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Original Research Article

Dose and Duration of N-acetylcysteine on Superoxide Dismutase, MCP-1, and Foam Cell in Atherosclerosis Rat Model Research

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ABSTRACT

Oxidative stress plays a key role in the complex process of atherosclerosis. N-acetylcysteine (NAC) is currently being investigated as a potential treatment for various conditions associated with oxidative stress and reduced glutathione (GSH) levels. NAC's potential as a primary preventive measure in individuals at risk of cardiovascular disease has not been investigated. Therefore, this research aims to examine the effect of NAC dosages of 600 mg and 1200 mg on Superoxide Dismutase (SOD) and monocyte chemotactic protein-1 (MCP-1) levels, as well as the number of foam cells in Wistar rats, used as atherosclerosis model, across administration periods of 2 weeks and 6 weeks. A total of 30 male Wistar Rattus norvegicus rats were divided into 6 groups (n = 5), including normal (N), atherosclerotic (DL) given atherogenic diet, atherosclerosis group with 600 mg NAC for 2 weeks (DLN6-2), atherosclerosis group with 600 mg NAC for 6 weeks (DLN6-6), atherosclerosis group with 1200 mg NAC for 2 weeks (DLN12-2), and atherosclerosis group with 1200 mg NAC for 6 weeks (DLN12-6). The results showed that dose and duration of NAC administration increased SOD levels and reduced MCP-1 levels and the number of foam cells in atherosclerotic Wistar rats. Based on these results, NAC may be used as a primary prevention atherosclerosis.

Keywords: *N-acetylcysteine*, Superoxide Dismutase, Monocyte Chemotactic Protein-1, Foam cells

Introduction

Acute myocardial infarction, unstable angina pectoris, and sudden cardiac death are all primarily caused by atherosclerosis, which involves multiple significant inflammatory components in its pathophysiology.[1,2] Another complicated inflammatory vascular disease known as atherosclerosis is typified by the generation of mediators, cytokines, endothelial activation, and cell influx.[3,4] Individuals suffering from dyslipidemia (DL) have increased levels of reactive oxygen species (ROS) in monocytes and oxidized low-density lipoprotein (ox-LDL), as well as intracellular ROS generation in cultured endothelial cells.[5,6] The development of atherosclerosis is significantly influenced by endothelial dysfunction.[7,8] Endothelial dysfunction significantly amplifies inflammatory processes by triggering the increase of adhesion molecules due to endothelial injury and facilitating the release of pro-inflammatory mediators,[9] such as monocyte chemoattractant protein-1 (MCP-1).[10] Thromboembolic and atherogenic processes are facilitated by MCP-1, which also draws leukocytes to areas of vascular injury and mediates their recruitment to the sub-endothelial region.[11] Foam cells are produced when monocytes. undergo differentiation into macrophages, after which they emit pro-inflammatory cytokines and absorb ox-LDL.[6] Elevated levels of MCP-1 increase the likelihood of atherosclerotic plaque rupture in people[12]. Atherosclerosis is characterized by elevated oxidative stress due to alterations in the expression and activity of ROS-producing enzymes and the weakening of natural antioxidant systems.[13] The fundamental components of antioxidant systems in blood vessels include catalase, thioredoxin, paraoxonase, glutathione peroxidase (GPX), and mitochondrial uncoupling proteins (UCP). Protecting vascular tissue from oxidative injury, these enzymes collaborate to neutralize ROS, maintain redox balance, and more. Each system targets specific ROS, with SOD converting superoxide to hydrogen peroxide, catalase and GPX reducing hydrogen peroxide, and UCPs regulating mitochondrial ROS production.[14] During atherosclerosis, ROS production is markedly elevated, while the activity of endogenous antioxidant systems is often downregulated, exacerbating oxidative stress and contributing to vascular damage.[15] The enzyme superoxide dismutase (SOD) transforms superoxide radicals into hydrogen peroxide, which is subsequently decomposed by catalase and GPX to minimize cellular harm.[13,15]

