Mellitus, Neovascularization, Fibroblasts, Epithelialization

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INTRODUCTION

Diabetes mellitus (DM) has now become an epidemic disease. In the last 10 years there has been a 2-3-fold increase in DM prevalence due to population ageing, overweight and lifestyle change.[1] People with diabetes mellitus are at high risk of complications, with impaired wound healing as the most common one. Factors that affect wound healing in diabetic patients include high blood glucose level, poor blood circulation, tissue hypoxia, diabetic neuropathy, altered immune response and infection. Wound healing impairment in DM is also caused by a altered leukocyte function due to glucose level abnormality as well as secondary ischemic conditions which reduce the tissue oxygenation and nutrients to the wound area. Putting together, all of these factors would lead to hypoxic conditions which then inhibit tissue healing of diabetic wounds.[2]

The advancement of nutritional therapy has led to the specific use of arginine supplementation for protein synthesis, production of NO and cell proliferation through its metabolism to ornitine and polyamine. The NO is considered to have positive effects in wound healing due to its angiogenesis enhancement, improved immune response, cell proliferation, matrix deposition and remodeling.[3] Nitric oxide will provide a supply of nutrients and tissue oxygenation to the injured area by promoting vasodilation and blood circulation around the wound area.[4]

The evidence of L-Arginine use for wounds healing is not well known in Indonesia. Therefore, this study evaluated the effect of L-Arginine toward would healing process in terms of neovascularization, the number of fibroblast and epithelialization. It is expected that with this study will provide necessary evidences for health care professionals who often deal with wound healing impairment in diabetic patients.[5]

METHODS

A randomized post-test only control group study was conducted using 36 wistar rats (Rattus norvegicus) rats that had been induced to have diabetes, aged 2-3 months, and had weight between 180-200 grams. Before treatment administration, the mice were adapted for 7 days in a cage and feed with standard food and drinks. To induce type 2 diabetes in experimental mice, all groups of mice were injected by nicotinamide (NA) at a dose 230 mg/kgbw and 50 mg/kgbw streptozotocin (STZ) single dose intraperitoneally. After the induction process, the samples that met the requirements of diabetes (PPG ≥ 135 mg/dl) were divided into 2 groups with 18 rats for each group. Each individual was anesthetized by 20 mg/kgbw ketamine and 5 mg/kgbw xylazin then a wound was made by removing a full thickness skin at the back of the mice using a pump biopsy.

All groups of mice were treated according to their groups with following sequence: on the 1st day since the treatment was started, group 1 was given 2cc aquades as a placebo by sonde 2x a day for 10 days. Group 2 was given L-Arginine at a dose of 2cc 4.5mg by sonde 2x a day for 10 days. On the 3rd day (3 days after injury and treatment), blood serum was taken from all rats for Nitric Oxide levels examination. On day 7, nine rats were sacrificed using an excess dose of 75 mg/kgbw ketamine intraperitoneally and the wound examined histopathologically by HE staining to evaluate neovascularization, fibroblast density and epithelialization. On the 10th day, the remaining nine rats were sacrificed using an excess dose 75 mg/kgbw ketamine intraperitoneally and the wound was examined histopathologically by HE staining to evaluate neovascularization, fibroblast count and epithelialization of the wound.

All data were analyzed using SPSS for windows. The data was analyzed using *Saphiro-Wilk test*, *Levene's test* and *Mann-Whitney test* for nitric oxide due to abnormal distribution of the data and *independent student t-test* for neovascularization, fibroblast and epithelialization.

Table 1. The results of statistical comparison between control and treatment group across all variables.

Variable	Control	Intervention	P
Nitric Oxide** Day 3 (μM)	0,751	0,804	0,949
	(0,214-4,937)	(0,322-1,716	
Neovascularization* (μM)			
- Day 7	5,33±2398	5,44±2833	0,382
- Day 10	4,22±1922	1,89±1364	0,009
Fibroblast* (cell):			
- Day 7	57,89±16,2	61,67±22,3	0,686
- Day 10	74,11±28,57	38,11±20,90	0,008
Epithelialization (μM):			
- Day 7	2272,86±1943,9	2211,17±1666,67	0,943
- Day 10	17492,52±1776,27	1519,03±2467,50	0,791

p = signifikansi
* = *independent T-Test*, presented in mean±sd
** = *Mann-Whitney Test*, presented in Median (min-max)

RESULTS

The results showed that administration of L-Arginine can accelerate wound healing compared to the control group through a decrease in neovascularization and fibroblast cell counts on day 10. The median (min-max) Nitric Oxide level of control group was not significantly different to intervention group in day three despite it was slightly higher in treatment group (control group: 0.751[0.214-4.397] vs intervention group 0.804[0.322-1.716];p=0.949). It also revealed that the mean of wound gaps area of control group was also not significantly different to intervention group in day seventh and day tenth. The average wound gaps on the seventh day in control groups was 2272.86±1943.9 versus 2211.17±1666.67 in intervention group (p=0.943), while at 10 days of treatment, the wound gaps in control group was 1792.52±1776.27 versus 1519.03±2467.50 in intervention group (p=0.791) (Table 1 and Figure 1).

