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The Effect of N-acetylcysteine on Brain Tissue of Rats Fed with High-Cholesterol Diet

[Yüksek Kolesterol Diyeti ile Beslenen Ratların Beyin Dokularında N-Asetil-sisteinin Etkisi]

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ABSTRACT

Objectives: The effect of N-acetylcysteine in rats fed a high-cholesterol diet on oxidative stress in the plasma and brain tissue of rats was investigated.

Methods: The animals were maintained on a basal diet (control, n=10) or a high-cholesterol diet (1 % w/w) for eight weeks. The rats fed high-cholesterol diet were separated to three group; high-cholesterol diet (n=10), low N-acetylcysteine (n=10) and high N-acetylcysteine groups (n=9). Low and high N-acetylcysteine groups received N-acetylcysteine at a dose of 50 and 100 mg/kg/day respectively via intraperitoneally for eight weeks. Malondialdehyde, glutathione, nitric oxide, cholesterol and triglyceride levels were analyzed in the samples. The results were analyzed by Kruskal-Wallis variance analysis and then a Mann-Whitney U test.

Results: When N-acetylcysteine was administered at a low dose, lipid peroxidation products in the brain significantly decreased compared with the high-cholesterol group, while glutathione content enhanced. On the other hand, when N-acetylcysteine was administered at a high dose, lipid peroxidation products in the brain and plasma significantly increased compared with the control group.

Conclusions: These results suggest that N-acetylcysteine has a dual effect. If the N-acetylcysteine dose was carefully selected, N-acetylcysteine may have a neuroprotective effect against oxidative stress and hypercholesterolemia.

Key Words: N-acetylcysteine, high-cholesterol diet, nitric oxide, lipid peroxidation, glutathione, plasma lipids

ÖZET

Amaç: Yüksek-kolesterol diyeti ile beslenen sıçanların plazma ve beyin dokusundaki oksidatif strese N-asetilsisteinin etkisi incelendi.

Gereç ve yöntemler: Hayvanlara sekiz hafta boyunca bazal diyet (kontrol), veya yüksek-kolesterol diyeti (% 1 w/w) verildi. Yüksek kolesterol ile beslenen sıçanlar yüksek-kolesterol diyeti (n=10), düşük doz N-asetilsistein (n=10) ve yüksek doz N-asetilsistein (n=9) olmak üzere üç gruba ayrıldı. Yüksek ve düşük N-asetilsistein gruplarına sırasıyla 50 ve 100 mg/kg/gün dozunda N-asetilsistein intraperitoneal olarak sekiz hafta boyunca uygulandı. Örneklerde malondialdehid, glutatyon, nitrik oksit, kolesterol ve trigliserit düzeyleri analiz edildi. Sonuçlar Kruskal-Wallis varyans analizi ve sonra Mann-Whitney U testi kullanılarak değerlendirildi.

Bulgular: Düşük doz N-asetilsistein uygulaması yapıldığında yüksek kolesterol diyeti grubundaki ratlara göre beyin dokusu glutatyon içeriği anlamlı derecede artarken lipid peroksidasyon ürünleri ise azalmıştı. Diğer yandan yüksek doz N-asetilsistein uygulananlarda hem beyin dokusu hem de plazma lipid peroksidasyon ürünleri kontrol grubuna göre anlamlı derecede yüksekti.

Sonuçlar: Bu sonuçlar N-asetilsisteinin çift etkiye sahip olduğunu düşündürmektedir. N-asetilsistein dozu dikkatli seçilirse oksidatif stres ve hiperkolesterolemiye karşı sinir dokusunu koruyucu etki gösterebilir.

Anahtar Kelimeler: N-asetilsistein, yüksek-kolesterol diyeti, nitrik oksid, lipid peroksidasyonu, glutatyon, plasma lipidleri

Introduction

It has been demonstrated that rats with chronic hyperlipidemia and hypercholesterolemia are related to the development of renal, cardiovascular and neurodegenerative diseases. The brain is especially sensitive to oxidative stress, due to the lipid composition of cell membranes and low levels of antioxidant enzymes (1). A high-cholesterol diet provides a relevant example of endogenous chronic oxidative stress due to the resulting hypercholesterolemia. It is known that a high-cholesterol diet induces ROS overproduction (2,3). Reactive oxygen substances (ROS) have been suggested to exert their cytotoxic effects by causing peroxidation of membrane phospholipids, which results in an increase in membrane fluidity, which increases permeability and the loss of membrane integrity (4,5). Our previous studies have shown that malondialdehyde (MDA) concentrations of both plasma and liver homogenates were increased in diet-induced hypercholesterolemic rats (6). End products produced during the lipid peroxidation process, including MDA, are very reactive, and capable of cross-linking of membrane proteins containing amino groups.

