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Effects of N-Acetyl Cysteine on Oxidative Stress Biomarkers in End-stage Renal Disease

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Authors' contributions

This work was carried out in collaboration between all authors. Author MS designed the study, performed the statistical analysis and wrote the protocol. Author BN wrote the first draft of the manuscript. Authors BN and BE managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background and Objective: Imbalance between the oxidants and antioxidants in biologic systems is called oxidative stress which is associated with wide range of diseases and malfunctions. Renal physiology, high blood flow and reabsorption mechanisms make kidneys susceptible organs to oxidative stress, especially in End-Stage Renal Disease (ESRD) patients; because of their decreased antioxidant capacity along with increased oxidant species. N-Acetyl cysteine (NAC) is a known synthetic antioxidant. Our aim of study was to investigate helpful antioxidant effects of NAC on oxidative stress biomarkers in ESRD patients.
Methods: This double-blind, randomized clinical trial was done on 130 ESRD patients undergoing hemodialysis. Effects of one month administration of 3 different doses of oral NAC (600, 1200 and 1800 mg/day) on oxidative stress biomarkers (Glutathione (GSH), Malondialdehyde (MDA),

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Catalase (CAT) and Superoxide Dismutase (SOD)) were assessed. Each group was divided into two sub-groups of drug and placebo, randomly.
Results: GSH concentrations increased significantly in all three groups (P<0.05). Decrease in MDA levels in doses of 600 mg/day and 1200 mg/day were significant (P<0.05). Significant decrease in activity of CAT was observed by 600 mg/day of NAC (P<0.05). Slight but not significant decrease in SOD activity levels were observed in these three groups (P>0.05).
Conclusion: Data demonstrate the protective effects of NAC in ESRD patients against oxidative stress. Also, according to the results, dose of 600 mg/day is preferred for this clinical application.

Keywords: N-Acetyl cysteine; oxidative stress; end-stage renal disease; glutathione; malondialdehyde; catalase; superoxide dismutase.

1. INTRODUCTION

Reactive Oxygen Species (ROS) have a central role in the etiology of numerous worldwide diseases [1-5]. ROS play an important role in the pathophysiological processes of a surprisingly wide variety of renal diseases. In renal disease patients, there are good evidences indicating that the interaction of blood with artificial membranes leads to activation of several cellular pathways, which include the plasma cascade systems of complement, coagulation and kinins together with activation of polymorphonuclear leukocytes (PMN), monocytes, lymphocytes and platelets. Thus, activation of these pathways promotes the production of ROS [6-11].

Oxidative stress (OS), results from excessive generation of oxidation-derived products and insufficient anti-oxidant defense mechanisms [12]. Sources of OS could react with cellular lipids, proteins, nucleic acids and carbohydrates; leading to deterioration of cellular structural architecture [3,13]. In this regard, utilization of some antioxidant drugs to elevate the antioxidant capacity of human body is essential.

Therefore, in order to protect renal disease patients against OS induced complications of hemodialysis, antioxidants, particularly use of N-Acetyl Cysteine (NAC) have been proposed. Some reports indicating its outstanding efficacy in renal protection, cardiovascular events, serum homocysteine level and oxidative stress-related inflammation in renal patients is available [14-17].

Despite widespread investigations in this area, few studies are done on advantages of oral NAC on plasma biomarkers. Also, because of the controversial data on the antioxidant enzymes activities, we have investigated the beneficial effects of three doses of NAC on these four OS parameters in Iranian End-Stage Renal Disease (ESRD) patients: Glutathione (GSH), Malondialdehyde (MDA), Catalase (CAT) and Superoxide Dismutase (SOD).

2. MATERIALS AND METHODS

2.1 Design and Setting

This study was a double-blind randomized clinical trial done in Baqiyatallah Hospital, Tehran, Iran. We selected 130 ESRD patients, randomly. This randomization was performed by using a table of random numbers. They were randomly allocated into one of six therapeutic groups. Neither patients nor physicians were informed of drug or placebo. All patients were on the same diet during the clinical trial.

2.2 Ethical Issues

This trial was conducted according to Declaration of Helsinki principles and the ethical committee of Baqiyatallah University of Medical Sciences approved the proposal of the study. Also, written informed consents were obtained from all patients.

