

Effects of N-acetyl-cysteine on endothelial function and inflammation in patients with type 2 diabetes mellitus

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Abstract

Endothelial dysfunction has been associated with premature vascular disease. There is increasing data that N-acetyl-cysteine (NAC) may prevent or improve endothelial dysfunction. The aim of this study was to assess the effects of NAC on endothelial function in patients with type 2 diabetes mellitus, a population at high risk for endothelial dysfunction. Twenty-four patients with diabetes mellitus were assigned randomly to initial therapy with either 900 mg NAC or placebo twice daily in a double-blind, cross-over study design. Flow-mediated vasodilation (FMD) of the brachial artery was assessed at baseline, after four weeks of therapy, after a four-week wash-out

period, and after another four weeks on the opposite treatment. Plasma and red blood cell glutathione levels and high-sensitivity C-reactive protein (CRP) were measured at all four visits. At baseline, FMD was moderately impaired ($3.7 \pm 2.9\%$). There was no significant change in FMD after four weeks of NAC therapy as compared to placebo ($0.1 \pm 3.6\%$ vs. $1.2 \pm 4.2\%$). Similarly, there was no significant change in glutathione levels. However, median CRP decreased from 2.35 to 2.14 mg/L during NAC therapy ($p=0.04$), while it increased from 2.24 to 2.65 mg/L with placebo. No side effects were noted during the treatment period. In this double-blind, randomized cross-over study, four weeks of oral NAC therapy failed to improve endothelial dysfunction in patients with diabetes mellitus. However, NAC therapy decreased CRP levels, suggesting that this compound may have some efficacy in reducing systemic inflammation.

forming NO adducts.⁹ The thiol N-acetyl-cysteine (NAC) may potentiate the activity of NO directly by forming more biologically active adducts, scavenging free radicals, and preventing NO oxidation and degradation. In addition, NAC stimulates the synthesis of glutathione, which acts as a nucleophilic scavenger and as an enzyme-catalyzed antioxidant in the event of oxidative tissue injury.¹⁰

The aim of this study was to evaluate the effect of NAC on endothelial function as measured by flow-mediated vasodilation (FMD) of the brachial artery and the degree of glutathione induction by NAC in patients with type 2 diabetes, a patient population at high risk for endothelial dysfunction.

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Introduction

The endothelium is central to the regulation of vascular tone, inflammation, platelet aggregation, and lipoprotein oxidation by the production of nitric oxide (NO).¹ The reduction of NO bioavailability is a consequence of endothelial dysfunction and thought to be an early event in the pathogenesis of atherosclerosis.² Hyperglycemia³ and insulin resistance⁴ have been associated with severe endothelial dysfunction in humans. Studies evaluating the effect of antioxidant therapy on endothelial function, including vitamin C and E and probucol, have been encouraging.^{5,7} However, long-term therapy with vitamin E does not seem to have any beneficial effect in improving endothelial function.⁸

Reduced thiols are molecules with a sulfhydryl group that have many biological functions, including scavenging oxygen-free radicals, acting as cofactors for enzymatic reactions, and modifying the half-life of NO by

Materials and Methods

General study design

We performed a prospective, randomized, placebo-controlled, double-blind, cross-over study of the change in vascular endothelial function after four weeks of treatment with NAC and four weeks of placebo in patients with type 2 diabetes mellitus. Endothelial function was assessed in each individual by FMD of the brachial artery at four different time points: prior to therapy, after four weeks of NAC or placebo, after a wash-out period of four weeks, and after four weeks of the opposite treatment. Compliance with the study medication was assessed by pill count. In addition, plasma and red blood cell total, reduced glutathione levels, and high-sensitivity C-reactive protein (CRP) were measured in each patient at all four different time points. The protocol was approved by the institutional review board, and all participants gave written informed consent.

Patient population

A total of 24 patients were included in the study. Subjects were enrolled if they were between the ages of 21 and 80 years and diagnosed with type 2 diabetes mellitus within the

past 10 years. Diabetes was defined according to the recommendations of the American Diabetes Association Expert Committee on the Classification and Diagnosis of Diabetes.¹¹ Only patients on a stable medical regimen for diabetes for at least two months prior to study entry were enrolled and patients were encouraged to continue on the same regimen throughout the study, unless urgent changes were required. Patients were on either insulin or oral anti-hyperglycemic therapy with a hemoglobin A1c of 6.0-9.5%.

