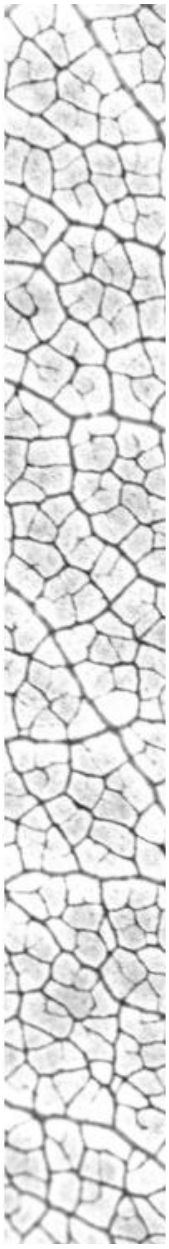

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N-acetyl-Cysteine effects on tissue malondialdehyde level and tissue edema on the Ischemia-Eperfusion injury of white rats' *(Rattus Norvegicus)* skeletal muscles

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Abstract

Background: N-acetylcysteine (NAC), as an antioxidant, has been proven to have protective effects on cells undergoing oxidative stress. In the case of skeletal muscles, NAC can reduce muscle fatigue by decreasing Na^+ K^+ pump activity during exercise, and improve skeletal muscle microcirculation in an experimental model of severe closed soft-tissue injury. **Purpose:** This study aims to determine NAC effects on the ischemia-reperfusion injury of skeletal muscles by measuring tissue malondialdehyde (MDA) levels and edema, as well as the effect of NAC dose on MDA levels. **Method:** Randomization was carried out to 30 white rats (Rattus norvegicus), which then divided into 3 treatment groups, i.e., K group as the control group (NaCl 0.9%), PTX (Pentoxyfilline) group, as well as P1, P2, and P3 treatment groups. ANOVA test was employed to identify the differences in MDA levels and water content among groups. **Results:** ANOVA test results indicated that there was a significant difference between the control group (K) and the treatment group given NAC (P1, 2, 3), $p = 0.007$, while there was no difference in edema indicating that there were no different data groups among the five treatment groups with a p-value = 0.616 ($p > 0.05$). **Conclusion:** NAC administration provides positive results by reducing tissue MDA levels with the recommended dose of 200 mg/kg of body weight. NAC administration does not reduce tissue edema, and there are no significant differences with the Pentoxifylline group.

Keywords: NAC, MDA, edema, ischemia-reperfusion of skeletal muscles

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INTRODUCTION

Ischemia-reperfusion, a condition of tissue ischemia with inadequate oxygen supply followed by reperfusion which triggers a series of complex inflammatory responses, becomes a crucial problem in the fields of clinical orthopedics and traumatology, as well as the reconstructive surgery related to limb salvage after ischemia. Ischemia-reperfusion injury causes poor clinical outcomes in patients undergoing replantation, free muscular flap, free myocutaneous flap, compartment syndrome, or all revascularization procedures, even if executed with the correct procedures. An acute ischemic limb is one of the most frequent emergencies of peripheral blood vessels. Skeletal muscle is the largest tissue component in the extremities, but most vulnerable to ischemia, so muscle damage is the most important aspect of the limb reperfusion syndrome. (Simelane, et al, 2016).

The reperfusion of ischemic tissue causes the formation of toxic reactive oxygen species (ROS). It is caused by hypoxanthine formed from degraded adenosine triphosphate (ATP) cells that cannot be catalyzed by xanthine oxidase in ischemic conditions. ROS can cause lipid peroxidation in cell membranes resulting in serious damage to the membrane, including the increase of membrane permeability and damage to its function for ion exchange by forming malondialdehyde (MDA) compounds.

One of the therapeutic strategies to prevent or overcome the injury of ROS-mediated reperfusion is antioxidant. N-acetylcysteine (NAC), as an antioxidant, has been proven to have protective effects on cells undergoing oxidative stress. NAC has been identified to have protective effects against ischemia-reperfusion injury in the brain, liver, kidneys, lungs, and myocardium. The NAC compound works as a substitute for

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glutathione, which plays a role in binding the toxic metabolites produced through paracetamol biotransformation in the liver.

In the case of skeletal muscles, NAC can reduce muscle fatigue by decreasing Na^+ K^+ pump activity during exercise. N-acetylcysteine has been shown to improve skeletal muscle microcirculation in an experimental model of closed soft-tissue injury. Another study also suggests that NAC administration before and after simulation of compartment syndrome can maintain skeletal muscle contractility, through the effect of reduced neutrophil activation and the resulting oxidant injury.

