Azhar Int J Pharm Med Sci 2023; Vol 3 (2): 61-69
(Research Article)

Open Acces

Print ISSN: 2735-4598
Online ISSN: 2735-4601

Possible Ameliorating Effect of N-acetyl Cysteine on Type II Diabetic Nephropathy: Clinical Trial

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Article history: Received 2022-08-10 Revised 2023-01-10 Accepted 2023-03-30

Abstract: Diabetic nephropathy is characterized by progressive rise in proteinuria. GFR decline, hypertension, and an increased risk of cardiovascular significant morbidity and mortality are all hallmarks of the condition. N-acetylcysteine (NAC) is a powerful antioxidant that efficiently removes a wide range of free radicals generated by oxygen species. NAC can help prevent nephropathy by reducing oxidative cellular damage and enhancing renal vascularization. Several studies have focused on the positive effects of antioxidant medicines such as NAC in decreasing the risk of atherosclerosis and its associated consequences. The object of this study is to see how NAC affects serum lipoprotein a Lp (a) and proteinuria levels in people with type II diabetes-induced nephropathy. This trial is a single-center, randomized, prospective, and placebo-controlled. The effectiveness of 1200 mg/day of NAC for 60 days in conjunction with conventional therapy for an interventional group is compared to no NAC treatment for the control group in this trial. Comparative data was based on the measurement of specific biomarkers including proteinuria, Lap (a), lipid profile, kidney function test, and blood pressure. NAC significantly reduced proteinuria levels in the experimental group after two months of therapy ($P<0.05$). However, there was no significant improvement in serum Lp (a) compared to the placebo group ($P>0.05$). The clinical efficacy of NAC in improving proteinuria levels, systolic blood pressure in diabetic nephropathy patients is superior to that of the control group. NAC had no effect on serum Lp (a) and serum creatinine levels.

Keywords: Diabetic nephropathy; N-acetylcysteine; Proteinuria; Lipoprotein (a) Lp (a).

1. INTRODUCTION

Diabetes mellitus is a vast group of illnesses marked by hyperglycemia. Diabetes is associated with long-term consequences, including microvascular issues such as retinopathy, nephropathy, nerve damage, and a higher chance of developing cardiovascular disease (CVD)[1]. According to the International Diabetes Federation (IDF), 451 million people globally had diabetes in 2017, with the figure anticipated to rise to 693 million by 2045[2].

About 40% of individuals with both types of diabetes (type 1 and type 2) experience diabetic nephropathy (DN), which is the primary cause of renal damage in patients beginning renal replacement therapy[3-5]. The most common clinical indication of DN is macroalbuminuria, which is defined as a urine albumin excretion rate of greater than 300 mg per day on two consecutive days of sterile urine collections. When the urine albumin-to-creatinine ratio is considered, macroalbuminuria in diabetic nephropathy exceeds 300 mg of albumin per gram of creatinine[6]. Despite the widespread interest in the etiology and treatment of DN, less attention is directed to the link between it and lipoprotein disorders, particularly lipoprotein (a)

Cite this article: Hamed T. A., Ibrahim M. E., Salem H. A. Possible Ameliorating Effect of N-acetyl Cysteine on Type II Diabetic Nephropathy: Clinical Trial. Azhar International Journal of Pharmaceutical and Medical Sciences, 2023; 3 (2):61-69. doi: 10.21608/AIJPMS.2023.155638.1159

(Lp a)[7]. In diabetic patients, Lp (a) has also been shown to be linked to hypertension[7].