Kim et al. (7) reported that the high-fat diet increased plasma nitric oxide (NO) concentrations up to 5 fold, and induced nitric oxide synthases (iNOS) mRNA expression in liver. Rahman et al.. (8) demonstrated that urinary NO excretion and renal NOS activity in the hypercholesterolomic rat was greater than normocholesterolomic control rats. The effect of hypercholesterolemic diet on NO concentration in brain was not known. NO, which is a short-lived free radical, important signaling molecule in the central nervous system (9). It can originate at least from four different sources in the central nervous system: the endothelium of cerebral vessels, the immunostimulated microglia and astrocytes, the nonadrenergic noncholinergic nerve, and the glutamate neuron. Neurons produce NO mainly by the constitutive Ca^{2+}-dependent a activity of neuronal NOS (nNOS). Glial cells synthesize NO mainly by calcium-independent iNOS. Endothelial cells produce NO by the constitutive Ca^{2+}-dependent activity of endothelial NOS (eNOS) (10).

Hypercholesterolemia depleted the glutathione (GSH) content of cerebral tissues (11,12). Glutathione provides major protection in oxidative injury by participating in the cellular defense systems against oxidative damage (13). Glutathione scavenges ROS and protects protein thiol groups from oxidation. Several reports indicate that tissue injury, induced by various stimuli, and are coupled with glutathione depletion. N-acetylcysteine (NAC) is a GSH precursor and direct antioxidant. As a potent antioxidant, NAC directly scavenges hydrogen peroxide (H_2O_2), hydroxyl free radicals, and hypochloric acid in vitro (14). According to the reports by Sprong et al. (15) and Wang et al (16), NAC behaves either as anti- or pro-oxidants depending on the dose and schedule of NAC administration. Low dose (275 mg/kg in 24 h) of NAC protected rats against LPS-mediated oxidative stress, whereas high dose NAC (900 mg/kg in 24 h) increased LPS-induced lung injury and mortality. Pretreatment with NAC protects against acute ethanol-induced liver damage. However, when administered after ethanol, NAC behaves as a pro-oxidant and worsens acute ethanol-induced liver damage.

Thus, the purpose of this study was to investigate the effects of NAC on cerebral oxidative stress induced by a high-cholesterol diet in rats. For the present study, we used the following parameters to evaluate high-cholesterol-fed effect and oxidative stress: (i) for hypercholesterolemia, we determined the changes produced in total cholesterol and triglyceride levels; (ii) for oxidative stress, we analyzed the degree of lipid peroxidation, reduced glutathione content and nitric oxide in brain; and (iii) for indication of atherosclerosis, we examined the structure of aortic tissue under light microscope.

Materials And Methods

Chemicals

All the reagents were of analytical grade, purchased from Merck (Darmstadt, Germany) and Sigma (St Louis, MO).

Animals

Ethical approval was obtained from the University of Trakya, Ethical Committee. Thirty nine female Sprague-Dawley rats were divided into 4 different groups randomly; as control (n=10), high-cholesterol (HC diet group, n=10), high-cholesterol plus 50 mg/kg/day NAC (Low NAC group, n=10) and high-cholesterol plus 100 mg/kg/day NAC (High NAC group, n=9). The control group was fed with standard basal rat chow, while other groups were fed with basal chow enriched with 1 % cholesterol for eight weeks (17). NAC (Assist-Husnu Arsan Chemical, Turkey) was also administered to the treatment groups intraperitoneally (50 or 100 mg/kg/day for low and high NAC groups respectively). At the end of study, the rats were anaesthetized by ketamine (50 mg/kg) plus xylazin (10 mg/kg), and their trunk blood was collected. Blood samples were centrifuged for 10 minutes at 1500 x g at 4 °C and the plasma was stored at –70 °C until the analysis time. Their brains were immediately removed, washed with ice-cold normal saline, and frozen at –70 °C until assayed.