In terms of vascularization status, the results showed that the mean of neovascularization between the two groups on day seventh were roughly similar with slightly higher value in the treatment group (Control: 4.33±2398 vs Treatment: 5.44±2833; p=0.382). However, the vascular density was significantly higher in the control group at 10 days post-treatment (Control: 4.22±1922 vs treatment: 1.89±1364; p=0.009). In addition, the mean number of fibroblast between the two groups on the 10th day after treatment (Control: 74.11±28.57 vs treatment: 38.11±20.90; p=0.008) was also significant higher in control group while it rather similar on the day 7 (Table 1 and Figure 2).

DISCUSSION

Impaired wound healing process is almost always occurred in diabetic patients[6]. In diabetes mellitus,

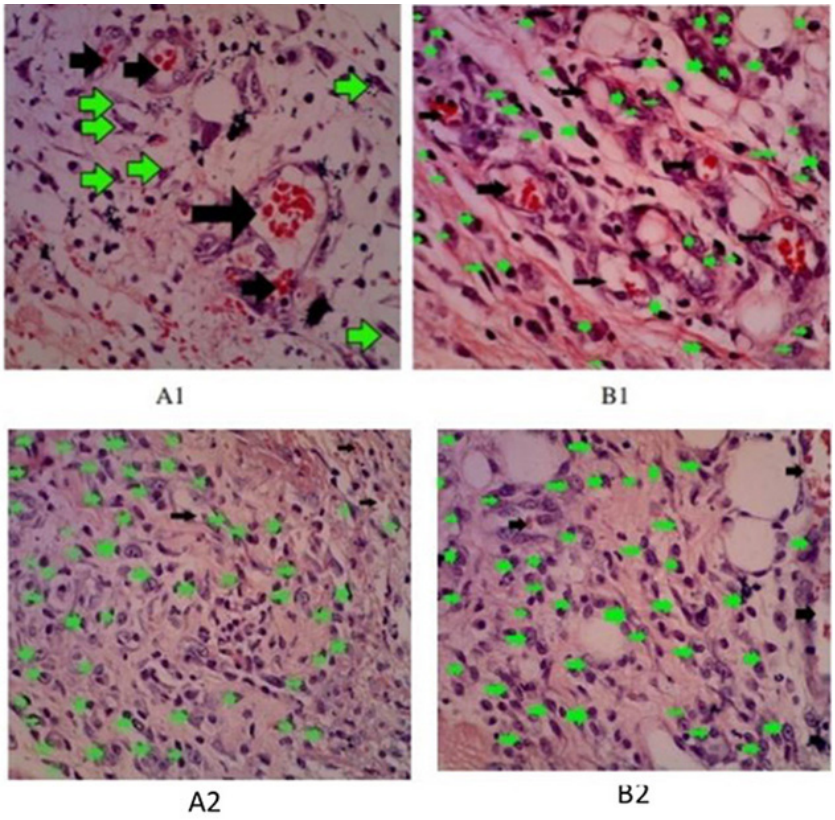

Figure 1. Skin histopathology examination, black arrows show neovascularization and green arrows show fibroblasts. A1 is control group (P0) and B1 is treatment group (P1) after 7 days of treatment. A2 is control group (P0) and B2 is treatment group (P1) after 10 days of treatment.

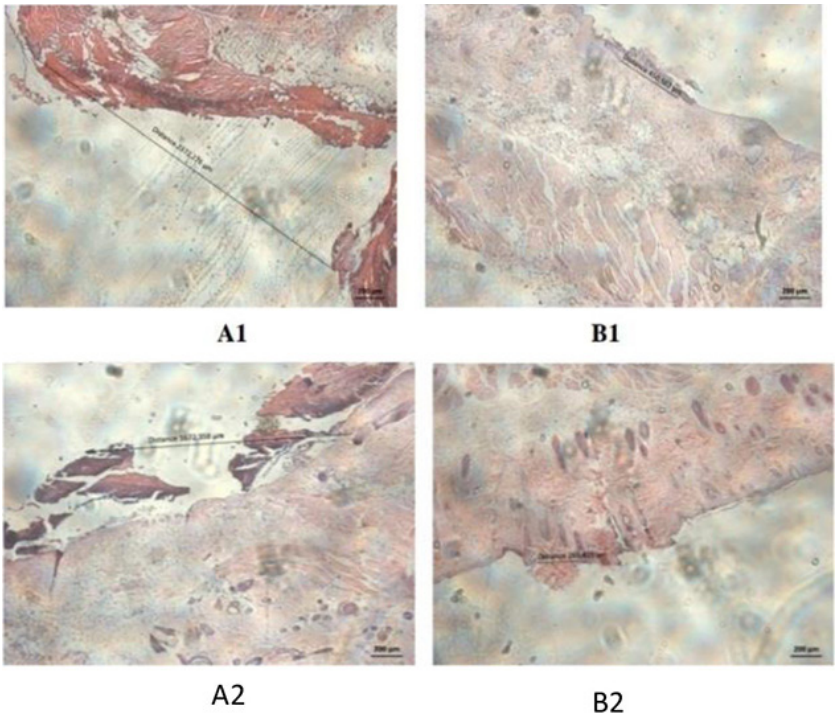

Figure 2. Skin Histopathology examination on day 7.
Arrows show tissue wound gap in: A1 is control group (P0) and B1 is treatment group (P1) after 7 days of treatment. A2 is control group (P0) and B2 is treatment group (P1) after 10 days of treatment.