Based on these facts, this study aims to determine the NAC effects on ischemia-reperfusion injury of skeletal muscles by measuring tissue MDA levels and edema, as well as the effect of NAC dose administration on MDA levels. Pentoxifylline was also used in this study as a comparison.

METHOD

In this study, 30 white rats were used with the criteria, i.e., the rats used were Wistar rats (Rattus norvegicus), aged 2–2.5 months with a body weight around 200-300 grams, and healthy as indicated by active motion. The rats were acclimatized under laboratory conditions for 7 days by giving ad libitum standard feeds and drinking water. Then, the first anesthetic was performed in the intramuscular with ketamine 5 mg/kg of rats' body weight, and the dose could be given repeatedly according to the time needed.

In the experimental rats, randomization was carried out, which then divided into 3 treatment groups, i.e., group K, a control group in which the white rats were given 4 hours ischemic treatment, then given 2 ml of saline solution (NaCl 0.9%) intravenously per 100 gram of rat's body weight per day, then 1-hour reperfusion was performed. PTX group, a comparison group in which rats were given 4 hours of ischemic treatment and Pentoxifylline injection 20 mg/kg of rat's body weight, then 1-hour reperfusion was performed. The P1 treatment group, in which the rats were given 4 hours of ischemic treatment and intravenous NAC injection 200 mg/kg of rat's body weight, then 1-hour reperfusion was performed. P2 treatment group, in which rats were given 4 hours of ischemic treatment and intravenous NAC injection 400 mg/kg of rat's body weight, then 1-hour reperfusion was performed. The P3 treatment group, in which the rats were given 4 hours of ischemic treatment and intravenous NAC injection 800 mg/kg of rat's body weight, then 1-hour reperfusion was performed.

MDA tissue levels were measured through gastrocnemius muscle tissue weighing 100 mg after 1-hour reperfusion. Measurement was carried out using TBA reagent, then, it was heated in a water bath at 80° C for 15 minutes. After chilling, 3000 rpm centrifugation was conducted for 15 minutes. The absorption intensity of supernatant was measured by a spectrophotometer with λ = 532 nm. MDA levels were calculated using the linear regression equation from the standard curve of the MDA solution.

Tissue edema was determined by measuring water content in percent. The tissue was taken after a 1-hour reperfusion and weighed (as a wet weight). Next, the tissue was dried with the freeze-drying method at -80^o C for 24 hours, then weighed and measured its water content. ANOVA test was employed to identify the differences in MDA levels and water content among groups.

Table 1. The Mean and Standard Deviation of Tissue MDA Levels

Group	Mean of MDA level (µmol/L)	SD (µmol/L)
K (0.9% NaCl)	9.8740	2.00298
PTX (Pentoxifylline 20mg/kg of BW)	7.5005	2.49672
P1 (NAC 200 mg/kg of BW)	5.8367	2.76624
P2 (NAC 400 mg/kg of BW)	5.3962	1.88571
P3 (NAC 800 mg/kg of BW)	4.7635	2.53845

MDA: malondialdehyde

Table 2. The normality test results of groups' data distribution on tissue MDA levels (µmol/L)

Group	p MDA level
K (NaCL 0.9%)	0.766
PTX (Pentoxifylline 20mg/kg of BW)	0.056
P1 (NAC 200 mg/kg of BW)	0.629
P2 (NAC 400 mg/kg BW)	0.400
P3 (NAC 800 mg/kg BW)	0.448

Table 3. The Homogeneity of variance test on Tissue MDA Levels

Levene Statistic	df1	df2	Sig.
0.615	4	25	0.656

MDA: malondialdehyde

RESULTS

Table 1 presents the test results of MDA levels in gastrocnemius muscle tissue of each group including the mean and standard deviation of tissue MDA levels for each group. **Table 2** contains the normality test results of data distribution. In the normality test, the data were normally distributed for the five groups with p> 0.05 (p = 0.766, p = 0.056, p = 0.629, p = 0.400, and p = 0.448). Meanwhile, **Table 3** shows the homogeneity of variance (p> 0.05). Based on the results in **Table 2** and **Table 3**, the ANOVA test could be performed. Based on ANOVA test results, it was found that there were at least two different data groups among the five treatment groups. It was identified by p <0.05 in which p = 0.007.