Preparation of tissue samples

The brain tissues were weighed and homogenized with 10 % (w/v) in ice-cold 150 mM KCl. The homogenate was centrifuged at 5000 x g for 10 min, and various analytical determinations were performed in the supernatant fraction (18).

Determination of malondialdehyde levels

Malondialdehyde was used as an indicator of lipid pe-

roxidation in plasma and brain tissue. MDA levels were measured according to the method of Ohkawa et al. (18). The results were expressed as μmol/L in plasma and nmol/mg protein in tissue samples.

Determination of glutathione levels

Glutathione was determined spectrophotometrically by using Ellman's reagent (19). For determination of GSH concentrations, a precipitating solution was added to the tissue homogenate to precipitate all proteins in the sample. After centrifugation at 11000 x g, for 15 minutes at 4 °C, clear supernatants were used for analysis. The results were expressed as nmol GSH/mg protein.

Determination of serum cholesterol and triglyceride levels

Total serum cholesterol and triglyceride levels were determined by an enzymatic method (Roche Diagnostics GmbH), using commercial kits (Roche/Hitachi 912 chemistry analyzer).

Determination of nitric oxide levels

In order to estimate the amount of NO production, NO metabolites in the supernatant were determined by a colorimetric method based on Griess reaction (20). The results were calculated using a calibration curve prepared with sodium nitrite in distilled water and were expressed as nmol/mg protein.

Determination of protein concentrations

The protein contents of the samples were determined according to Lowry et al. (21).

Histological analysis

Aortic tissues were examined under a light microscope for morphological alterations indicative of atherosclerosis. Aortic tissues were placed in 10 % (v/v) formaline solution and processed routinely by embedding in paraffin. Briefly, 3 μm-thick sections were cut and stained with hematoxylin and eosin and examined under a light microscope (Olympus- BX-51). An experienced pathologist, who was unaware of the treatment conditions, made histological assessments. Atherosclerotic lesion was assessed by a semiquantitative inflammation score (22):

0, no inflammatory cells;

1, light localized infiltration;

2, dense but localized infiltration;

3, dense infiltration.

Statistical analysis

The results were presented as mean±standard deviation (x±SD). Kolmogorov-Smirnov Goodness of Fit Test was used to test whether the distribution of parameters was normal or not. A Kruskall-Wallis test was used for analysis, and then the Mann-Whitney *U*-test was used to compare groups. Correlations between parameters were examined by the Spearman Correlation test.

The results of the morphometric analysis of all four groups were quantitatively analyzed with the Kruskal-Wallis one-way ANOVA by ranks, and then post hoc comparisons between groups made by using the Wilcoxon runk-sum test.

A value of p less than 0.05 was considered as statistically significant.

Results

Table 1 shows the total cholesterol (TC), triglyceride (TG) and MDA concentrations of plasma at the end of the experiment (after eight week treatment). The plasma TC and TG concentrations in the HC diet group were found to be significantly higher when compared with those in the control group ($p<0.01$, both). The plasma TC level in the high NAC group was significantly lower than the HC diet group ($p<0.01$). There were no significant differences observed in TC concentrations between the low and high NAC groups. The plasma TG level in the high NAC group was significantly lower than both the HC diet and the low NAC groups ($p<0.01$

Table 1. The plasma levels of cholesterol, triglyceride and malondialdehyde (MDA) at groups

	Control diet (n=10)	HC diet (n=10)	Low NAC (n=10)	High NAC (n=9)	P#
Cholesterol (mmol/L)	1.50±0.18	1.89±0.15 a**	1.63±0.32	1.56±0.16 b**	χ2=14.4, p<0.01
Triglyceride (mmol/L)	0.69±0.17	1.13±0.27 a**	0.98±0.39	0.56±0.24 b** c*	χ2=16.5, p<0.01
MDA (μmol/L)	3.66±0.43	5.21±0.77 a**	2.93±0.18 a** b**	4.53 ±0.42 a* c**	χ2=31.8, p<0.001