The higher GSH level that results in less ROS generation is intimately linked to NAC's antioxidant qualities.[16] However, NAC can also bind directly to oxidants such as hydrogen peroxide, hyperchloric acid, and hydroxyl radicals.[17] The use of various antioxidants in the treatment of atherosclerosis is considered because the disease is caused by systemic inflammatory processes, oxidative stress, fat accumulation, cell death, and arterial fibrosis.[18]

There has been a lot of research on the effects of NAC in suppressing atherosclerosis process, but investigations comparing dose and duration of NAC treatment in increasing antioxidant activity and suppressing the inflammatory process in atherosclerosis have never been conducted. Therefore, this research aimed to examine the effects of NAC at doses of 600 mg and 1200 mg on SOD and MCP-1 concentrations and the number of foam cells in atherosclerosis model rats with duration of therapy of 2 weeks and 6 weeks.

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Materials and Methods

Animals and grouping

This study was conducted at the Experimental Animal Laboratory of the Faculty of Veterinary Medicine at Syiah Kuala University. This study was ethically approved by the Medical Research Ethics Commission of the Faculty of Veterinary Medicine, Syiah Kuala University, and registered with number 240/KEPH/VII/2023 on July 31, 2023. The Federer formula was used to determine the treatment sample size.[19] Laboratory experimental research used a completely randomized design with posttest only with a control group design method. In addition, 30 male white rats (*Rattus norvegicus*) Wistar strain, aged 5 weeks, weighing 75 to 100 g, divided into six groups (n = 5) randomly were used. The groups consisted of Normal (N, rats fed a standard diet or normal control), atherosclerotic (DL) given atherogenic diet, atherosclerosis group with 600 mg NAC for 2 weeks (DLN6-2), atherosclerosis group with 600 mg NAC for 6 weeks (DLN6-6), atherosclerosis group with 1200 mg NAC for 2 weeks (DLN12-2), atherosclerosis group with 1200 mg NAC for 6 weeks (DLN12-6).

Induction of rat atherosclerosis model and administration of N-acetylcysteine (NAC)

After rats in the control group had adjusted to their new environment for two weeks, regular feeding was carried out. In contrast, rats in the atherogenic group were fed an atherogenic diet *(containing 0.2% cholic acid, 2% egg yolk, 5% goat fat, and 92.8% maize rice)* at their leisure for eight weeks, [20,21] and NAC administration started in the ninth week.[20] In this phase, rats were still given a diet according to the research plan. In the N6-2 group, the NAC dose was given at 600 mg for 2 weeks, while N6-6 was given for 6 weeks. The N12-2 group was given a NAC dose of 1200 mg for 2 weeks and the N12-6 group was given for 6 weeks.

Collection of blood and aortic tissue

After the week following the injection of NAC, a cardiac puncture was used to collect blood and aortic tissue. Euthanasia was carried out by injecting ketamine 15-20 mg/body weight (Ilium ketamine; Troy Laboratories, Australia) intraperitoneally before blood collection. The collected blood was put in a tube with a label. A microcentrifuge was then used to centrifuge the blood for ten minutes at 3000 rpm. While the aorta tissue was prepared histopathologically using the frozen paraffin method and stained with hematoxylin-eosin, the acquired plasma was promptly stored at -80°C for SOD and MCP-1 analysis.

Measurement of SOD level by enzyme-linked immunosorbent assay

Bioenzy of Jakarta, Indonesia used Rat T-SOD ELISA kit (Catalog Number BZ-22188718-EB) to measure the SOD concentration. To measure the level of SOD, the following steps were performed: first, 90 µL of plasma sample was added to the microplate well without enzyme; next, the plasma sample with enzyme solution was added; and finally, a control enzyme solution was introduced. After 10 seconds of shaking, the microplate was closed and placed in an incubator set to 37°C for 50 minutes. In addition, 180 µL of the chromogenic agent was added and shaken briefly for 10 seconds and left standing at room temperature for 10 minutes. Absorbance at 450 nm was measured using an ELISA reader for microplates (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories Inc.).

