ORIGINAL ARTICLE

# N-Acetylcysteine Improves Renal Fuction and Reduces Tissue Malondialdehyde Levels in Glycerol-Induced Acute Kidney Injury of Wistar Rats

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**ABSTRACT**

**Introduction:** The etiology of myoglobinuric acute kidney injury involves oxidative injury brought on by the Fenton reaction and myoglobin redox cycle. Renal tubules may be harmed, and lipid peroxidation compounds with vasoconstrictor characteristics may be produced. N-acetylcysteine (NAC) is an antioxidant shown to improve renal microcirculation and have protective effects in various models of renal damage. The aim of the study was to demonstrate the protective impact of NAC in glycerol-induced rats by measuring tissue malondialdehyde (MDA) level and renal function test (RFT), and to determine the correlation between the protective effect and NAC dose.

**Methods:** This study measured tissue malondialdehyde (MDA) and renal function to examine any protective effect of NAC in a glycerol-induced rat model and to determine whether the effect was dose-related. Five groups of male Wistar rats were used: 1) saline control group, (2) glycerol (50%, 8mL/kg, i.m) plus saline i.v group, 3) glycerol plus NAC (100 mg/kg)-treated group, 4) glycerol plus NAC (200 mg/kg)-treated group, 5) glycerol plus NAC (400 mg/kg)-treated group. At 24 hrs, after glycerol injection, rats were sacrificed, cardiac blood was taken for renal function measurement, and renal tissues were removed for thiobarbituric acid MDA level assessment.

**Results:** Our study revealed that glycerol administration significantly amplified renal tissue MDA, serum creatinine, and BUN (blood urea nitrogen) levels. However, NAC administration dampened the MDA increment and renal function deterioration ($p<0.05$). Moreover, tissue MDA, BUN, and serum creatinine levels were significantly correlated to NAC dose ($r=0.485$; $r=0.491$; $rs=0.544$, respectively; all $p<0.05$), indicating that NAC protection declines by dose increments.

**Conclusion:** In this glycerol-induced acute kidney injury rat model, the administration of intravenous NAC 100 mg/kg reduced lipid peroxidation and improved renal function. Nevertheless, the protective effect was diminished in higher doses.

**Keywords:** Myoglobin; acute kidney injury; N-acetylcysteine; MDA; health risk

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## INTRODUCTION

Acute kidney injury (AKI) is a clinical condition distinguished by a rapid decline in kidney function and a high mortality rate (Makris & Spanou, 2016). AKI can arise from a variety of causes, including nephrotoxic substances exposure (Purnomo et al., 2021). AKI can also be induced by rhabdomyolysis (Gaut & Liapis, 2021). Rhabdomyolysis is a condition in which myoglobin, intracellular proteins, and electrolytes seep into the circulation from injured skeletal muscle. This might be exacerbated by AKI, the major cause of death in rhabdomyolysis cases (Hebert et al., 2023). Significant progress has been made in the treatment of rhabdomyolysis-induced AKI. However, the mortality rate remains high (Chavez et al., 2016).

The mechanisms of myoglobinuric AKI in rhabdomyolysis involve oxidative injury and renal vasoconstriction. Myoglobin is thought to cause renal oxidative injury in one of two ways: First, the Fenton reaction is catalyzed by the release of free iron from myoglobin. Second, the myoglobin redox cycle causes lipid peroxidation (Hebert et al., 2023). The renal tubular cell's cytoplasmic oxidative stress may induce peroxidation of lipids, proteins, and DNA, which may result in acute tubular necrosis (Hasanatuludhhiyah et al., 2015; Ratliff et al., 2016). Additionally, myoglobin-induced oxidative damage may intensify vasoconstrictor mediators in circulation, resulting in a reduction in renal blood flow. All of these issues could harm renal function (Liu et al., 2017).

The administration of antioxidants was proven to confer a protective effect in rhabdomyolysis-induced AKI (Honore et al., 2018; Zamorskii et al., 2019). N-acetylcysteine is an antioxidant that operates directly as a free radical scavenger and indirectly as a precursor of glutathione, an endogenous antioxidant (Ezeriņa et al., 2018). The protective effect of NAC has been demonstrated in many models of renal injuries. Additionally, it has been shown to improve renal microcirculation (Ergin et al., 2016, 2021; Huang et al., 2019). The antioxidant properties of NAC are also proposed to reduce the generation of lipid peroxidation product, F2-Isoprostane, which may bring about renal vasoconstriction

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(Bauer et al., 2014; van 't Erve et al., 2017).