Based on **Table 5**, there were significant differences between the control group given NaCl 0.9% (K) and the treatment group given NAC 200 mg/kg of rat's BW (P1), between K group and the treatment group given NAC 400 mg/kg of rat's BW (P2), as well as between K group and the treatment group given NAC 800 mg/kg of rat's

Table 4. ANOVA Test Results for Tissue MDA Level Variable

Variable	F	P
Tissue MDA Levels	4.545	0.007

MDA: malondialdehyde

Table 5. The Test Results of Multiple Comparisons through Post Hoc Test on Tissue MDA Levels Among Groups

Group	Mean difference	Significance	Note
K-PTX	2.37350	0.094	Insignificant
K-P1	4,03733*	0.007	Significant
K-P2	4,47783*	0.003	Significant
K-P3	5,11050*	0.001	Significant
PTX-P1	1.66383	0.234	Insignificant
PTX-P2	2.10433	0.135	Insignificant
PTX-P3	2.73700	0.056	Insignificant
P1-P2	0.44050	0.749	Insignificant
P1-P3	1.07317	0.439	Insignificant
P2-P3	0.63267	0.647	Insignificant

MDA: malondialdehyde

Table 6. The Mean and Standard Deviation of Water Content

Group	Mean of water content (%)	SD (%)
K (NACL 0.9%)	75.2442	1.46128
PTX (Pentoxifylline 20mg/kg of BW)	75.9070	1.82777
P1 (NAC 200 mg/kg of BW)	74.9605	1.72020
P2 (NAC 400 mg/kg of BW)	76.1440	1.22943
P3 (NAC 800 mg/kg of BW)	75.2338	1.11743

Table 7. The Normality Test Results of Group's Data Distribution on Water Content

Group	p water content
K (NACL 0.9%)	0.453
PTX (Pentoxifylline 20mg/kg of BW)	0.076
P1 (NAC 200 mg/kg of BW)	0.008
P2 (NAC 400 mg/kg of BW)	0.134
P3 (NAC 800 mg/kg of BW)	0.061

BW (P3). There was no significant difference between the control group given NaCl 0.9% (K) and the groups with Pentoxifylline 20 mg/kg of rat's BW (PTX). There was no significant difference between the PTX group and the treatment groups with all three doses of NAC (P1, P2, P3). There was no significant difference among the three treatment groups given NAC with three different doses (P1, P2, P3).

The result of tissue edema level or water content in the tissue is presented in **Table 6**. The mean of water content in the K group (NaCl 0.9%) reached 75.2442 with a standard deviation of 1.46128. The mean of water content in the PTX (Pentoxifylline) group amounted to 75.9070 with a standard deviation of 1.82777. The mean of water content in the P1 group (NAC 200mg/kg rat's BW) reached 74.9605 with a standard deviation of 1.72020. The mean of water content in the P2 group (NAC 400 mg/kg of rat's BW) amounted to 76.1440 with a standard deviation of 1.29243. The mean of water content in the P3 group (NAC 800 mg/kg of rat's BW) reached 75.2338 with a standard deviation of 1.11743.

In the normality test, the data were normally distributed for the four groups. ($p = 0.766$, $p = 0.056$, $p = 0.629$, $p = 0.400$, and $p = 0.448$). It was identified by $p > 0.05$. Due to the normal data distribution for all four groups, the ANOVA test could be performed. The results of the normality test can be found in **Table 7**.

Table 8. The Homogeneity of Variance Test on Water Content

Levene Statistic	df1	df2	Sig.
0.765	4	25	0.558

Table 9. ANOVA Test Results for Water Content Variable

Variable	F	P
Tissue MDA Levels	0.675	0.616

In the normality test, the data were normally distributed for four groups with $p > 0.05$ ($p = 0.766$, $p = 0.056$, $p = 0.629$, $p = 0.400$, and $p = 0.448$). Based on **Tables 7** and **8**, it is clearly identified that the data were normally distributed and had a homogeneity of variance ($p > 0.05$), so that the ANOVA test could be performed. The ANOVA test results as presented in **Table 9** suggested that there were no different data groups among the five treatment groups with a p-value = 0.616.

DISCUSSION

The study results proved that NAC could reduce lipid peroxidation, which was characterized by a decrease of MDA levels in muscle tissue undergoing ischemia-reperfusion injury. It indicated that NAC had the protective effects on cell membranes against free radical attack. This effect might be due to the NAC's role as an antioxidant directly or indirectly. Multiple comparisons through the Post Hoc Test suggested that there was no significant difference in tissue MDA levels in the control group (K) and PTX group with Pentoxifylline 20 mg/kg of rat's BW intravenously. In this study, Pentoxifylline was not proven to reduce MDA levels in skeletal muscle tissue.