wound tissue will experience prolonged hypoxia and nutrient deficiency which lead to impaired chemotaxic and phagocytic of leukocyte, especially macrophage in the initial phase of wound healing resulting in prolonged inflammatory phase. Chronic tissue inflammation resulted in inefficient angiogenesis, re-epithelialization, and decreased collagen synthesis.[7]

L-Arginine is one of the most metabolically versatile amino acids. In addition to its role in the synthesis of nitric oxide, L-Arginine serves as a precursor for the synthesis of polyamines, proline, glutamate, creatine, agmatine and urea.[8] Several human and experimental animal studies have indicated that exogenous l-arginine intake has multiple beneficial pharmacological effects when taken in larger doses than normal dietary consumption.[9] During injury process, the level of L-Arginine becomes increasingly low due to reduced oxygenation and nutrients delivery to the injured area and.[10] L-Arginine promotes fibroblasts proliferation, neovascularization and epithelialization in diabetic wounds that will increase blood circulation and increasing oxygen supply to wound tissue.[11]

Based on the results of the analysis on day 3 it was found that nitric oxide levels did not increase on day 3. In experimental animal studies, wound treatment with administration of L-Arginine increased nitric oxide in the inflammatory phase and gradually decreased in the proliferation and remodeling phases.[12] In other animal studies, it was explained that nitric oxide had an important role in the wound healing process by stimulating collagen production by fibroblasts, matrix deposition, remodeling and angiogenesis.[7] In diabetic conditions, it is known that the level of nitric oxide tend to be lower than normal. Therefore, although the initial goal of this study was to enhance nitric oxide levels, but the result failed to show any significant differences between treatment and control group in term of nitric oxide concentration on day 3. This phenomenon could be caused by lower rate of nitric oxide synthesis in diabetic rats and prolongation of inflammatory phase or it is also possible that measurement on day 3 was too early because profound angiogenesis only observed on day 10.

The density of fibroblast was also found to be increased in this study. Increasing fibroblast density is known to induce by increased secretion of FGF, released from platelet degranulation. Fibroblasts play an important role in this phase. fibroblasts are connective tissue that synthesize and secrete collagen, proteoglycans and glycoproteins needed

for wound healing. Fibroblasts also produce growth factors that induce angiogenesis, endothelial cell proliferation, and migration. At the end of the proliferation phase, epithelization will occur, ie epithelial cells at the edges of the wound will proliferate and form new surfaces similar to the surface damaged by injury.[12]

Fibroblasts are the main cells during the proliferation phase which play a role in providing the extracellular matrix as a framework for migrating keratinocytes. Denser fibroblasts lead to denser and more compact extracellular matrix that stimulates the epithelialization process by keratinocytes.[13] Therefore, the greater the number of fibroblasts in the wound would result in faster epithelialization process than the control wound group. The main goal of epithelialization is complete wound healing, when the entire wound surface is covered by the epithelial layer.[14] When the wound surface is closed, fibroplasia with granulation tissue formation will also stop and the maturation process begins.[15] This phenomenon can be observed on the day 10 of experiment when the number of fibroblasts in the treatment group decreased compared to the 7th day because on the 10th day, the fibroblasts proliferation was already stopped and their number decreased significantly. On the other hand, epithelialization did not appear to be different on the 7th day and appeared to begin to decline on the 10th day but, again, there was no significant difference. The reason could be due to narrowing of the wound and also due to constant hyperglycemia during the study that resulted in complete slowing of the wound healing process. Another possibility is that the epithelialization assessment on the 10th day could be too early, considering the slowed healing process in diabetic wound.

Examination of post prandial glucose in mice is difficult to do since not all mice can be regulated fasting and eating at the same time. In the initial and final GDPP examination, the results were not different between the control group and the treatment group with blood glucose values between 150-220 mmol / L. The results of the GDPP analysis also showed no difference between the two groups which could mean that the wound healing process was not caused by a decrease in blood glucose. However, in this study, it could not prove that wound healing was caused by blood sugar levels but was due to L-Arginine enhancing nitric oxide.

CONCLUSION

Oral L-Arginine supplementation did not significantly increase nitric oxide level and failed to produce any significant effect of epithelialization. However, the supplementation significantly lowered neovascularization and the number of fibroblasts on day tenth in diabetic rats.

CONFLICT OF INTEREST

All authors declared that there is no conflict of interest regarding this publication

AUTHOR CONTRIBUTION

All authors contributed equally in the writing of this article

FUNDING

This study was self-funded without any contribution from third party.

ETHIC APPROVAL

This study had been ethically approved by ethical commission of Faculty of Medicine Udayana University with approval letter number 416/KE-PH-Lit-2/VII/2018

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