The glycerol induction in an animal model is a method mainly employed to study the effect of various drugs on parameters of rhabdomyolysis-induced AKI (Mousleh et al., 2018; Yuqiang et al., 2022). The hypertonic glycerol solution injected intramuscularly may produce myolysis, hemolysis, and hypovolemia, exposing the kidney to abundant heme protein and myoglobin (Reis et al., 2019; Soares et al., 2002). Few studies have demonstrated the beneficial effect of NAC on glycerol-induced acute kidney injury animal models, nevertheless, none examined NAC of minimal three different doses as required to establish a dose-response curve (Fernández-Fúnez et al., 2002; Kim et al., 2010). Since the pharmacological effect of any active chemical should be confirmed by a dose-response relationship, a study that explores the dose-response relationship of NAC on an oxidative stress marker and renal function parameters could provide essential findings accordingly. Therefore, this study aimed to demonstrate the protective impact of NAC in glycerol-induced rats by measuring tissue malondialdehyde (MDA) level and renal function test (RFT), as well as to determine the correlation between the protective effect and NAC dose.

## METHODS

This study involved 35 male Wistar albino rats (Rattus norvegicus) weighing between 160 and 200 g. The rats were acclimatized within 1 week prior to the experiment. They were provided standard rat food before the glycerol induction but were not given access to water for 16 hours. The Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine at Universitas Airlangga approved all animal experiments (No. 213-KE). Rats were randomly divided into five groups, and they were kept at 23- 24°C on a 12:12 hours light-dark cycle. The healthy control group 1 (C1) was the rats given normal saline (NS) intravenously and NS (8 ml/kg BW) intramuscularly (the sham group). Glycerol solution (50% v/v, 8 ml/kg) was injected intramuscularly into the rats of the other four groups. The control group 2 (C2) was given NS intravenously. NAC was provided to the other three NAC groups at doses of 100 mg/kg BW (NAC1), 200 mg/kg BW (NAC2), and 400 mg/kg BW (NAC3). A single dose of N-acetylcysteine (Hidonac, Zambon) was administered intravenously via a tail vein 30 minutes before glycerol injection. A glycerol solution was injected intramuscularly into one hind limb. Normal saline and glycerol were injected under light ether anesthesia. After inducing rats with glycerol, they were watched for 24 hours without any nutritional or water limitations. Rats were euthanized after the follow-up period. Blood was sampled by heart puncture, and the kidney was extracted.

Renal tissue samples were analyzed using the thiobarbituric acid (TBA) reagent to determine the MDA concentration. The samples were homogenized in a cold PBS solution. The homogenate was centrifuged for 15 minutes at 3000 rpm. 1 mL of 15% TCA and 0.37% TBA in 0.25 N HCl and the supernatant were combined. After that, the substance was heated for 15 minutes at 80 °C. After cooling, it was centrifuged for 15 minutes at 3000 rpm. Using a spectrophotometer, the supernatant's absorbance was calculated at 532 nm. The MDA level was determined using the linear regression curve of the standard solution.

To measure of renal function, blood urea nitrogen (BUN) and serum creatinine levels of intracardiac blood samples were tested using an autoanalyzer kit.

SPSS 17.0 (IBM, USA) was used to analyze the data, which was then provided as mean±SD. Priorly, Shapiro-Wilk and Lavene's tests were performed to examine the normality and homogeneity of the data. For the normally distributed and homogeneous data, the ANOVA test was used to compare means, followed by post hoc LSD (least significant difference) testing. Pearson's correlation test was utilized to examine the relationship between NAC dosage and those measures. Nonparametric procedures were performed for the data which was not normally distributed. We used the Kruskall-Wallis test, followed by the Mann-Whitney post hoc test. The correlation between NAC dosage and those measures was investigated using Spearman's correlation test. At a p=0.05 level, statistics were declared significant.

## RESULTS

### The effect of NAC on Tissue MDA Levels

The mean ± SD of MDA levels of each group is presented in Figure 1. The data on MDA tissue levels were both homogeneous and normally distributed. Consequently, the ANOVA test was used followed by LSD for the post hoc test. Our investigation showed glycerol induction markedly raised tissue MDA levels ($p<0.05$). The administration of NAC could prevent the increment of the MDA level. There were differences in MDA levels between the glycerol group (C2) and the glycerol+NAC (100 and 200 mg/kg BW) groups (NAC1 and NAC2). The tissue MDA levels of the healthy control group (C1) and glycerol+NAC (100 and 200 mg/kg BW) groups did not differ significantly ($p>0.05$).