To avoid confounding factors known to affect endothelial function and minimize confounding that arises from the cross-over design, patients were excluded if they met any of the following criteria: uncontrolled diabetes requiring frequent dose adjustments of medical therapy or noncompliance with the medical regimen, congestive heart failure, anginal symptoms, exercise-induced ischemia (if prior stress testing were available), ischemic EKG changes at rest, prior myocardial infarction or coronary revascularization procedures (including percutaneous coronary interventions and coronary artery bypass grafting), and peripheral vascular disease. Other exclusion criteria were significant comorbidities such as renal or hepatic insufficiency.

A general physical examination was performed by a study physician prior to enrollment. Subjects were studied at all visits after a 12-hour fast. Participants were asked not to take their diabetes medications (sulfonureas) for 12 hours prior to any of the studies, and those participants taking insulin were asked to omit the rapid-acting insulin on the morning of each visit.

Laboratory data

Baseline laboratory data included plasma glucose, total serum cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides (Synchron CX analyzer, Beckman/Coulter, Brea, CA, USA). The hemoglobin A1c was determined in whole blood using ion-exchange HPLC (Tosoh 2.2, Tokyo, Japan). CRP was measured using a commercially available kit from Diagnostic Products Corporation (Los Angeles, CA, USA). This High Sensitivity CRP Kit (Catalog #LKCR1) was run on an Immulite Analyzer and is based on a solid-phase, chemiluminescent immunometric assay methodology. Functional or active PAI-1 was quantitated using an ELISA kit (Catalog #PI 90) from Oxford Biomedical Research (Oxford, MI, USA). Levels of interleukin-6 (IL-6; Catalog #D6050), soluble intracellular adhesion molecule (ICAM-1) (Catalog #BBE 1B), and soluble vascular cell adhesion molecule (VCAM-1) (Catalog #DVC00) were quantitated using ELISA kits from R&D Systems (Minneapolis, MN, USA). GSH was measured

by a commercially available assay (Cayman Chemical, Ann Arbor, MI, USA) that utilizes a carefully optimized enzymatic recycling method, using glutathione reductase, for the quantification of GSH. The sulfhydryl group of GSH reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent), and produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is produced concomitantly, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of GSH in the sample.

Medical therapy

Patients were maintained on their usual medical regimen throughout the study period. Changes to medications were made only if absolutely necessary at the discretion of the treating physician. After the baseline evaluation, all patients were randomized to NAC 900 mg orally twice per day or placebo for a period of four weeks. Following a four-week wash-out period, the opposite therapy was administered

for another four weeks. NAC was provided by BioAdvantex Pharma Inc. (Mississauga, ON, Canada) and tested for purity. Patient compliance was assessed by pill count and telephone confirmation and found to be >90%.

Endothelial reactivity testing

To assess the endothelium-dependent reactivity in the macrocirculation, FMD of the brachial artery was measured by using high-resolution ultrasound with a 10.0-MHz linear array transducer and an HDI Ultramark-9 System (Advanced Technology Laboratories, Bothel, WA, USA). All measurements were conducted in accordance with recently published guidelines.¹² Measurements were made in the right arm in a temperature-controlled room (22°C) after a 15-minute equilibration period with the subject resting in the supine position. Studies were performed after a 12-hour fast. Blood pressure and heart rate were monitored in the contralateral arm before and after the brachial artery measurements and prior to and after nitroglycerin administration. Once an acceptable image of the brachial artery was obtained, the arm was immobilized in that position throughout the study. Subsequently, a pneumatic tourniquet was placed around the forearm distal to the target vessel and inflated

Table 1. Baseline characteristics of the study population.

	24 patients
Female gender	13 (54%)
Active smoker	3 (13%)
Former smoker	8 (33%)
Dyslipidemia*	21 (88%)
Systemic hypertension	17 (71%)
No. of years of diabetes mellitus	5.1±2.4
Body mass index	33.3±7.0
Total cholesterol (mg/dL)	159±45
Low-density lipoprotein cholesterol (mg/dL)	91±26
High-density lipoprotein cholesterol (mg/dL)	42±11
Triglyceride (mg/dL)	193±118
Hemoglobin A1c (%)	7.3±0.9
Fasting glucose (mg/dL)	154±63

*Defined as total cholesterol >200 mg/dL or on lipid-lowering therapy.