#Made by Kruskall-Wallis

a: compared with control diet group made by Mann-Whitney U test

b: compared with HC diet group made by Mann-Whitney U test

c: compared with low NAC group made by Mann-Whitney U test

*p<0.05, **p<0.01 and ***p<0.001

Table 2. The brain tissue levels of malondialdehyde (MDA), glutathione (GSH) and nitric oxide (NO) at groups

	Control diet (n=10)	HC diet (n=10)	Low NAC (n=10)	High NAC (n=9)	P#
MDA (nmol/mg protein)	6.19±0.57	9.51±1.03 a**	6.91±0.63 b**	8.61±0.79 a** b* c**	χ^2=30.2, p<0.001
GSH (nmol/mg protein)	54.56±9.06	20.76±7.18 a**	36.77±18.74 b*	36.68±6.96 a** b**	χ^2=26.4, p<0.001
NO (nmol/mg protein)	5.13±0.59	4.68±0.54	5.18±1.24	3.06.60 ±0.38 a** b** c**	χ^2=21.5, p<0.001

#Made by Kruskall-Wallis

a: compared with control diet group made by Mann-Whitney U test

b: compared with HC diet group made by Mann-Whitney U test

c: compared with low NAC group made by Mann-Whitney U test

*p<0.05, **p<0.01 and ***p<0.001

and p<0.05, respectively). Plasma malondialdehyde levels were significantly increased in the cholesterol-fed rats as compared to the control group (p<0.01). Plasma malondialdehyde levels were significantly decreased in the low NAC treatment groups when compared with the cholesterol-fed rats (p<0.01). However, plasma MDA level in high NAC group was significantly higher than the low NAC and control groups (p<0.01, and p<0.05, respectively).

As shown in Table 2, when MDA levels measured in brain tissue homogenates of HC group were compared with those obtained from control group, a significant increase was observed (p<0.01). The low and high NAC groups showed a significant decrease in the MDA levels of brain homogenates when compared with HC diet group (p<0.01 and p<0.05, respectively). However, the high NAC group still showed significantly higher values in the MDA levels of brain homogenates compared to low NAC treatment group (p<0.01). The GSH content of brain homogenate in the HC diet group was significantly low when compared to the control group (p<0.01). But, the GSH content of brain tissue homogenates in the both NAC groups were significantly higher than the HC diet group (p<0.05 and p<0.01, respectively). There were no significant differences in NO concentrations between the control, HC diet and low NAC groups in brain tissue homogenates. However, NO levels of brain tissue homogenates in the high NAC group was lower than all groups (p<0.01, all).

Correlation between parameters

There were positive correlations between the level of plasma total cholesterol and plasma triglyceride (r=0.726, p<0.001), plasma MDA (r=0.326, p<0.05) and brain tissue MDA (r=0.499, p<0.01) levels; however, a negative correlation was found between plasma total cholesterol and brain tissue GSH levels (r=-0.494, p<0.01). Plasma triglyceride was also negatively associated with brain tissue GSH content (r=-0.486, p<0.01). Plasma MDA was positively associated with brain tissue MDA level (r=0.682, p<0.001), although it was negatively correlated with brain tissue GSH (r=-0.414, p<0.01) and NO content (r=-0.409, p<0.01). There were negative correlations between the level of brain tissue MDA and GSH (r=-0.666, p<0.001) and NO content (r=-0.327, p<0.05).

Histological results

Cellular infiltrates as a marker for an inflammatory reaction were more distinct in HC diet group compared to animals in the control group (p<0.05). Cellular infiltrates were reduced in animals treated with both NAC doses when compared to the HC diet group (mean inflammatory score in HC diet group: 1.1, in both NAC groups: 0.0, p<0.05) (Figure 1).

There is a strong correlation between the morphometric results with the plasma MDA and total cholesterol levels (p<0.001 and p<0.01, respectively).