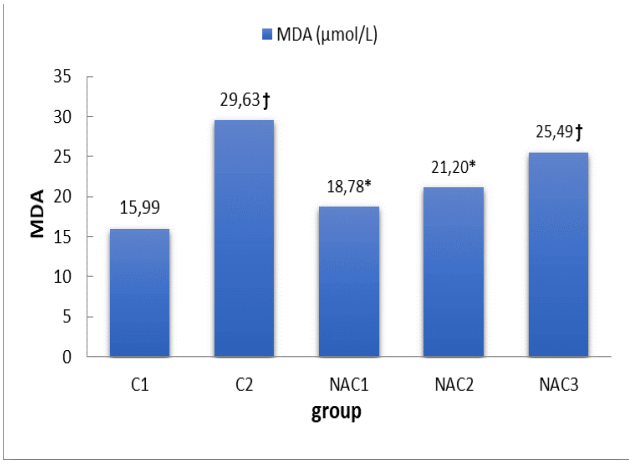


Figure 1. The mean of tissue MDA level

† p <0,05 significant difference with the healthy control group (C1) (LSD)
* p <0,05 significant difference with glycerol group (C2) (LSD)

### The Effects of NAC on BUN and SC Levels

Tests for homogeneity and normality of BUN and serum creatinine levels were performed. The data were not normally distributed, thus the Kruskall-Wallis test was employed and subsequently, the Mann-Whitney test was utilized for the post-hoc analysis. Glycerol induction also significantly increased BUN and serum creatinine levels ($p<0.05$). Intravenous administration of NAC also preserved renal function. The BUN and serum creatinine levels of glycerol+NAC (100 & 200 mg/kg BW) treated groups were significantly lower than those of the glycerol group ($p<0.05$). However, the levels remained significantly higher than those of the healthy control group ($p<0.05$).

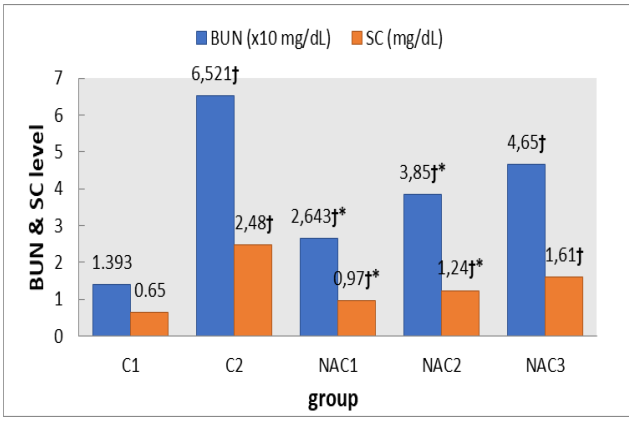


Figure 2. The mean of BUN and SC level

† p <0,05 significant difference with the healthy control group (C1) (Mann Whitney-U post hoc test)
* p <0,05 significant difference with the glycerol group (C2) (Mann Whitney-U post hoc test)

### Correlation between NAC Doses with MDA, BUN, and SC Levels

We further tested the correlation between three groups of different NAC doses with the three parameters. The data of MDA concentration and SC level was shown to be normally distributed, whilst the distribution of BUN level data was otherwise not normal. The Pearson correlation test revealed statistically significant correlations between NAC dosage and renal tissue MDA ($r=0.485$, $p<0.05$) and SC levels ($r=0.491$, $p<0.05$). The Spearman correlation test revealed a statistically significant correlation between NAC dosage with BUN ($rs=0.544$, $p<0.05$). Thus, the NAC dose that provided the greatest protective effect in our study was 100 mg/kg BW. Greater doses of NAC, i.e. 200 mg/kg BW. demonstrated diminished protective effect, whilst 400 mg/kg BW had no protective effect instead.

## DISCUSSION

In this study, glycerol induction impaired renal function, which was indicated by a significant increase in BUN and SC. In groups treated with NAC 100 and 200 mg/kg BW, BUN and SC were significantly lower than in the glycerol group, though considerably higher than in the healthy control group. In the NAC 400 mg/kg BW group, the renal function parameters were not significantly different from the glycerol group. Our finding was not similar to a study by Fernandez-Funez et al., which could not prove the protective effect of NAC against renal function impairment produced by glycerol induction (Fernández-Fúnez et al., 2002). This was probably related to different administration routes. In their study, NAC was given intraperitoneally. Fernandez-Funez et al. used a higher glycerol dosage, 10 mL/kg, which was injected into both hind limbs of rats. The period of water limitation was likewise greater in their study. In addition, muscle destruction could be more severe in their study. Thus, a more significant amount of myoglobin is released into circulation. A longer duration of water restriction could exaggerate dehydration. Therefore, more severe hypovolemia could amplify renal impairment.