2.3 Follow Up

All patients were visited by nephrologists before, during and after the study and clinical symptoms were entered into the questionnaire in order to assess the clinical conditions of each patient if needed.

2.4 Data Collection

For the present study, 130 eligible patients were divided randomly into 6 groups; 3 groups were treated with 600, 1200, 1800 mg/day of N-acetyl cysteine and the three other groups were treated with the same doses of placebo for 30 days. We named these groups by their doses; 50 patients in 600 mg/day, 50 patients in 1200 mg/day and 30 patients in 1800 mg/day were called group1, group2 and group3, respectively. In each group, half of them received NAC and others received placebo.

All of the patients were instructed to use their prescribed drugs after lunch.

ESRD Patients which were under three times a week hemodialysis (HD) were included in this study. Patients were not allowed to use any other antioxidant therapy for at least one month before and during the course of the trial. Folic acid, calcium carbonate and erythropoietin were taken by all the patients.

Exclusion criteria were using any kind of other antioxidant drugs, deterioration in clinical conditions of patients during the course of the study, cigarette smokers or substance abusers and using less than 80% of the medications. Also, NAC administration was discontinued in the case of occurrence of any severe NAC side effects.

In NAC groups, five patients canceled their participation in the study and two patients died. In placebo groups, four patients canceled their participation, three patients discontinued because of the hospitalization and two patients died.