The chemical structure of the Lp (a) molecule is close to that of low-density lipoprotein (LDL), with a disulfide link connecting apolipoprotein a (Apo a) to apolipoprotein B-100. It has also been linked to diabetic microvascular complications. An elevated blood level of (LP a) in diabetic and also in non-diabetic individuals is a significant risk factor for atherothrombogenesis[8,9] and is regarded as a distinct risk factor for cardiac diseases[10] and the progression of diabetic nephropathy, particularly in type 2 diabetic patients with proteinuria[7]. N-acetylcysteine (also known as N-acetyl-cysteine, NAC) is a natural plant phytochemical antioxidant found in onions[11]. The fundamental molecular mechanism of NAC is that it is an antioxidant and reducing agent. Its indirect antioxidant activity is due to NAC's capacity to act as a reduced glutathione (GSH) precursor, which is a substrate of several antioxidant enzymes and a well-established direct antioxidant. Furthermore, when endogenous cysteine (Cys) and GSH are low, NAC can act as a direct antioxidant for oxidant species like NO2[12].

NAC treatment can lower Malondialdehyde (MDA) levels as well as cardiac events in hemodialysis patients[7]. Also, because of its capacity to dissolve disulfide bonds, NAC can lower Lp (a) levels, which helps ameliorate diabetic nephropathy. It also has antioxidant properties, which can help to slow the advancement of DN[7]. NAC can also be utilized as a cardio protective agent[13].

2. METHODS

2.1. Study design: Prospective clinical-based study

The clinical study was carried out as a single-center, prospective, placebo-controlled, randomized trial in which allocation ratio was 1:1. The study was carried out after approval of the Medical Ethics Committee of Benha University, Egypt, number (MS15-9-2019) and was registered and posted on the wall of the "ClinicalTrials.gov" public website. The ClinicalTrials.gov Identifier: NCT04531163. The trial was operated in conformance with the Helsinki Declaration.

The outpatient clinic of the internal medicine department, Benha University asked for volunteers to take part in the trial. An Arabic-language informed written consent that signed by persons included in the study to state their full understanding of the study objectives, and their consent to share in the study. The education of patients was deemed an important part of the study. The etiologies of disease, risk factors, modifications in lifestyle, treatment guidance, and instructions to complete treatment have been clarified to each patient.

The study was carried out on 60 patients who were diagnosed with type 2 diabetes as defined by American Diabetes Association (ADA)[14]. Random sampling was the method to recruit patients into the study according to the inclusion criteria; if the patients had an established diagnosis of Type II Diabetes Mellitus, his age is over 35 years, if the patients had proteinuria equal or more than 30 mg /dl, and absence of heart and liver diseases. Both men and women were eligible to participate in the study. The exclusion criteria were pregnant women, cigarette smokers and patients on lipid-lowering medications, and nursing women. The clinical pharmacist created a computer list to assign patients to the medication or control groups in a blinded random manner. The study medications were dispensed at each bi-monthly visit. Patients were not informed to which group they were allocated. NAC dosing and duration of treatment were chosen based on previous research trials.

Patients were categorized randomly into two groups; Interventional group (group I) (n=30) their treatment was with NAC (Acetylcysteine 600 mg sachets 2 times per day orally) - that was obtained from Al-Motaheda pharmaceutical company, Egypt for two months in addition to their routine medications and control group (group II) (n=30): which treated with routine medications only. All patients were subjected to full medical history according to a predesigned sheet and lab tests were done at the beginning and after the treatment period.

2.2. Lab test determination

2.2.1. Lp (a)

By using enzyme-linked immunosorbent assay (ELISA) technique using Imubind Lp (a) ELISA kit (Tint Eliza Lp (a); Biopool, Umea, Sweden)[15] (catalog number # EH297RB) using a method reported by (Aoki et al., 1993)[15]. The principle of this method is that, the Lp (a) present in the sample is bounded to Lp (a) antibodies on the micro-test well during incubation. Peroxidase conjugated Lp (a) antibodies (catalog number NBP1-39589) are then added tagging the bound Lp (a), thus forming a "sandwich", antibody-Lp (a) conjugated antibody. A

sample incubation time of one hour is used to ensure quantitative "sandwich" formation of the Lp (a) in the sample. The unbound conjugate antibody is washed away and peroxidase substrate is added. The amount of yellow Lp (a) now is present in the sample[15].