Tissue MDA levels decreased gradually according to the dose increase. The greatest decrease in MDA levels was triggered by NAC 800 mg/kg of rat's BW, while the lowest decrease was by NAC 200 mg/kg of rat's BW. However, multiple comparison results through the post hoc test showed no significant difference among the three doses. The drug use was not only assessed in terms of its efficacy, but also its safety, suitability, and cost factors. NAC is a drug with an identified safety profile. NAC has long been used for acute paracetamol intoxication, both orally and intravenously, with a loading dose of i.v 150 mg/kg of body weight. Oral NAC is also widely used as a mucolytic for various respiratory disorders. Clinical trials suggested that the dose up to 6.4 g/m2 of body surface area/day only caused minor gastrointestinal disorders. NAC had low toxicity in experimental animals, oral LD50> 10 g/kg of rat and mouse's body weight, and LD50 after intravenous administration of 2.8 g/kg BW for rats. This study indicated that a dose 200 mg/kg of BW had contributed to a significant effect on tissue MDA reduction, thus this

dose was recommended. The higher the dose, the closer it was to the toxic dose.

Tissue edema was assessed based on water content parameters. After being taken as much as 100 mg for tissue MDA examination, gastrocnemius muscle substances were weighed and determined as wet weight. Furthermore, the tissue was immediately dried by the freeze-drying method at -80ºC for 24 hours, then weighed and defined as dry weight. This method was simple and reliable to identify the fluid increase in the tissue. The results of the statistical calculations indicated that there was no significant difference between the control group and the NAC given groups. These results differed from the previous study which showed the effects of edema decrease on severe CSTI injury models by administering intravenous NAC 400 mg/kg of rat's BW. The effect differences with this study might be due to the severe CSTI treatment given of the previous study in such a way that resulted in blunt trauma on non-lethal muscles without causing compartment syndrome. Hence, this treatment did not trigger ischemia-reperfusion injury.

This study result is in line with the previous study on NAC protective effects on ischemia-reperfusion injury in the lungs after transplantation. The NAC protective effects on the lungs were reflected in improved oxygenation, decreased lipid peroxidation, increased glutathione levels, and improvement in peak airway pressure. However, there was no significant difference in myeloperoxidase activity and the ratio of wet weight to dry weight between the control group and groups with NAC. The previous study suggested that the insignificant decrease in pulmonary edema might be due to the length of observation time.

Interstitial edema on the ischemia-reperfusion injury was triggered by vascular barrier damage caused by activated leukocyte attachment to the vessel wall, resulting in extravasation. The other study, examining the NAC protective effects on the striated muscle of compartment syndrome model on rats, reported that NAC did not decrease neutrophil infiltration (myeloperoxidase activity) acutely, but decreased at 24 hours. Based on the results obtained, it cannot be concluded that NAC does not affect tissue edema in ischemia-reperfusion injury of skeletal muscles. A longer observation period is needed to determine NAC effects. In this study, the observation was conducted 1 hour after NAC administration and 1-hour reperfusion. In addition, the NAC in this study was given one dose. In acute paracetamol intoxication, NAC 150 mg/kg of BW as loading dose, followed by a further dose of 50 mg/kg of BW every 4 hours for 72 hours.

Several matters limited to this study. The lack of observation time to determine NAC effects, in which MDA examination and tissue edema were performed after 1-hour reperfusion. NAC was only given once as a loading dose. Multiple administration is required or continued with slow infusion to determine NAC effects further. Histological and morphological analysis is needed to determine muscle tissue damage and inflammatory cells in muscle tissue undergoing ischemia-reperfusion injury directly, to prove the NAC protective effects. Further research is required to examine NAC effects on systemic inflammation caused by ischemia-reperfusion injury of skeletal muscles that can trigger MODS.

CONCLUSION

Intravenous NAC administration provides positive results by reducing tissue MDA levels in ischemia-reperfusion injury of white rats' skeletal muscles. The tissue MDA levels on ischemia-reperfusion injury in white rats' skeletal muscles are not affected by NAC given dose, therefore the recommended dose is 200 mg/kg of body weight. NAC intravenous administration does not reduce tissue edema on the ischemia-reperfusion injury of white rats' skeletal muscles.

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