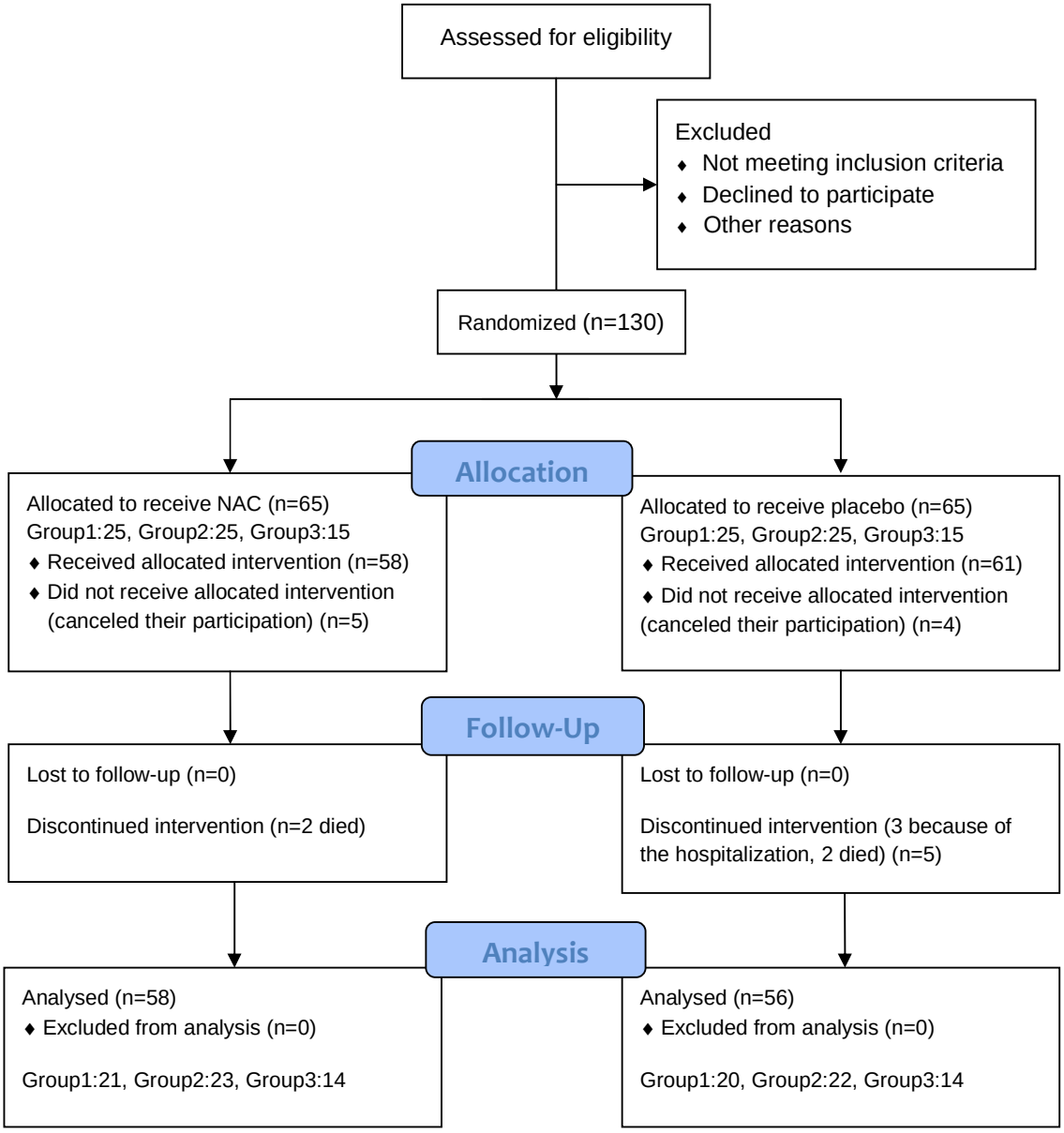


Fig. 1. CONSORT patient flow chart. Screening and randomization

2.5 Data Analysis

In order to find out whether or not N-acetyl-cysteine was more effective than placebo in improving the OS biomarkers, we compared the concentration variations of biomarkers in each NAC and placebo groups and also the differences between these three groups.

All analyses were done using SPSS for windows 15.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using the Kruskall Wallis and Mann-Whitney-*U* test. Differences were considered significant when the probability was $P< 0.05$.

2.6 Biochemical Methods

Venous blood samples were collected just before hemodialysis. All blood samples were taken after 10-12 hours of fasting in the morning (between 08:30 AM and 10:30 AM). Samples were collected in vacuum tubes containing EDTA as anticoagulant. Plasma samples were separated from blood cells and stored at -80℃ and were used for measuring the levels of GSH and MDA and activities of Catalase (CAT) and Superoxide Dismutase (SOD).

2.7 GSH Assay

GSH levels were determined using modified version of Tietze method [18]. Cellular proteins were precipitated by addition of 5% of sulfosalicylic acid and removed by centrifugation at 3000×g for 15 min. GSH levels were assayed in the supernatant as follows: 800 µl of 0.3mM solution of Na_2HPO_4 and 100µl of 0.04% solution of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.1% sodium citrate were added to 100 µl of the protein-free cell lysate supernatant. The absorbance of the solution was monitored at 412 nm for 5 minutes using Beckman UV spectrophotometer. The value for each sample was read from the standard curve and was expressed in nmol/ml.

2.8 MDA Assay

MDA concentrations were measured by TBARS (thiobarbituric acid reactive substances) assay tests according to modified version of Satho's method [19]. In brief, 500 µl of plasma was mixed with 10% solution of Trichloroacetic Acid (TCA) in a centrifuge tube and shaken gently. Then 1 ml of 0.67% solution of Thiobarbituric Acid (TBA) in 2M sodium sulfate was added to the tube and heated in a bath of boiling water for half an hour. After cooling in cold water, the resulting chromogen was extracted with n-butyl alcohol by vigorous shaking. The organic phase was separated by centrifugation at 3000×g for 15 minutes and its absorbance was recorded at a wavelength of 532 nm. The level of absorbance was converted into nmol/ml using 1,1,3,3-tetra-ethoxypropane as standard.

2.9 CAT Activity Assay

The CAT activity was determined following the decomposition of H_2O_2 according to the Cohen method [20,21]. The reaction starts by mixing H_2O_2 (6 mM) with plasma and phosphate buffer (50 mM at pH 7.0) and stops by adding H_2SO_4 (6 N) after 3 minutes. The last step is adding $KMnO_4$ (0.01 N) and finally absorbance can be measured by means of spectrophotometer at 480 nm. The activity of enzyme is expressed as units per liter.