2.2.2. Proteinuria

For the evaluation of proteinuria, a dipstick urine test (type 3A dipstick, Dialab, Neudorf, Germany) (REF804P) was applied. The test's guiding idea is "Protein Error of PH Indicators." If the PH is kept constant, protein can alter the color of various acid-base markers. The primary colors can range from "yellow," which has a detrimental effect, to "yellow-green, green, and green-blue," which has a positive result. Compared to the other urine proteins, albumin has 99 percent specificity. Over 20 mg/dl [16] of urine protein is considered normal. For assessing proteinuria using the dipstick technique, a mid-stream fresh urine sample is taken in the morning. The test strip was gently submerged for one second in urine before being pulled over the bottle's rim to drain any remaining excess urine. After 60 seconds, the strip is compared to the color scale.

2.2.3. Serum creatinine

Serum creatinine (Scr): Buffered Kinetic Jaffè reaction without deproteinization. The chemicals were obtained from Diamond diagnostics company, Budapest, Hungary.

Principle: Creatinine reacts with picric acid (catalog number # BK-443340D) in alkaline media to form a yellow-red complex. The absorbance of the colored compound is measured at wavelength 492 nm. And it's directly proportional to the creatinine concentration in the specimen[17]. Specimen and working solution were pipetted in test tubes, mixed, and after 30 seconds the absorbance A1 was read. After exactly 2 min. the absorbance A2 was read. Same procedures were done for standard.

$$A1\text{-}A2 = A_{specimen} \text{ or } A_{standard}.$$

The concentration of creatinine in specimen: $\frac{A\ specimen}{A\ standard} \times 2$ (mg/dl)[17,18].

2.2.4. Systolic blood pressure

Patient blood pressure was measured in the first day three times and the mean value was obtained prior to treatment initiation, written down, and recorded in the patient follow-up sheet. This process was repeated each bi-monthly visit. And the values were recorded. A mercury sphygmomanometer was used to monitor blood pressure.

During each bimonthly periodical visit, the following parameters were considered:

1- Assurance of drug use technique 600mg every 12 hours orally.
2- The occurrence of any adverse effects.
3- Follow-up sheets were updated.

At the first and last visit, the following parameters were considered:

1. History and clinical examination.
2. Blood sample collection for laboratory investigations.

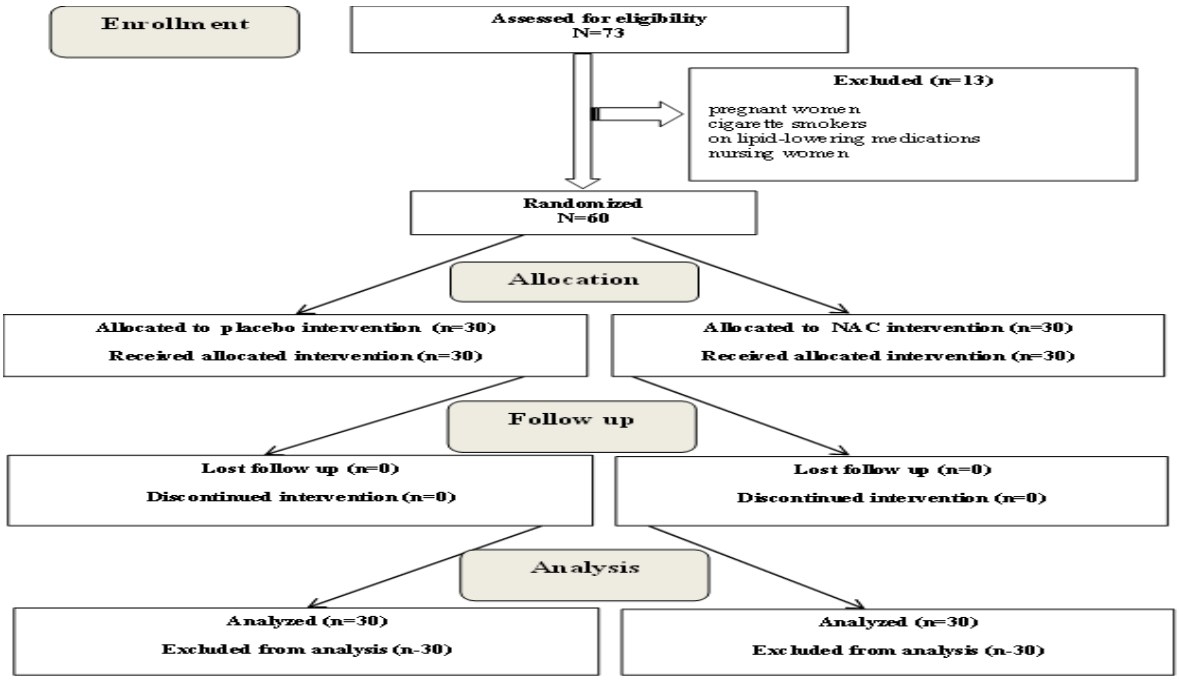

Figure 1. Study flow chart.

3. RESULTS

3.1. Statistical methods

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Shapiro-Wilk test was done to test the normality of data distribution. The Student T Test was performed to determine the statistical significance of the differences between the means of the two research groups. The Mann Whitney test was employed to determine the statistical significance of a non-parametric variable difference between two research groups. To investigate the association between two qualitative variables, the Chi-Square test was performed. Paired sample t test (for parametric) or Wilcoxon signed rank sum test (for non-parametric) was used to assess changes in parameters over time. Correlation analysis was used to assess the strength of association between two quantitative variables. Linear regression analysis was used for prediction of risk factors. All reported p values were two-tailed and $p<0.05$ was considered to be significant.

Table 1. Comparison of demographic, clinical and laboratory parameters among studied groups.

			Placebo	**Experimental**	***P1***
			N=30	**N=30**	
Age (years)		**mean±SD**	43.8±7.6	44.8±8.6	0.625
Males		**N (%)**	18(60%)	18(60%)	1
Females		**N (%)**	12(40%)	12(40%)	
BMI (kg/m2)		**mean±SD**	29.8±2.9	29.2±3.4	0.458
Duration of DM (years)		**Median**	12.5(6-30)	12.5(6-29)	0.772
SBP (mmHg)	**Pretreatment**	**mean±SD**	141.4±4.1	143.4±6.6	0.173
	Post	**mean±SD**	140.5±5.2	133.5±5.5	**<0.001**
	P2		0.313	**<0.001**	
Proteinuria	**Pretreatment**	**Median**	271.5(129-462)	276.5(158-331)	0.237
	Post	**Median**	267(123-435)	239(134-293)	**0.009**
	P2		0.304	**<0.001**	
Serum creatinine (mg/dl)	**Pretreatment**	**Median**	1.9(1.2-2.7)	1.9(1.2-2.8)	0.958
	Post	**Median**	1.9(1.2-2.3)	1.8(1.3-2.9)	0.619
	P2		0.622	0.419	
Lipoprotein a	**Pretreatment**	**Median**	16(11-22)	16(11-25)	0.976
	Post	**Median**	17(12-21)	16(11-26)	0.738
	P2		0.262	0.145	

BMI: body mass index, DM: Diabetes Mellitus, SBP: systolic blood pressure, P1: comparison between placebo and experimental groups; p2, comparison between pre and post treatment levels.

Seventy three cases were allocated to the study, 13 were excluded, 60 were randomized, into 2 groups, 30 cases received NAC, and 30 cases received placebo treatment. Both groups were matched for age, gender, BMI, SBP, DM duration and baseline laboratory parameters.

After treatment, SBP and proteinuria improved significantly in NAC group but not in placebo group. Serum creatinine and Lp (a) levels were not affected significantly after treatment in both groups.