Measurement of MCP-1 level by enzyme-linked immunosorbent assay

A Rat MCP-1 ELISA kit (Cat. BZ-22184055-EB, Bioenzy, Jakarta, Indonesia) was used to quantify MCP-1 concentration. After 100 µL of plasma sample and the standard were added to the well, the sample was incubated at 37°C for one hour to begin measuring MCP-1. Subsequently, 90 µL of substrate reagent was added, and the mixture was allowed to incubate at 37°C (outside of direct sunlight) for 15 minutes. Fifty microliters of stop solution were added to halt the reaction. After five minutes, the absorbance of the sample at 450 nm was measured using a microplate ELISA reader (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories Inc.).

Measurement of aortic foam cells

The initial steps in investigating aortic foam cells involved hematoxylin-eosin staining and histological preparations using the frozen paraffin method. An Olympus CX216 light microscope was used to view foam cells at 400x magnification. In three aortic cross-sections, the total number of cells was counted from the field of view. The aorta cross-section's tunica intima to tunica medium layers contained foam cells with pale features and blue nuclei.

Statistical analysis

In this research, the number of aortic foam cells, MCP-1, and *SOD* concentration data were the parameters measured. To assess the normality and homogeneity of data distribution (p-value > 0.05), the Shapiro-Wilk and Levene tests were applied. Subsequently, one-way analysis of variance (ANOVA) was used to determine the effects of varying doses and durations of NAC administration on these parameters across all research. Post-hoc analysis (Duncan test) was then conducted to identify statistically significant differences between the parameters in all groups, and Pearson correlation tests were used to examine the correlation of each parameter within each group. These statistical tests were performed using SPSS version 21 for Windows.

Results and Discussion

The DL group had considerably lower SOD levels than the N group ($P<0.01$), as shown in Table 1. Moreover, in contrast with the PC group, the SOD levels of rats in the DLN6-2, DLN6-6, DLN12-2, and DLN12-6 groups were noticeably stronger ($P<0.01$). There was no statistically significant difference between the N and DLN6-2 groups concerning SOD levels, suggesting that the 600 mg of NAC administered for two weeks (DLN6-2) restored SOD levels to those of the N group (Table 1). A higher dose of NAC (1200 mg) was able to increase SOD levels higher both with treatment for 2 weeks and 6 weeks. Duncan test (Figure 1) also showed that SOD levels in the DLN6-6 group were significantly higher than those in the DLN6-2 group, while the SOD levels in the DLN12-6 group were not different from those in the DLN12-2 group. Duration of NAC treatment affects increased SOD levels, specifically at dose of 600 mg; namely at 6 weeks, the SOD levels were higher than at 2 weeks, but the time of NAC treatment at 1200 mg did not affect both 2 and 6 weeks.

The Pearson correlation test (Table 2) shows a strong and highly significant positive correlation ($P < 0.01$) between dose and SOD levels for both 2 weeks and 6 weeks, with correlation coefficients of 0.927 and 0.741, respectively. In addition, it also proves that the higher dose, the more significant increase in SOD level. This can also be proven by the results of linear regression analysis with r square (R2) values of 0.860 and 0.549 at 2 weeks and 6 weeks. (Table 2).

Table 3 shows that duration of NAC treatment is positively correlated with SOD levels, which means that the more extended treatment of NAC causes an increase in SOD levels in both those given dose of 600 mg and 1200 mg. In the treatment of NAC at dose of 600 mg, duration of treatment showed a robust positive correlation with a value of $r = 0.909$. This was very significant ($P <0.01$) (Table 3), while at dose of 1200 mg, duration of treatment had a strong positive correlation with a value of $r = 0.741$ and was significant ($P <0.05$). This proves that duration of NAC treatment has a positive relationship with levels of SOD in the blood plasma of rats. This is further supported by the results of the linear regression analysis, which yielded R-squared (R^2) values of 0.826 and 0.549, respectively. This shows that the strength of the relationship between duration of treatment and the increase in SOD levels in the blood plasma of rats is 82.6% for 2 weeks and 54.9% for 6 weeks (Table 3).