2.10 SOD Activity Assay

SOD activity was measured by inhibition of NADH oxidation [22]. TDB buffer (Triethanolamine (0.1 M) – Diethanolamine (0.1M) – 1.38% HCl buffered at pH=7.4) was added into cuvettes, followed by NADH (0.27 mM), EDTA (5 mM), $MnCl_2$ (2.5 mM) and 0.1 ml of sample. 2-mercaptoethanol (3.75 mM) was added to the mixture and the decrease in absorbance was monitored after 5 minutes. One unit of activity is defined as the amount of enzyme that inhibits the oxidation of NADH by 50% at 25℃.

3. RESULTS

Table 1 shows demographic and general biochemical characteristics of patients in the study. As can be seen in the first to third rows of the table, there were no significant differences between age, sex and body mass index (BMI) of the patients in drug and placebo groups ($P>0.05$). Also, NAC administration didn't affect the results of kidney function and complete blood count (CBC) tests.

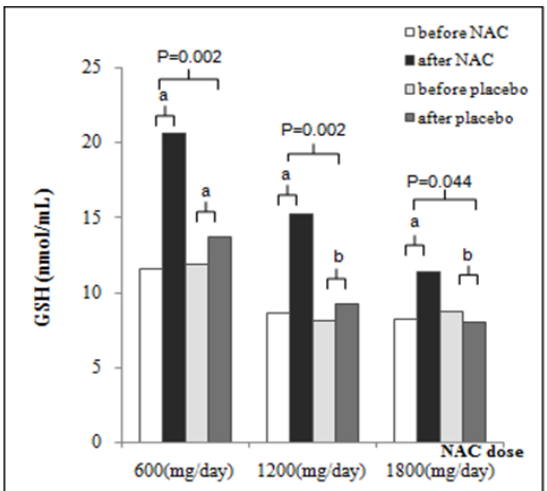
Among all patients in these three groups, 34 (31%) had history of kidney diseases and 29 (27%) had experienced kidney transplantation one or two times.

Predominant causes of ESRD in these patients were hypertension (37.3%), diabetes mellitus (27.3%), glomerulonephritis (6.4%) and obstructive uropathy (1.8%). 22% of patients had

a history of cardiovascular diseases and 4.5% a cerebrovascular accident.

3.1 GSH Concentration

As can be seen in Fig. 2, significant increases were observed in plasma levels of GSH in drug groups in comparison with placebo groups (P<0.05). Also, by doing Kruskal Wallis test, significant differences in GSH plasma levels were observed in comparison of three different groups with each other (P<0.05).



	600(mg/day)	1200(mg/day)	1800(mg/day)
Before NAC	11.68±1.86	8.65±0.98	8.19±1.48
After NAC	20.60±2.58	15.26±1.09	11.41±1.48
Before placebo	11.94±1.42	8.12±1.1	8.74±2.13
After placebo	13.75±1.17	9.23±1.2	8.02±1.01

Fig. 2. Effects of different doses of NAC on plasma levels of GSH

Data are expressed as mean±S.E.M
a: Significant difference (P<0.05),
b: Not significant (P>0.05)
NAC: N-Acetyl Cysteine, GSH: Glutathione,
SEM: Standard Error of Mean

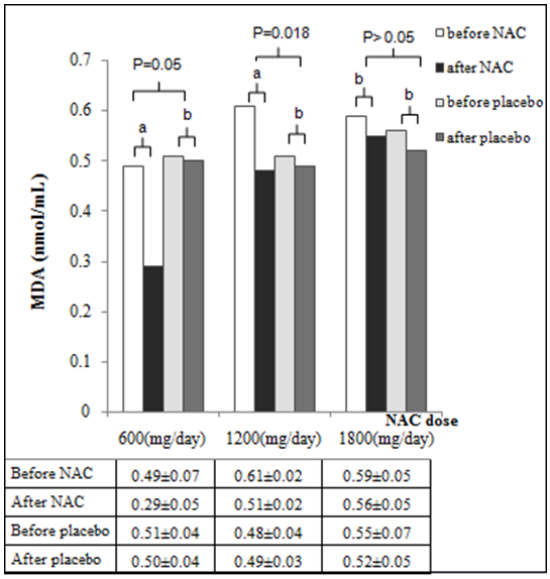
3.2 MDA Concentration

Fig. 3 shows effects of different doses of NAC on MDA plasma levels. In doses of 600 mg/day and 1200 mg/day we can see significant differences in comparison with placebo groups (P<0.05).