Percentage change in proteinuria was calculated. No significant correlation was found between baseline data with percentage change in proteinuria.

Regression analysis was conducted for prediction of Proteinuria change using age, gender, BMI, duration, baseline SBP, proteinuria, creatinine, lipoprotein a and NAC treatment as covariates. Only receiving NAC treatment was considered favorable predictor of better improvement in proteinuria.

Table 2. Correlation of baseline data with percentage change in proteinuria.

	Proteinuria percentage change	
	Rs	***P***
Age	0.204	**0.119**
BMI	0.073	0.579
Duration of DM	0.184	0.159
Baseline SBP	0.165	0.209
Baseline Proteinuria	0.093	0.478
Baseline Serum creatinine	0.160	0.222
Baseline lipoprotein a	0.056	0.670

BMI: body mass index, DM: Diabetes Mellitus, SBP: systolic blood pressure, rs: correlation coefficient.

Table 3. Regression analysis for prediction of Proteinuria change.

	Proteinuria percentage change	
	B	***P***
Age	0.283	0.119
Gender	0.363	0.904
BMI	0.264	0.579
Duration of DM	0.288	0.159
Baseline SBP	0.335	0.209
Baseline Proteinuria	0.013	0.478
Baseline Serum creatinine	4.248	0.222
Baseline lipoprotein a	0.173	0.670
NAC treatment	10.313	**<0.001**

BMI: body mass index, DM: Diabetes Mellitus, SBP: systolic blood pressure, β: regression coefficient.

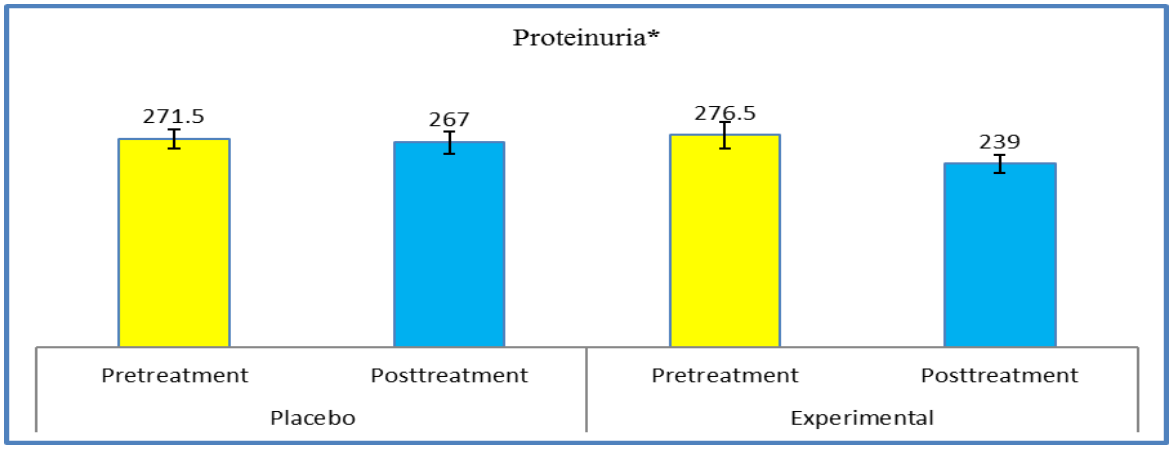

Figure 2. Proteinuria before and after treatment between studied groups. Columns represent means, error bars represent standard errors.

