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Correlation of neutrophils lymphocytes ratio with femur muscle damage due to acute limb ischemia in white Wistar rats

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ABSTRACT

Background: Acute limb ischemia (ALI) is a sudden decrease in limb perfusion that threatens limb viability and requires urgent evaluation and management. Assessment determines whether the limb is viable or irreversibly damaged. Neutrophil-lymphocyte ratio (NLR) is a useful marker for cardiovascular disease to delineate the prognosis. Endothelial dysfunctions related to atherosclerotic plaques are usually associated with states of neutrophilia together with lymphopenia processes.

Methods: This study was a post-test only control group performed on Wistar strain white rats. Thirty rats were divided into 5 groups, Control group (K), treatment group one (P1) examined NLR and femoral muscle tissue after 3 hours of ALI, P2 after 4 hours of ALI, P3 after 5 hours of ALI and P4 after 6 hours of ALI. Muscle tissue was histologically examined.

Results: The duration of ALI increases the NLR value in Wistar strain white rats, the NLR value examined 6 hours after the rats experienced ALI increased significantly compared with the NLR values examined in the control group, the group examined 3 hours, 4 hours and 5 hours after the rats had ALI ($p = 0.004$). Necrotic muscle tissue very strongly correlated with the duration of ALI ($r = 0.860$, $p<0.001$). NLR value is strongly correlated with muscle tissue damage ($r = 0.634$, $p<0.001$).

Conclusion: The duration of ALI increases the NLR value in wistar strain white rats, NLR values increased significantly after 6 hours of ALI. Necrotic muscle tissue occurs after six hours of ALI. NLR value is strongly correlated with muscle tissue damage. Revascularization must be done within 6 hours to prevent permanent damage.

Keywords: neutrophil-lymphocyte ratio, acute limb ischemia, tissue damage, Wistar rats.

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INTRODUCTION

Acute limb ischemia (ALI) is defined as the recent onset (< 14 days) of decreased limb perfusion that endangers life and/or limb. This is a medical emergency with management options ranging from urgent revascularization to limb amputation. The best patient outcome requires tailoring the treatment to the individual patient.[1]

Since the onset of ALI, there is conversion of aerobic muscle metabolism to anaerobic metabolism with increased production of lactate. Ischemic injury results in the loss of endothelial integrity, interstitial edema and a tense necrotic limb. Systemically, there is release of inflammatory mediators (such as interleukins-1, 6, 8, tumor necrosis factor and monocyte chemotactic protein-1) and activation of the complement cascade. Multiorgan dysfunction may ensue, manifested by acute lung injury, acute renal failure and myocardial dysfunction. Unchecked, these events become life-threatening.[1]

There are two components to the reperfusion syndrome, which follows extremity ischemia. The local response, which follows reperfusion, consists of limb swelling with its potential for aggravating tissue injury and the systemic response, which results in multiple organ failure and death. It is apparent that skeletal muscle is the predominant tissue in the limb but also the tissue that is most vulnerable to ischemia. Physiological and anatomical studies show that irreversible muscle cell damage starts after 3 h of ischemia and is nearly complete at 6 h.[3,4]

The Neutrophil-to-Lymphocyte ratio (NLR) is an easy to perform test from the white blood cell count. An increase in NLR has been associated with vascular end points reflecting inflammation in atherosclerotic lesions. Atherosclerosis is a global threat and vascular end points, like myocardial infarction or critical limb ischemia (CLI), is a leading cause of death in industrialized countries.[5,6]

The neutrophil to lymphocyte ratio (NLR) reflects the balance of the neutrophilia of inflammation and the relative lymphopenia of a cortisol-induced stress response, and it is a stronger univariate predictor of outcome than the neutrophil or lymphocyte count.[7] The states of lymphopenia disclosed by the whole blood count are associated with atherosclerosis progression, and the decrease in lymphocytes may be caused by apoptotic processes triggered during atherosclerotic lesions. On the other hand, the

quantitative increase in neutrophils is also related to the atherogenic process, acting through lipid mediation, necrosis and inflammation, secreting chemokines and cytokines.[8]

There are a number of problems with current PAD management strategies, such as the difficulty in selecting the appropriate treatments for individual patients. Many patients undergo repeated attempts at revascularization surgery, but ultimately require an amputation. There is great interest in developing new methods to identify patients who are unlikely to benefit from revascularization and to improve management of patients unsuitable for surgery. Circulating biomarkers that predict the progression of ALI and the response to therapies could assist in the management of patients.[2]

This study aims to determine NLR to assess muscle tissue damage in the development of ALI, correlation between duration of ALI and NRL value and correlation between duration of ALI and muscle tissue damage.