Also significant differences in MDA levels are seen between groups (p<0.05).

3.3 Activity of Antioxidant Enzymes

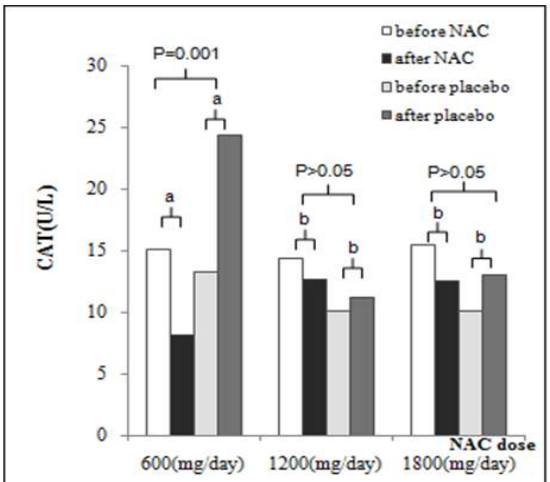
Fig. 4 shows effects of different doses of NAC on CAT activity levels. In 600mg/day dose we can see significant differences in comparison with placebo group (P<0.05).



	600(mg/day)	1200(mg/day)	1800(mg/day)
Before NAC	0.49±0.07	0.61±0.02	0.59±0.05
After NAC	0.29±0.05	0.51±0.02	0.56±0.05
Before placebo	0.51±0.04	0.48±0.04	0.55±0.07
After placebo	0.50±0.04	0.49±0.03	0.52±0.05

Fig. 3. Effects of different doses of NAC on plasma levels of MDA

Data are expressed as mean±S.E.M
a: Significant difference (P<0.05),
b: Not significant (P>0.05)
NAC: N-Acetyl Cysteine, MDA: Malondialdehyde,
SEM: Standard Error of Mean



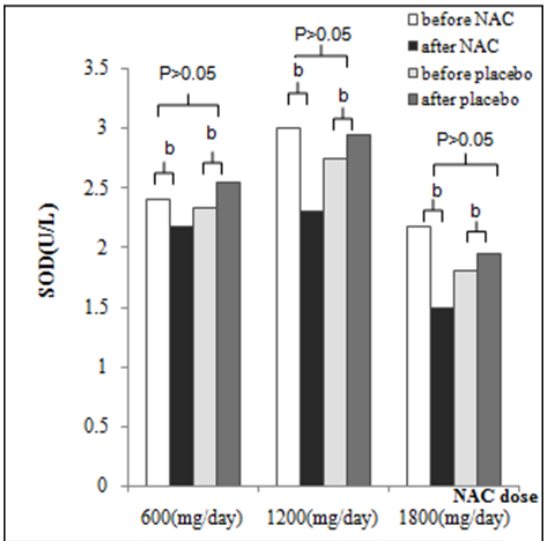
	600(mg/day)	1200(mg/day)	1800(mg/day)
Before NAC	15.18±2.5	14.43±2.2	15.43±2.8
After NAC	8.18±1.47	12.72±1.8	12.61±1.58
Before placebo	13.26±1.80	10.11±2.7	10.16±1.8
After placebo	24.42±3.10	11.16±1.3	13.09±3.12

Fig. 4. Effects of different doses of NAC on plasma levels of CAT

Data are expressed as mean±S.E.M
a: Significant difference (P<0.05),
b: Not significant (P>0.05)
NAC: N-Acetyl Cysteine, CAT: Catalase,
SEM: Standard Error of Mean

Also significant differences in CAT activity levels are seen between groups (P<0.05).

Fig. 5 shows a slight but not significant decrease in SOD activity levels by different doses of NAC. Also differences between groups were not significant (P>0.05 for both).