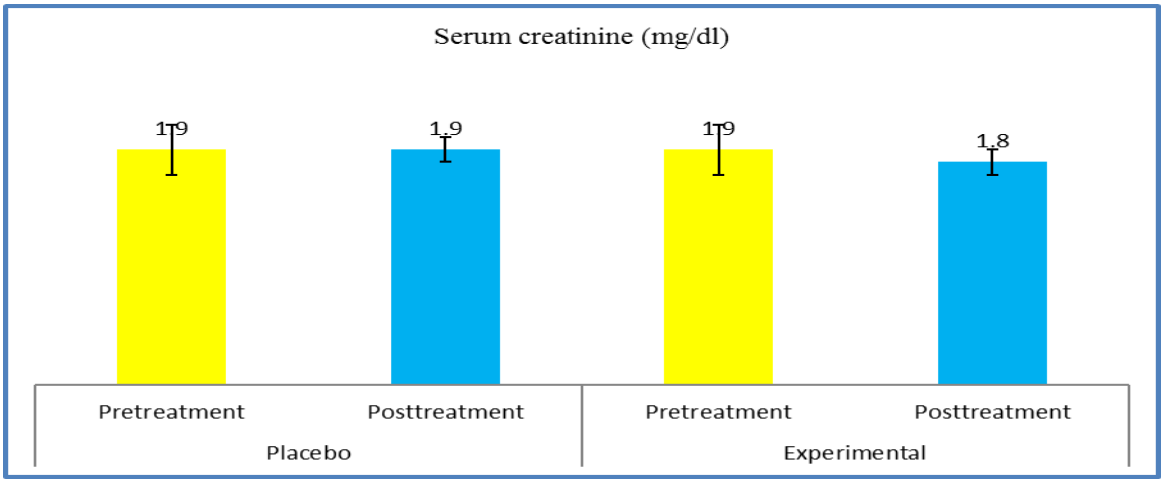

Figure 3. Creatinine before and after treatment between studied groups. Columns represent means, error bars represent standard errors.

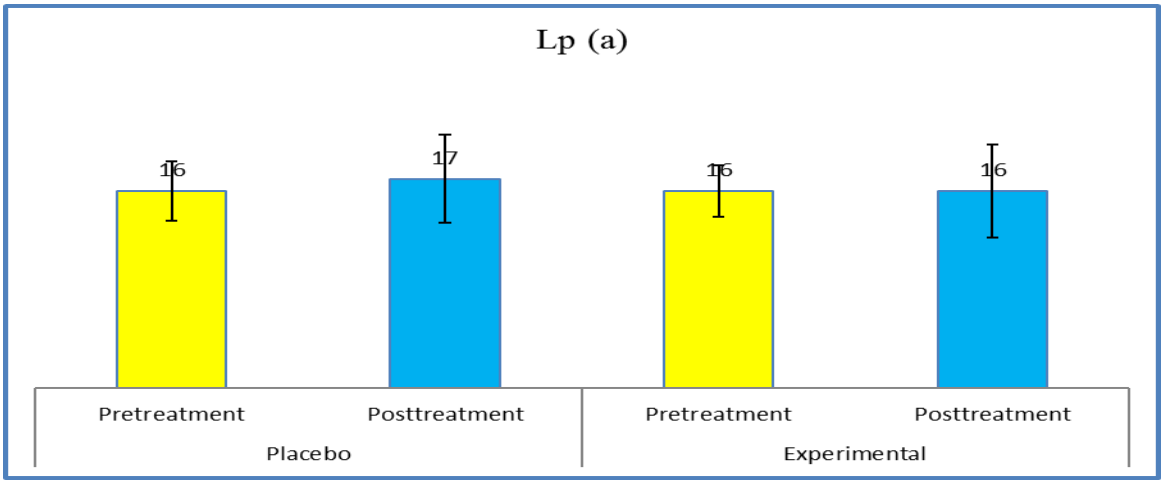

Figure 4. Lipoprotein a before and after treatment between studied groups. Columns represent means, error bars represent standard errors.