METHODS

Study design and animal model

This study was a post-test only control group performed on Wistar strain white rats. Thirty rats were divided into 5 groups, control group (C), treatment group one (P1) examined NLR and femoral muscle tissue after 3 hours of ALI, P2 after 4 hours of ALI, P3 after 5 hours of ALI and P4 after 6 hours of ALI. before the treatment of rats was weighed and adapted for 1 week, homogenization was carried out in a cage, the temperature in the cage was set at room temperature, every day the rats were fed in the form of 20 grams of pellets and drinking water was given in ad libitum.

Acute limb injury initiation process

The process of making ALI is carried out by a veterinarian. Mice are anaesthetised with ketamine 30 mg/kg-body weight intra-muscular in the left quadriceps femoris muscle, mice breathe spontaneously during the procedure. Perform aseptic and antiseptic actions in the right femur area. Perform incisions around the right femoral artery. Identifying the right femoral artery. Perform proximal portion of the right femoral artery ligation after the inguinal ligament using nylon 7.0.

Blood sampling and tissue histology analysis

Blood sample was taken from retro-orbital plexus in the eye, the microhematocrit is scratched on the medial canthus eye under the eyeball towards the optic foramen, then the microhematocrit is rotated to injure the retro-orbital plexus. Blood is collected in eppendrof which has been given EDTA. Calculation of the ratio of neutrophils and lymphocytes.

The quadriceps femoris muscle tissue was taken 1x1 cm then fixed with 10% formaldehyde buffer and paraffin block was made and then stained with HE staining. The sample was dried and examined by a light microscope with a magnification of 100x.

Statistical analysis

The effect of the duration of ALI with NLR values was analyzed by different analysis of Kruskal-Wallis and post hoc Mann Whithney. To determine the correlation of the duration of ALI with damage to muscle tissue and the correlation of NLR values with tissue damage were analyzed using spearman correlation analysis. Values are considered significant if $p < 0.05$.

RESULTS

Clinical manifestation of rats' right foot showing changes in skin color after proximal right femoral artery ligation. (Figure 1)

The NLR values in groups P1, P2 and P3 were not different from the NLR values in the control group, but the NLR values in the P4 group increased compared to the other four groups. The data distribution of NLR values on the duration of ALI is normally distributed and has different variances so that to determine the effect of the duration of ALI on the NLR value using the Kruskal Wallis hypothesis test. The results of the Kruskal Wallis test shows p=0.004 which means that there are at least two groups that have NLR values that are significantly different (Table 1).

Mann-Whitney post-hoc analysis was used to see groups that had significantly different NLR values. The results of the Mann-Whitney post-hoc analysis showed that the NLR value at P4 where ALI had occurred for 6 hours had a significantly increased NLR value compared to the other four groups (Table 2).

Histological observations using hematoxylin-eosin staining found that there were 7 normal tissues, 17 tissues that had pre necrosis and 6 tissues that had undergone necrosis. Necrosis was found

Table 1 NLR value in each group

		n	Median (minimum-maximum)	mean ±SD	p
NLR	C	6	0.65 (0.37-1.06)	0.65 ± 0.24	0.004
	P1	6	0.74 (0.29-0.90)	0.62 ± 1.08	
	P2	6	0.55 (0.29-0.90)	0.57 ± 0.24	
	P3	6	0.62 (0.42-0.86)	0.62 ± 0.18	
	P4	6	2.94 (2.28-11.13)	4.94 ± 3.72	

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Table 2 Analysis post-hoc Mann-Whitney

NLR	p
C vs P1	0.229
C vs P2	0.522
C vs P3	0.873
C vs P4	0.004*
P1 vs P2	0.262
P1 vs P3	0.297
P1 vs P4	0.004*
P2 vs P3	0.631
P2 vs P4	0.004*
P3 vs P4	0.004*

Table 3 Tissue damage in each group

		Normal	Pre-necrosis	Necrosis
Tissue Damage	C	6	0	0
	P 1	0	6	0
	P 2	1	5	0
	P 3	0	6	0
	P 4	0	0	6