Table 2. Baseline medical therapy of the study population.

Insulin therapy	3 (13%)
Metformin therapy	16 (67%)
Thiazolidenedione therapy	4 (17%)
Diet-controlled alone	2 (8%)
Lipid-lowering therapy	15 (63%)
Angiotensin converting enzyme inhibitor	15 (63%)
Aspirin therapy	15 (63%)

to 50 mmHg above the subject's systolic blood pressure for a period of five minutes. One minute after sudden deflation, the brachial artery dimensions were measured by ultrasound and compared with the baseline vessel dimensions. Endothelium-independent vasodilation was assessed by studying brachial artery diameter changes three minutes after the administration of 0.4 mg of sublingual nitroglycerin. This test was performed 15 minutes after the reactive hyperemia test and after obtaining a new baseline reading. Previous research showed satisfactory reliability and repeatability of this technique.¹³

Statistical methods

Continuous variables are described as mean±standard deviation while categorical variables are described as proportions. The primary endpoint of the study was the change in FMD (expressed as % change) after four weeks of NAC and placebo therapy, respectively, and was compared using the sign-rank test. Secondary endpoints included changes in glutathione and CRP levels during NAC and placebo therapy. Similar to the primary endpoint analysis, a Wilcoxon signed-rank test was used to determine statistical significance of any observed differences. Comparisons across groups were made using the Student's *t*-test. The study had 80% power to detect a 3.3% change in FMD at *p*<0.05. Results were considered statistically significant at *p*<0.05. All statistical analyses were performed using Stata version 8.2 (Stata Corp., College Station, Texas, USA).

Results

Patient characteristics

The mean duration of diabetes was 5.1±2.4 years (range 1-10 years). Baseline characteristics of the study population are presented in Table 1. Five (21%) patients were on insulin therapy, 2 (8%) were controlled with diet alone, while the remainder (71%) were on oral anti-diabetic therapy (Table 2).

NAC and FMD

At baseline, FMD demonstrated moderately impaired endothelial function (3.7±2.9%). There was no significant change in FMD after four weeks of NAC therapy as compared to placebo (0.05±3.6 vs. 1.2±4.2%; Figure 1). As expected, endothelial-independent vasodilation (induced by nitroglycerin) was significantly larger than FMD at baseline (8.5±4.1 vs. 3.7±2.9%, *p*<0.001). NAC therapy did not affect endothelial-independent vasodilation significantly (9.0±4.8 vs. 9.1±4.8%). There was no effect of treatment order. No side effects were noted during the treatment period.

Laboratory testing

There was no significant change in total plasma glutathione levels from baseline to posttherapy (3.1±0.6 vs. 2.9±0.6 μM). Similarly, total (106.9±47.7 vs. 107.9±50.9 μM) and reduced (94.8±38.5 vs. 95.2±38.4 μM) red blood cell glutathione levels (referred to as packed red blood cells) did not change significantly during treatment.

Median CRP decreased from 2.35 mg/L (25-75% quartiles: 1.02-6.87 mg/L) to 2.14 mg/L (25-75% quartiles: 0.97-7.01 mg/L, *p*=0.04;

Figure 2) during NAC therapy, while it numerically increased from 2.24 mg/L (25-75% quartiles: 1.82-6.85 mg/L) to 2.65 mg/L (25-75% quartiles: 1.51-6.50 mg/L, *p*=0.40) with placebo. When comparing the median change in CRP during NAC versus placebo therapy, there was a nonsignificant trend toward a CRP decrease with NAC (-0.18 vs. 0.21 mg/L, *p*=0.14). When the analysis was repeated after excluding one patient who had an infection at the baseline measurement of the placebo phase (CRP of 41.2 mg/L), the overall change