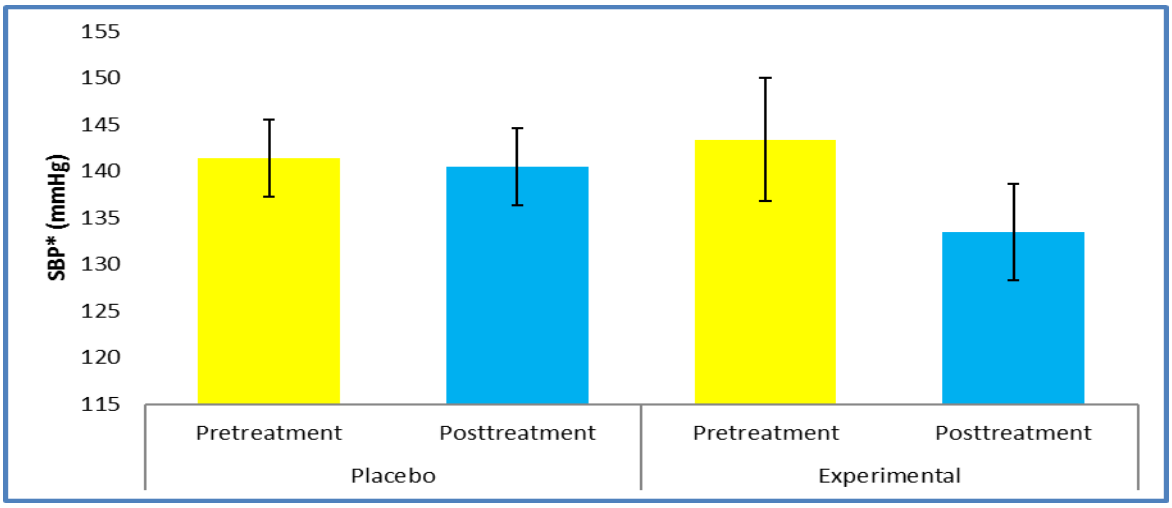

Figure 5. SBP among studied groups before and after treatment between studied groups. Columns represent means, error bars represent standard deviations.

4. DISCUSSION

Oxidative stress complicates the pathogenesis and evolution of diabetic nephropathy. There is evidence that it plays an important role in the pathophysiology of kidney injury[19]. NAC, a powerful antioxidant that is a synthetic precursor of reduced glutathione, has been investigated here for its nephro-protective effects. The possible impact of NAC on proteinuria lowering may be linked to its ant oxidative characteristics. In this study, NAC has been demonstrated to have a considerable proteinuria reducing effect. The positive outcomes of NAC on the kidney seem to be attributable to oxidative stress regulation via glutathione recovery (GSH). NAC acts as a synthetic precursor for GSH which plays a role as a reactive oxygen species scavenger which in turn attenuates oxidative stress. This was evidenced by Nogueira GB et al.[19]. They located that NAC shows significant improvement not only in renal glomerular filtration rate but also in proteinuria[19]. On the contrary, a pilot study done by Mohammad G. Saklayen et al.,[20] examined the use of NAC in patients with DN to reduce oxidative stress and proteinuria. The study revealed that N-acetylcysteine had no effect on total oxidative stress and did not diminish proteinuria. This result did not support our results and this may be due to the use of lower doses of NAC and for shorter periods so it could not decrease the oxidative biomarkers significantly.

Lp (a) consists of low-density lipoprotein attached to the large glycoprotein apo (a) by one disulfide bond or more. It contributes to microvascular complications in diabetic patients and is a significant risk factor for atherothrombogenesis. Our study showed that NAC cannot significantly

decrease Lp (a). The drug dose and the length of NAC therapy are both significant considerations influencing the potency of this medication and this may suppose the inability of NAC to make a significant reduction in the level of Lp (a). According to our results, Hamid Rouhi and Forouzan Ganji[6] reported that NAC has no meaningful effect on serum Lp (a) reduction. Similar results were reported by O. Wiklund et al.[21] who concluded that NAC appears to have minimal capacity to reduce serum Lp (a). However, previous studies have suggested that, due to the disulfide bond reducing property of NAC, it can decrease high levels of Lp(a)[22]. But this did not go with our study results. This may be due to the duration of therapy of NAC being for two months only.