Effect of NAC in MCP-1 in Atherosclerosis Model Rats

According to Table 1, there was a significant difference in MCP-1 levels between the DL and N groups ($P<0.01$). Figure 2 shows that the

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Table 1: Effect NAC in All Group

Serum	Group	n	Mean ±(SD)
SOD	N	5	0.27 ± 0.06
	DL	5	0.03 ± 0.01
	DLN6-2	5	0.26 ± 0.02
	DLN6-6	5	0.32 ± 0.02
	DLN12-2	5	0.34 ± 0.02
	DLN12-6	5	0.38 ± 0.05
MCP-1	N	5	23.16±2.92
	DL	5	50.26±8.43
	DLN6-2	5	26.50±7.09
	DLN6-6	5	27.49±1.97
	DLN12-2	5	23.04±3.66
	DLN12-6	5	15.25±2.35

Table 2: Pearson correlation test (the relationship between dose of NAC treatment to SOD and MCP-1)

	Weeks	Pearson Correlation/ Dose	Linear Regression	P value
SOD	2	0.927	0.860	0.001
	6	0.741	0.549	0.001
MCP-1	2	-0.0334	0.111	0.419
	6	0.956	0.914	0.000

Table 3. Pearson correlation test (the relationship between duration of NAC treatment to SOD and MCP-1)

	Mg	Pearson Correlation/ Dose	Linear Regression	P value
SOD	600	0.909	0.826	0.002
	1200	0.741	0.549	0.036
MCP-1	600	0.109	0.01	0.737
	1200	-0.826	0.681	0.01

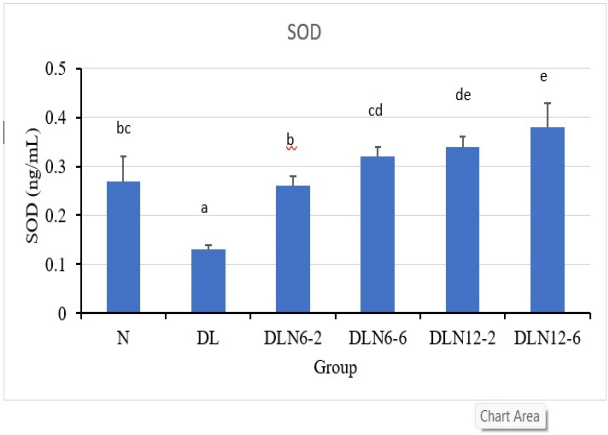

Figure 1. Histogram of Duncan's test results of the SOD levels (ng/ml) of rats (*Rattus norvegicus*) in the treatment groups N, DL, DLN6-2, DLN6-6, DLN12-2, DLN12-6. Different superscripts indicated significant differences.

MCP-1 levels were significantly lower in the DLN6-2, DLN6-6, DLN12-2, and DLN12-6 groups compared to the DL group (P<0.01). The levels of MCP-1 were not significantly different in the DLN6-2, DLN6-6, and DLN12-2 groups (P>0.05), but there was a significant drop in the DLN12-6 group (p<0.01). This shows that high doses of NAC are more effective in reducing MCP-1 levels. The Pearson correlation test (Table 2) shows that dose negatively correlates with MCP-1 levels. In the treatment of NAC for 2 weeks, dose has a relatively weak negative correlation (r = -0.334), which is not significant (P > 0.05). However, with a 6-week treatment, there is a strong negative correlation, and it is very significant (P<0.01). This can also be proven by linear regression analysis with r square (R2) values of 0.111 and 0.914 at 2 weeks and 6 weeks, meaning that the strength of the relationship between the decrease in MCP-1 levels and dose in NAC treatment for 2 weeks is only approximately 11.1%. However, in NAC treatment for 6 weeks, the strength of the relationship between dose and MCP-1 levels reaches 91.4%.