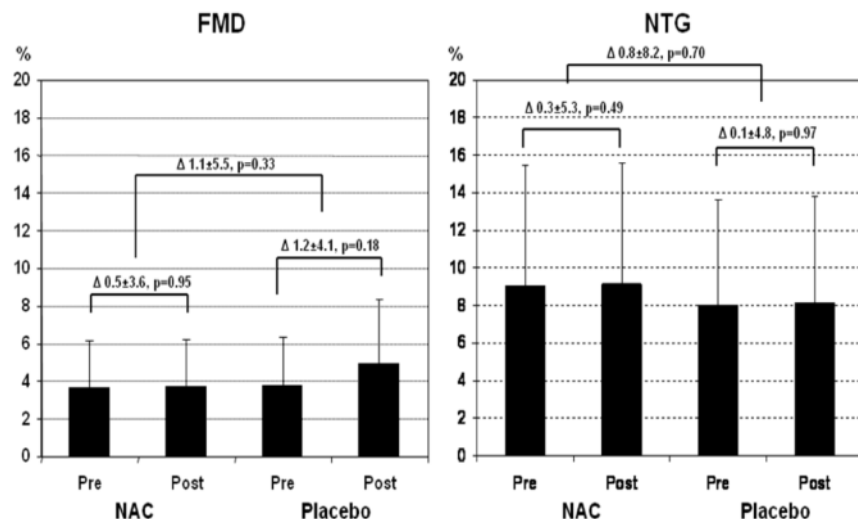


Figure 1. Changes in brachial artery flow-mediated vasodilation (FMD) and nitroglycerin (NTG) induced vasodilation before and after therapy with N-Acetyl-cysteine (NAC) and placebo.

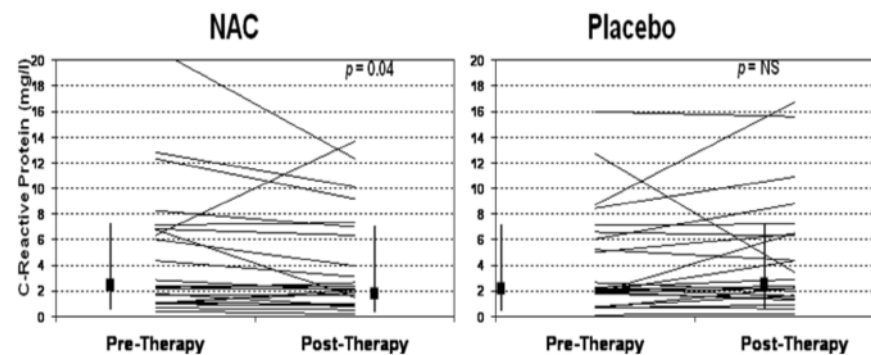


Figure 2. Individual changes of C-reactive protein before and after treatment with NAC and placebo.

Table 3. Inflammatory and endothelial markers.

Marker	Pre-NAC	Post-NAC	<i>p</i>	Pre-placebo	Post-placebo	<i>p</i>
IL-6	3.3 (3.0-3.6)	3.2 (2.8-3.6)	0.27	3.2 (2.9-3.5)	3.0 (2.7-3.4)	0.28
VCAM-1	732 (593-870)	717 (580-854)	0.52	709 (578-840)	703 (571-836)	0.82
ICAM-1	262 (230-295)	258 (225-292)	0.88	264 (235-293)	250 (218-281)	0.02
PAI-1	36.5 (21.9-51)	38.6 (20.6-56.6)	0.88	40.9 (24.4-57.5)	49.1 (27.9-70.3)	0.13

Data presented as median (95% confidence interval).

in CRP was -0.18 mg/L during NAC and 0.23 mg/L during placebo therapy ($p=0.053$).

Other inflammatory and endothelial markers, including IL-6, PAI-1, ICAM-1, and VCAM-1, did not change significantly throughout the treatment period (Table 3).

Discussion

This double-blind, randomized cross-over study showed no improvement in FMD after four weeks of NAC therapy compared with placebo. This could be explained by the lack of glutathione induction, which has been shown to have favorable effects on endothelial function. However, NAC did have a modest effect in decreasing CRP, suggesting that this compound may have some efficacy in reducing systemic inflammation.