Table 4 Correlation of the duration of ALI to tissue damage

		Duration of ALI
Tissue Damage	r	0.860
	p	<0.001
	n	30

Table 5 NLR values are grouped according to tissue damage

		N	Median (minimum-maximum)	Mean ± SD	p
NLR	Normal tissue	7	0.59 (0.29-1.06)	0.60 ± 0.26	<0.001
	Pre-necrotic tissue	17	0.71 (0.35- 1.08)	0.67 ± 0.19	
	Necrotic tissue	6	2.94 (2.28- 11.13)	4.94 ± 3.72	

Table 6 Correlation test between NLR value and tissue damage

		Tissue Damage
NLR	r	0.634
	p	<0.001
	n	30

in the group that had ALI for 6 hours (Table 3). Histology of quadriceps femoris muscle histology in the control group and each treatment group can be seen in Figure 2.

Test of the spearman correlation of the duration of ALI to tissue damage showed that the correlation between the duration of ALI and tissue damage was significant. The value of the spearman correlation 0.860 shows a positive correlation with a very strong correlation strength (Table 4).

NLR values in the group that experienced tissue necrosis increased significantly when compared with the pre necrosis and normal tissue groups. The NLR value in the pre necrosis group was not different from the NLR value in the normal tissue group (Table 5).

Spearman Correlation test between NLR value and tissue damage showed that the correlation between NLR value and tissue damage was significant with the spearman correlation value of 0.634 indicating a positive correlation with strong correlation strength (Table 6).

DISCUSSION

Acute limb ischemia (ALI) is defined as a sudden decrease in the blood supply of a previously stable leg resulting in resting pain and other features of severe ischaemia and threatening limb viability. This may be subdivided into acute (onset 24 hour) and sub-acute (onset 24 hour–2 weeks). The classical clinical presentation of ALI is pain, paralysis, paraesthesia, pallor, pulselessness and perishing cold leg. At presentation, the presence or absence of sensory and motor function, mottling (blanching or non-blanching) and muscle tenderness will determine whether the limb is viable, threatened or non-viable. All cases require emergency referral to a vascular specialist for definitive management and in patients with classical acute limb ischemia it is important that revascularization is accomplished within 6 h to prevent irreversible muscle damage.[9-11]

This study found that the value of NLR in the group that occurred ALI after 3 hours, 4 hours and 5 hours was still in the normal range and not different from the NLR value of the control group. The NLR values examined 6 hours after ALI increased significantly compared to the NLR values examined in the control group, the group examined 3 hours, 4 hours, and 5 hours with p = 0.004, this indicates that the increase in NLR values in ALI occurred after 6 hours duration of ALI. A period of 6 hours after ALI occurs in total acute ischemia where neutrophils will increase and lymphocytes decrease.

In the ALI condition, ischemic injury occurs which causes loss of endothelial integrity, interstitial edema which systemically causes the release of inflammatory mediators which causes the release of cortisol and catecholamines and causes an increase in neutrophil values and decreased lymphocyte count.[1]

Physiological and anatomical studies show that irreversible muscle cell damage begins after 3 hours of ischemia and is almost complete at 6 hours.[3,4]

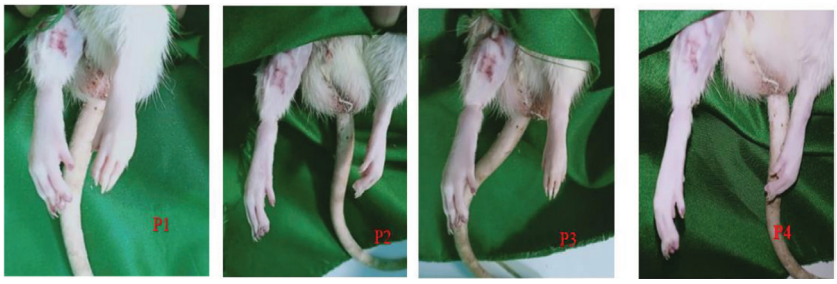

Figure 1 Clinical manifestation of rats' right foot showing changes in skin color after proximal right femoral artery ligation