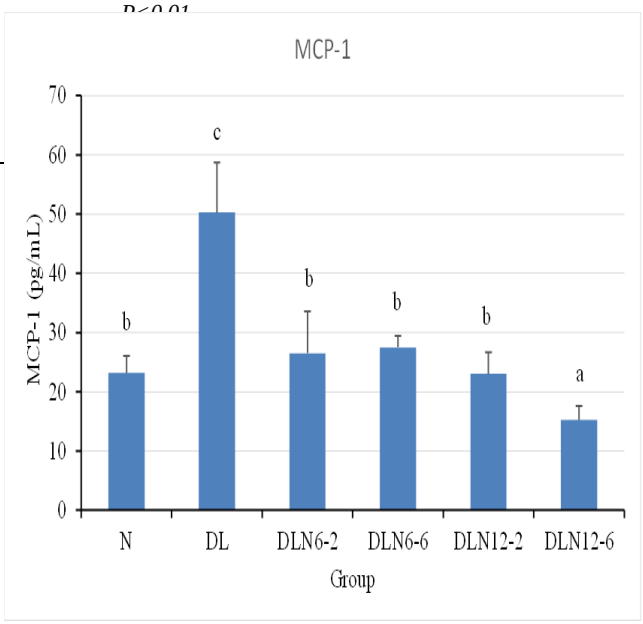

Figure 2. Histogram of Duncan's further test results of the average MCP-1 levels (pg/ml) of blood plasma of rats in the treatment groups N, DL, DLN6-2, DLN6-6, DLN12-2, DLN12-6. Different superscripts indicated significant differences.

Table 3 shows that duration of NAC treatment is negatively correlated with MCP-1 levels. In the treatment involving 600 mg NAC, duration only showed a low correlation (r = 0.109) and was not significant (P> 0.05). However, in the treatment involving 1200 mg, duration had a robust negative correlation (r = - 0.826) and was significant (P <0.05). This shows that at higher doses (1200 mg), the longer the treatment duration, the more effective it is in reducing MCP-1 levels. This can also be observed from the results of linear regression analysis with an r square (R2) value of 0.681, which means that the strength of the relationship between duration of treatment and decrease in MCP-1 levels with high doses is 68.1%.

Effect of NAC in aortic foam cells of atherosclerosis rat model

The calculation of the number of foam cells in the tunica intima-tunica media in each treatment group is shown in Table 4.

In animal models of oxidative stress, NAC has been shown to be an efficient antioxidant reducing damage and guarding against harm to other organs both in vitro and in vivo. Previous study used a 400–600 mg daily dose of NAC and a treatment period ranging from 4–32 weeks, showed that NAC was superior to a placebo after 12 weeks in terms of reducing the risk of exacerbation and COPD symptoms.[22] According to this research, the levels of SOD, MCP-1, and foam cells were all affected by a 600 mg dose and two weeks of NAC administration. There was a strong association between the length of treatment and the 600 mg NAC dosage (r = 0.86). This was significant (P< 0.05) to increase SOD, the same with the treatment of 1200 mg, duration of treatment had a positive correlation (r = - 0.826) and (P <0.03). This proves that at a high dose (1200 mg), the longer the treatment is administered, the

greater the increase in SOD levels, as showed by an R-squared (R^2) value of 0.860.
According to the results, previous study examined how NAC affected SOD in young female rats suffering from nephrotoxicity

Table 4. Results of measurement on the number of foam cells in the aorta

Group	Mean (±sd)	P-Value
N	2.16 ±0,57[a]	0.000
DL	38.25±2.09[f]	
DLN6-2	32.25± 2.42[e]	
DLN6-6	25.50± 1.03[d]	
DLN12-2	20.41±0.56[c]	
DLN12-6	6.91±2.45[b]	

Different superscripts indicated significant differences.