A few reports have studied the effect of NAC on vascular endothelial function. In a recent study by Andrews *et al.*, NAC was administered into the coronary and femoral arteries of patients with and without atherosclerosis.¹⁴ NAC potentiated acetylcholine-mediated vascular dilation, indicating its usefulness in improving endothelial function. In another study, intravenous infusion of NAC showed significant vasodilation in small epicardial arteries and augmented coronary blood flow.¹⁵ In an animal model of diabetes mellitus, the oral administration of NAC completely prevented the development of endothelial dysfunction independent of the degree of glycemic control.¹⁶

The present study failed to show any beneficial effects of NAC on endothelial function in this high-risk diabetic patient population, despite striking endothelial dysfunction at baseline in the majority of patients. The most likely explanation for the lack of effect is that NAC did not increase glutathione levels sufficiently. Glutathione acts as a nucleophilic scavenger and as an enzyme-catalyzed antioxidant in the event of oxidative tissue injury.¹⁰ Oxygen-free radicals, responsible for inactivating NO, are scavenged from plasma or endothelial cells by both NAC and glutathione, thereby increasing the bioavailability of NO. In particular, intracellular reduced glutathione has a vital role in protecting endothelial cells from oxygen-free radicals¹⁷ and improves endothelial vasomotor response to acetylcholine when directly infused into the coronary artery.¹⁸ Recently, a low level of red blood cell glutathione peroxidase-1 activity has been associated with an increased risk of cardiovascular events in a patient population with established coronary artery disease, suggesting the importance of glutathione in cardiovascular disease prevention.¹⁹ Glutathione is synthesized intracellularly from the amino acids

glycine, glutamate, and cysteine. Since the former two are abundantly available in the intracellular space, an adequate supply of cysteine into the cell becomes the rate-limiting step in the synthesis of glutathione.

In a preliminary animal study, we demonstrated that oral NAC administration substantially increased both glutathione and cysteine levels.²⁰ It is unclear why NAC supplementation failed to increase plasma and red blood cell glutathione levels in this study but one explanation could be that glutathione cannot be increased to supra-normal levels. Previously published studies have demonstrated that NAC increases glutathione in patients with decreased levels but no data exists on increasing glutathione above normal levels. For example, in one study in patients with the human immune-deficiency virus, a condition associated with substantially decreased glutathione levels, the oral administration of NAC replenished glutathione to normal levels.²¹ Another potential explanation for the lack of increase in glutathione levels could be the reduced bioavailability of NAC during supplementation.

There are a number of reports indicating that therapy with NAC may have beneficial effects in suppressing systemic inflammation, a condition that is closely linked to the development of atherosclerosis.²² For instance, VCAM-1 enables the attachment of circulating cells to the endothelium and thus promotes intravascular accumulation of various inflammatory cells.²³ NAC effectively decreased VCAM-1 expression in a diabetic population that is prone to elevated plasma soluble VCAM-1 concentrations.²⁴ Similarly, in patients undergoing liver transplantation, high-dose NAC administration significantly inhibited the increase in both ICAM-1 and VCAM-1 after reperfusion.²⁵ In cell cultures, Faruqi *et al.* showed that NAC inhibited IL-1-induced mRNA as well as cell surface expression of both E-selectin and VCAM-1, suggesting potent anti-inflammatory effects of NAC.²⁶ This study demonstrated no significant effect of NAC on ICAM-1, VCAM-1, and IL-6 but showed a modest decrease in CRP levels after four weeks of therapy. However, after the exclusion of one patient with severely elevated CRP secondary to an infection, there was only a trend toward CRP reduction with NAC ($p=0.053$). Given that relatively few therapies have been shown to reduce CRP to date,²⁷ NAC may represent an addition to the armamentarium of agents that can lower systemic inflammation. Whether the extent of CRP reduction observed in this study translates into clinically measurable benefits is unknown, but a recent study of 134 patients with end-stage renal disease suggests that NAC may be a clinically useful agent.²⁸ In that study, patients were randomized to either NAC or placebo and found to have a 40% lower incidence of cardiac events, ischemic strokes, and

peripheral vascular disease in the NAC treated group after a median follow-up of 14 months. A larger study linking the reduction in CRP with clinical outcomes may provide useful information in the future.

In conclusion, a four-week course of oral NAC therapy did not improve endothelial dysfunction in patients with diabetes mellitus and clinically absent cardiovascular disease. However, NAC therapy significantly decreased CRP levels in this challenging patient population. Further study is warranted to confirm this finding.

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