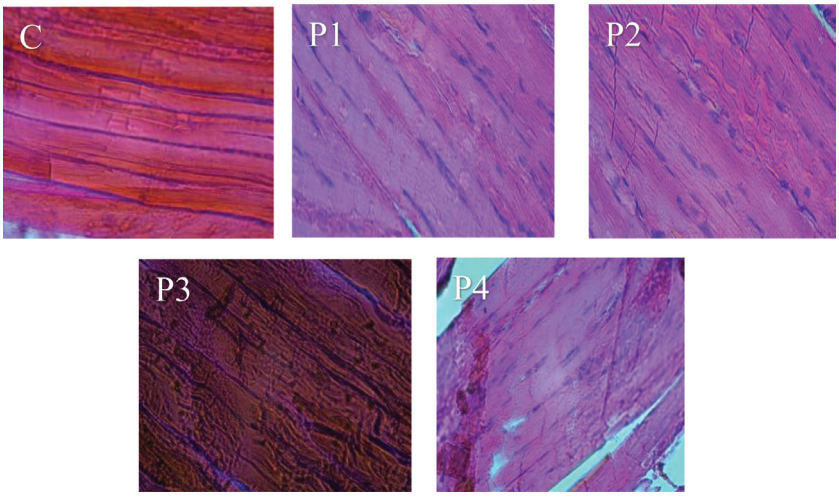

Figure 2 Microscopic description of muscle tissue. Normal tissue. The nucleus of the cell is located peripherally, intact sarcolemma and sarcoplasm (C). Prenecrosis tissue. The cell nucleus appears enlarged and is in the middle of sarcoplasm, intact sarcolema and sarcoplasm (P1, P2, P3). Necrotic tissue, inflammatory cell infiltration and fragmented sarcoplasm (P4).

Hayes et al. found that after 6 hours of ischemic 20% of pre-ischemic ATP persisted and resulted in muscle necrosis. Other researchers have also confirmed that after 4-6 hours of irreversible ischemic muscle changes occur. Labbe et al. using a model similar to Hayes et al. found that the level of necrosis in muscles occurs after 3 hours by 2%, after 4 hours by 30%, and after 5 hours ischemia occurs by 90% of total muscle. They also noted that greater muscle necrosis occurs in the middle part of the muscle and evaluates the level of ischemic damage clinically unreliable.[3] After 6 hours of ischemia the damage to the skeletal muscle tends to be irreversible with little hope of a rescue strategy.[4]

In this study it was found that the pre necrosis process began 3 hours after the occurrence of ALI, the longer the occurrence of ALI the tissue damage will be more severe and the tissue becomes necrotic at 6 hour after ALI. This study is in accordance with Hanan et al. who shows that revascularization must be carried out within 6 hours to prevent permanent muscle damage or before necrosis of the muscles.[10] Acute total ischemia would cause extensive tissue necrosis within six hours unless previous revascularization surgery were performed. Acute non-total ischemia can be treated medically but patients with irreversible ischemia require immediate amputation.[14]

Severe ischemia where there is no immune infection and disease, an increase in systemic NLR can represent an irreversible ischemic condition that can cause end points (for example, amputation).[12,13] NLR is also associated with damage to ischemic tissue, morbidity and mortality after acute ischemia in the system cardiovascular.[14]

In this study it was found that the value of NLR increased significantly in necrotic tissue and this occurred after 6 hours of ALI, while the NLR values in normal and pre necrosis tissue were not significantly different. Increasing the value of NLR correlates strongly with tissue damage.

Using NLR in patients with cardiovascular risk will be useful to describe the patient's prognosis. Endothelial dysfunction is associated with neutrophilic processes that coincide with lymphopenia. Antagonism between the causes and protective factors of the inflammatory process supports the emergence of injury to the vascular endothelium.[8]

This study has not been able to determine the cut-off value of NLR that shows necrotic tissue in ALI, so that the value of NLR can help clinicians determine appropriate treatment options for patients diagnosed with ALI.

CONCLUSION

The value of NLR increased significantly after 6 hours of ALI. The longer the occurrence of ALI, the more damage the quadriceps femoris muscle tissue. Necrosis occurs at the 6th hour after the occurrence of ALI. The NLR value has a strong correlation with damage to the muscle tissue of the femur. NLR values increased significantly in necrotic tissue.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the manuscript.

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AUTHOR CONTRIBUTION

All authors are contributed equally to the content of the study, including data gathering, statistical analysis and data synthesis.

ETHICAL CONSIDERATION

This study has been approved by the Ethical Committee Faculty of Medicine Universitas Syiah Kuala, Banda Aceh, Indonesia with reference number 17/EA/FK-RSUDZA/2019.

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