Before NAC	2.41±0.27	3.05±0.35	2.17±0.4
After NAC	2.17±0.31	2.3±0.36	1.49±0.30
Before placebo	2.33±0.31	2.74±0.39	1.8±0.39
After placebo	2.54±0.26	2.94±0.40	1.95±0.43

Fig. 5. Effects of different doses of NAC on plasma levels of SOD

Data are expressed as mean±S.E.M
a: Significant difference (P<0.05),
b: Not significant (P>0.05)
NAC: N-Acetyl Cysteine, SOD: Super Oxide Dismutase, SEM: Standard Error of Mean

4. DISCUSSION

There is a clear correlation between reduced antioxidant capacity and increased deterioration of OS [14]. Several studies are done on OS in patients with renal insufficiency. However, few of them have prospectively analyzed the effectiveness of thiol-containing antioxidants like N-acetyl cysteine on biomarkers of OS in ESRD patients.

NAC is a famous artificial precursor of GSH. For a long time this drug is used as a mucolytic agent [23]. Antioxidant effects of NAC is illustrated by different animal models and human trials [14,17,24-28]. Beneficial effects of oral NAC on cardiovascular system in ESRD patients [15], reduced oxidative stress-related inflammations in patients on hemodialysis [16], renoprotective effects in toxicity of cisplatin, cyclosporine, mercuric chloride, cadmium, lithium, gentamicin and amphotericin, decrease of increased rate of mononuclear leucocyte apoptosis in HD patients and its efficiency against acute paracetamol toxicity are observed [23].

Our data demonstrate significant beneficial effects of NAC in protection of ESRD patients against OS injuries.

Increase in Glutathione levels, which plays a fundamental role in detoxification of xenobiotics and maintenance of redox status of cells, decrease in MDA levels, which forms by degradation of polyunsaturated lipids by ROS and decrease in activity of antioxidant defense enzymes like CAT and SOD, as a maker of lipid peroxidation, show a decrease in ROS along with improvement in antioxidant sources of cells.

Glutathione is a fundamental intracellular antioxidant which acts directly by scavenging ROS. GSH is able to remove free radicals leading to formation of oxidized glutathione form (GSSG) [29]. Therefore, lack of glutathione makes cells susceptible to oxidative stress. A decline in cellular levels of GSH has been considered to be indicative for OS [30,31]. Previous studies have shown decreased level of GSH in renal disease patients [8,32]. Interestingly, we found that treatment with NAC restored renal glutathione along with antioxidative effects by direct ROS scavenging, according to the previous studies. Our data are in line with the observation that NAC strongly increased GSH levels and increased antioxidant sources in renal diseases patients [32]. Also Nitescu et al. observed elevated GSH concentrations after administration of 200 mg/kg of NAC intraperitoneally in renal ischemia-reperfusion rats [25].

Our data show that plasma MDA levels are significantly decreased in ESRD patients after administration of NAC. Previous studies indicated that MDA levels in hemodialysis patients is significantly increased [8,33]. Trimarchi et al. [34] showed antioxidant therapy in hemodialysis patients caused a significant decrease in MDA plasma concentration. Sehirli et al. [32] also founded decreased MDA level in renal ischemia/reperfusion rats after administration of NAC. In contrast to our data Dashti-Khavidaki et al. [35] reported no significant alterations in the concentration of MDA by NAC administration.