Our findings suggested that NAC might reduce lipid peroxidation, measured by MDA levels in renal tissue. Through its direct and indirect antioxidant activities, this medication protects cell membranes from free radicals (Eman Ali Abdelrazik et al., 2022; Ezeriņa et al., 2018). NAC's reduced thiol group has been shown to directly scavenge free radicals produced by myoglobin degradation and the myoglobin redox cycle in the kidney. The primary antioxidant impact of NAC, however, is derived from its function as a precursor to cysteine, hence increasing the production of reduced glutathione (GSH) (Hebert et al., 2023; Kerksick & Willoughby, 2005). Glutathione (GSH) is the major antioxidant defense both intracellular and extracellular (Ribas et al., 2014)

Our findings regarding NAC's ability to reduce MDA were consistent with those of research by Elshiekh et al., which demonstrated that in rats with oxidative stress brought on by renal ischemia-reperfusion injury, NAC decreased tissue MDA level (Elshiekh et al., 2019). The protective effect of NAC on the cell membrane is essential. The membrane is severely damaged by lipid peroxidation, which increases membrane permeability, disruption of secretory function, and ion exchange (Andrés Juan et al., 2021). Lipid peroxidation may also cause the inactivation of membrane-bound receptors and enzymes (Gaschler & Stockwell, 2017). Finally, membrane disintegration produced by lipid peroxidation may induce cell death. Lipid peroxidation products are involved in the pathogenesis of glycerol-induced AKI via three mechanisms: 1) propagating lipid peroxidation, which is accelerated by speeding up the rate of ferryl-Mb generation; 2) inducing oxidative injury in the kidney; and 3) producing vasoconstriction (Hebert et al., 2023). An inhibitor of lipid peroxidation has been proven to confer a protective effect against rhabdomyolysis-induced AKI (Semenovich et al., 2022). As an antioxidant, NAC may decrease lipid peroxidation by products like F2-isoprostane, a powerful vasoconstrictor. Numerous studies also show that NAC contributes to improved renal circulation (Heyman et al., 2003; van 't Erve et al., 2017). As a result, it is reasonable to assume that NAC may, in part, preserve renal blood flow and thus maintain the glomerular filtration rate (GFR), as demonstrated by lowered SC and BUN levels in NAC-pretreated glycerol-induced rats.

The strength of our study compared to previous studies is that our study used NAC with 3 different doses, whereas previous studies used only 1 dose of NAC. According to our research, adding more NAC had a smaller protective effect and even lack of it at the highest dose. Our result is coherent with the notion of the "antioxidant paradox", that providing high doses of antioxidants in oxidative stress-related conditions could not have any positive effects, either for preventive or therapeutic purposes. Oxidative stress may indeed cause tissue damage directly. However, the reactive species are also involved in the adaptation process against tissue damage (Halliwell, 2013). It may promote the endogenous antioxidant system and other body defense or repair systems, for example, heat shock protein, proteasome, autophagy, and heme oxygenase. A high-dose antioxidant may change into a pro-oxidant (Sotler et al., 2019).

There were certain restrictions on how the TBARS method might be used to examine the MDA level in our investigation. This method lacks specificity. Chromogenic compounds may be formed by other aldehydes other than MDA. Besides that, the free metal ion is needed to produce color formation. As a result, MDA measurement in our study may not be as accurate given that myoglobin breakdown caused the release of free iron (Moselhy et al., 2013). Moreover, the administration of NAC in this study was intended for AKI prevention. It might be beneficial to

prevent rhabdomyolysis in certain conditions, for instance, strenuous physical activity. However, for the setting of traumatic rhabdomyolysis "crush injury" or acute intoxication of medicines or toxins, there should be further studies exploring its therapeutic benefits.

## CONCLUSION

Intravenous NAC administration confers a protective effect in our model of glycerol induction, as indicated by a decrease in lipid peroxidation and renal function parameters. A dose increment of NAC resulted in a diminished protective effect. The NAC dose which provided the most significant protective effect in our study, was the lowest, i.e., 100 mg/kg BW.

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## CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

## ETHICS CONSIDERATION

This study was approved by the Animal Care and Use Committee (ACUC) with agreement number 213-KE.

## FUNDING DISCLOSURE

This research was self funded.

## AUTHOR CONTRIBUTION

All author have contributed to all process in this research, including preparation, data gathering and analysis, drafting and approval for publication of this manuscript.

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