They observed that the administration of 100 mg/kg/day of NAC improved kidney function and increased SOD concentration.[23] Another study showed that NAC can raise SOD by providing diabetic rats with a high-fat diet for 12 weeks along with 2 mmol/l NAC dissolved in drinking water.[24] NAC's free thiol group reacts with reactive nitrogen species (RNS) and ROS to produce its direct antioxidant action. Indirectly, it also aids in lowering oxidative stress and ROS generation by boosting SOD activity and recovering GSH.[25]
In the treatment involving 600 mg NAC, duration of treatment only showed a low correlation (r = 0.109). This was not significant (P> 0.05) to reduce MCP-1, but in the treatment involving 1200 mg, duration of treatment 6 weeks had a robust negative correlation (r = - 0.826) and was significant (P <0.05) to reduce MCP-1. This proves that at a high dose (1200 mg), the longer the treatment is administered, the more it can reduce MCP-1 (r square (R2) value of 0.681). The research on elderly rats with atherosclerosis showed that NAC treatment for a minimum of three months can lower MCP-1 levels.[26] The results are consistent with those of Qingyi Zhu's investigation, which found that greater doses of NAC can inhibit the levels of anti-inflammatory MCP-1 over an extended time.
NAC has complicated and poorly understood effects on inflammation, atherosclerosis, and ROS production. It has long been believed that NAC works as an antioxidant by either restoring intracellular GSH reserves or breaking down disulfide bonds.[27]
In this research, NAC treatment lowered foam cell, showing that NAC reduced atherosclerosis at least partially due to decreased in vivo ROS production. NAC treatment also dramatically decreased the atherosclerotic plaque in hyperlipidemic rats. In hyperlipidemic rats,

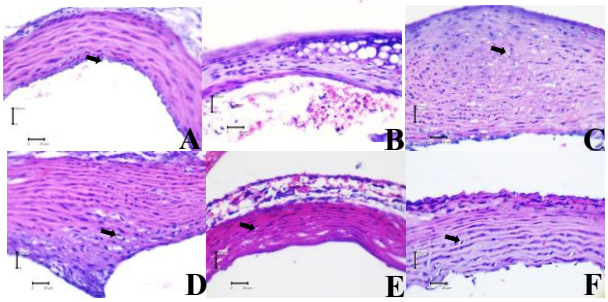

Figure 3. Cross-sectional images of the aorta. (A) N group (B) DL group, (C) DLN6-2 group, (D) DLN6-6 group, (E) DLN12-2 group, (F) DLN12-6 group. Arrows show pale foam cells with blue nuclei.

NAC inhibited the formation of ROS both intracellularly and extracellularly.[5] Other studies reseached the beneficial effects of NAC in reducing airway inflammation induced by lipopolysaccharide (LPS). Using oral doses of NAC at 200 mg, 600 mg, and 1200 mg daily, they used a validated ex vivo model of Acute Exacerbation of Chronic Obstructive Pulmonary Disease (AECOPD)[27,28]. Furthermore, another study showed that duration of treatment significantly influences NAC's anti-inflammatory activity.[28] Their results showed that NAC has long-lasting anti-inflammatory and antioxidant benefits when administered chronically at
low levels. On the other hand, high doses of NAC administered acutely produce a strong anti-inflammatory and antioxidant effect.[28]

Conclusion

In atherosclerosis model rats, NAC can raise SOD, lower MCP-1, and reduce the number of foam cells, according to the results of the data analysis. In Wistar rats given an atherogenic diet, long-term NAC therapy is increasingly beneficial in reducing atherosclerosis. This research can be the basis for conducting further research on other organs with variations in the dose and duration of NAC administration. Further studies are needed to fully elucidate its mechanisms, optimize its therapeutic applications and to determine the lethal dose of NAC.

Conflict of interest

The author reports no conflicts of interest in this work.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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