Table 1. Demographic and general biochemical characteristics of patients

Parameters		Group1 (600 mg/day)		Group2 (1200 mg/day)		Group3 (1800 mg/day)	
		NAC (n=21)	Placebo (n=20)	NAC (n=23)	Placebo (n=22)	NAC (n=14)	Placebo (n=14)
Demographic characteristics							
Age (Mean±SD) year		53±17.97	54.45±20.65	52.69±17.01	49.86±16.19	55.85±10.87	60.42±11.77
Sex	Male/Female	13/8	9/11	16/7	14/8	4/10	6/8
BMI (kg/m^2)	Before study	23.30±4.06	21.87±4.67	24.38±5.28	22. 25±2.5	24.88±3.8	24.64±3.9
Complete blood count							
Platelets (1000/mm^3)	Before study	157±94	170±86	155±73	161±54	144±53	2 13±89
	After study	134±76	135±95	163±65	170±58	163±96	123± 13
WBC (1000/mm^3)	Before study	5.8±0.94	5.4±1.3	6.950±2.6	7.4±5.1	5 .8±1.9	7.3±1.6
	After study	5.4±1.4	5.4±1.6	6.5±2.09	6.4±1.4	6.2±1. 2	6.1±0.2
Hemoglobin (g/dL)	Before study	10.6±1.84	10.8±2.12	11.5±1.27*	11.4±2.09	10.6±2.1	11.4±1.1
	After study	10.5±2.14	10.6±2.02	12.1±2.01*	11.4±2.5 6	14.6±4.2	10.6±0.2
Kidney function							
Blood urea nitrogen (mg/dL)	Before study	94.7±57.61	78.6±49.21	57.9±16.7	54.5±1 2.72	47.4±14.32	48.2±7.4
	After study	101.5±40.48	104.3±83.28	61.3±26.37	60.3 ±16.67	61±7.07	38.5±13.43
Serum creatinine (mg/dL)	Before study	8.73±3.42	7.5±2.99	9.4±2.3	10.27±2.9	9.88±3.64	10.4±2.7*
	After study	8.16±2.70	7.6±2.47	9.6±2.77	10.46±3.2	1 0.1±5.85	9.3±3.2*

Data are expressed as Mean ± SD

**P<0.05 for differences between before and after study, **P<0.05 for differences between drug and placebo groups.*

NAC: N-Acetyl Cysteine, BMI: Body Mass Index, WBC: White Blood Cell, SD: Standard Deviation

SOD enzyme catalyzes the dismutation of the superoxide anion radicals into H_2O_2, which is readily degraded by CAT into water. ROS such as singlet oxygen, hydroxyl radicals, superoxide and H_2O_2 are known to be cytotoxic agents because of their ability to induce lipid peroxidation in membranes. In biological systems, the antioxidant enzyme, CAT, protects SOD from inactivation by H_2O_2. Reciprocally, the SOD protects CAT from inhibition by superoxide anion [36-38].

Our data demonstrate that administration of oral NAC in these three groups slightly (but not significant) reduced activity of SOD enzyme. This reduction can be explained by reducing of superoxide anions in plasma after antioxidant administration. As previous studies mentioned [27,39] NAC is able to clean the cell from superoxide anion radicals by direct scavenging; therefore reduction of SOD activity seems logical. Researches of Zaniew et al. [40] and Ruiz Fuentes et al. [41] showed administration of oral NAC has no significant effects in SOD activity in renal disease patients. Beside these researches, Caylak and his colleagues found that SOD activity significantly decreased in lead-exposed rat erythrocytes after administration of NAC [42].

In this study CAT enzyme activity was increased due to renal injury, just like previous studies [8,43]. Our results show that CAT enzyme activity decreases after administration of oral NAC. It is reported that NAC directly reacts with H_2O_2 and breaks it down [27,39]. Hence, reducing of CAT enzyme activity can be because of this ability of NAC. Fuentes et al. [41] observed no differences in CAT activity after oral administration of NAC in patients with stable renal function after transplantation.

Our study was an outpatient clinical trial; thus patients took medications themselves. Results are dependent to adherence of the patients in following the instructions. This could be the most potent limitation of the study.

5. CONCLUSION

A significant increase in GSH concentrations, as a marker of antioxidant defense, in all three groups; a significant decrease in MDA levels, as a marker of destructive processes like lipid peroxidation in groups 1 and 2 and a significant decrease in CAT activity levels, as a marker of destructive processes like lipid peroxidation in group 1 were observed. A slight but not significant decrease in activity levels of SOD was also seen.

These results approve the effectiveness of oral NAC against OS in ESRD patients. Several destructive cascades which are caused by OS are known; hence this protective role of NAC could have obvious clinical benefits for ESDR patients. Long-term clinical trials are needed to investigate beneficial clinical effects of NAC in these patients.

Protective effects of NAC supplementation against OS was seen in all three doses after 30 days of administration. Therefore, according to the results of this study, 600mg/day of NAC is more cost-benefit and also have lower dose-dependent side effects in comparison to higher doses. Further studies are needed on the effectiveness of different doses of NAC to determine the optimal dosing schedule for these patients.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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