Also, Oxidative stress is a major contributor in cardiovascular complications. Excessive generation of reactive oxygen species causes loss of nitric oxide bioavailability, endothelial dysfunction, increased lipid peroxidation, plaque instability, and atherosclerosis. Many approaches are designated to ameliorate hypertension including cysteine-rich supplements as NAC to stop elevation in blood pressure. In this paper, we established significant improvement in systolic blood pressure in the NAC treated group. The proposed mechanism is that NAC can improve insulin sensitivity, reduce oxidative stress, minimize advanced glycation end products, increase glutathione accumulation, and modulate nitric oxide and other vasoactive mediators and thus, lower blood pressure[23]. Similar results are also shown by Hildebrandt W et al. and Vasdev S et al.[24,25]. In diabetic individuals, combining NAC with L-arginine increases nitric oxide synthesis and lowers systolic blood pressure[26]. On the other hand Marcin Renke et al.[27] found that using NAC treatment has no discernible changes in blood pressure as the antioxidant properties of NAC were not settled in their trial.

In this research, NAC does not have significant lowering effect on serum creatinine. The NAC effect on serum creatinine and renal function was also studied by Louis Moist et al.[28]. This double-blind randomized controlled trial showed that NAC has no effect on creatinine level. However, another study was established to evaluate the efficacy of NAC in reducing serum creatinine in diabetic nephropathy subjects[19]. The study concluded that NAC can significantly lower serum creatinine levels and increase creatinine clearance. This result contrasts with our results because treatment with NAC in their study was initiated early but results were not significant in the late treatment group with NAC. This protective effect is probably due to oxidative stress control through the recovery of nitric oxide bioavailability. These findings show that NAC may be effective as an adjunctive treatment in the early stages of the illness, and that continued treatment may even postpone problems in the later stages.

5. CONCLUSIONS

The findings of this research demonstrate that NAC therapy in conjunction with conventional diabetic treatment could decrease the proteinuria level and systolic blood pressure (SBP) but does not decrease serum Lp (a) and serum creatinine levels in patients with type II diabetic nephropathy with proteinuria. Further works are needed on a larger scale, for longer periods, and carried on a large number of patients to assure the ameliorating effect of NAC in patients with diabetic nephropathy.

Funding: The authors did not receive support from any organization for the submitted work.

Acknowledgments: My deep appreciation Clinical Pharmacy department, Faculty of Pharmacy for Girls, Al-Azhar University, for the expert guidance, valuable suggestions, advices, endless help and interest in the progress and performance that make the completion of this work possible. I want to express my deep indebtedness, gratitude and sincere appreciation to Internal Medicine department, Faculty of Medicine, Benha University and Benha University Hospitals doctors, nursesand laboratory staff for facilitating all procedures, helping in the progress and performance of this work, sustained support, continued encouragement and valuable instructions.

Conflicts of Interest: The authors declare no conflict of interest.

Ethical Statement: The study was carried out after approval of the Medical Ethics Committee of Benha University, Egypt, number (MS15-9-2019) and was registered and posted on the wall of the "ClinicalTrials.gov" public website. The ClinicalTrials.gov Identifier: NCT04531163. The trial was operated in conformance with the Helsinki Declaration. Informed consent was obtained from all patients involved in the study.

Author Contribution: Conceptualization, Tasneem A. Hamed and Hoda A. Salem.; Methodology, Tasneem A. Hamed and Mohamed E. Ibrahim.; Data

collection and literature research: Tasneem A. Hamed and Hond A. Salem; writing—original draft preparation, Tasneem A. Hamed; writing—review and editing, Tasneem A. Hamed and Hoda A. Salem; supervision, Hoda A. Salem and Mohamed E. Ibrahim. All authors have critically revised the final version, read and agreed to the published version of the manuscript.

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