Discussion

The present study demonstrated that the significant increase in oxidative stress levels in rats fed with a high-cholesterol diet, correlation occurs among hypercholesterolemia and brain oxidative stress. Plasma lipid peroxidation levels correlated with brain tissue lipid peroxidation levels. This data suggest that plasma lipid peroxidation levels can reflected to brain tissue oxidative stress. These results were in agreement with previous reports that found that a high-cholesterol diet induced hypercholesterolemia and oxidative stress (11,12,23). Depletion of GSH is one of the primary factors that

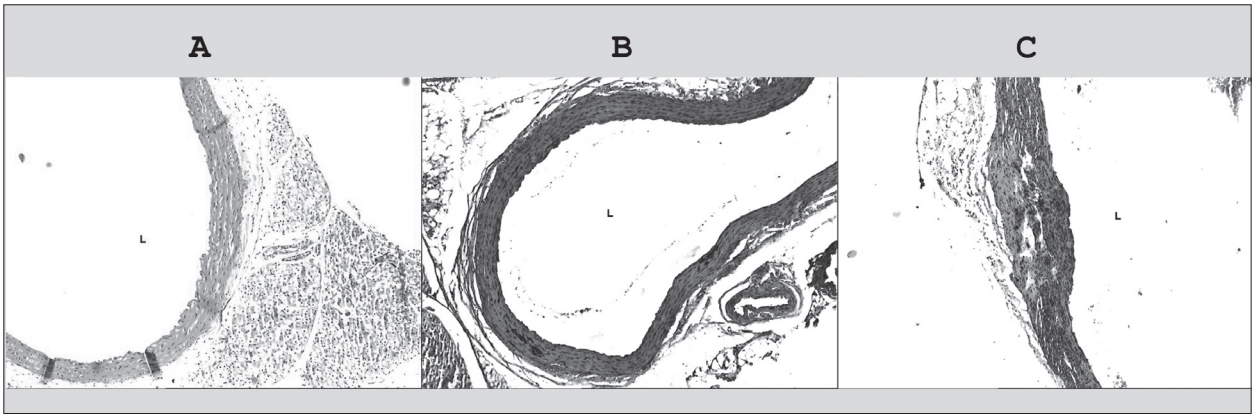

Figure 1. Aortic tissue of three hypercholesterolemic diet animals with (A and B) and without N-acetylcysteine therapy (C). Atherosclerotic intimal thickening and cellular infiltration was found at HC diet group. Cellular infiltrates were reduced in animals treated with low (A) and high (B) NAC doses (L; lumen, hematoxylin and eosin staining, magnification; X50).

permit lipid and protein oxidation. The present study showed depletion of brain tissue GSH content and this depletion was negatively associated with enhanced lipid peroxidation at cholesterol-induced oxidative stress.

We showed depletion of brain tissue GSH, which was restored by both doses of NAC treatment. But, high dose NAC treatment was not higher restored than low dose treatment. Likewise, it was demonstrated that low dose NAC administration improved lipid peroxidation induced with a high-cholesterol diet. It is suggested that NAC may also exert its antioxidant effect indirectly by facilitating GSH biosynthesis and supplying GSH for glutathione peroxidase-catalysed reactions (14). Jayalakshmi et al. (24) showed that in a glutathione-depleted hippocampal cell culture, NAC prevented neuronal injury after hypoxia-induced oxidative stress. Our results were in agreement with this literature.

We observed that a low dose of NAC has a protective effect against cholesterol induced lipid peroxidation. On the other hand, we have also observed increased levels of lipid peroxidation at high dose of NAC administration compared to a low dose of NAC. The protective effect of NAC can be attributed to its sulfhydryl group. The antioxidant activity of NAC primarily involves two mechanisms (14): (1) NAC acts as a free radical scavenger via sulfhydryl group, and (2) NAC acts as a precursor of GSH to facilitate intracellular GSH synthesis. Sprong et al. (15) is demonstrated that continuous intravenous infusion of low dose NAC treatment decreased H_2O_2 concentrations. However, in vitro, high dose NAC treatment increased hydroxyl radical generation in a system with Fe(III)-citrate and H_2O_2 by reducing ferric iron to its catalytic, active Fe^{2+} form. When administered at high dose, NAC may interact with ROS and generate thiyl radicals, which, in turn, may impart a pro-oxidant function. Actually, the presence of metals, such as Cu(II), and the presence of ROS, such as H_2O_2, potentiate "autooxidize" process of NAC (25). In this study shown that high dose NAC was not further increased GSH concentration. Furthermore, as it has undergone auto-oxidation, sulfhydryl group no longer acts as an "antioxidant".

Our group observed that high cholesterol diet was not effect of brain tissue NO concentrations. However, NO level was decreased at high dose of NAC administration compared to all groups. It has been demonstrated that antioxidants with thiol group exert neuroprotection from NO-induced toxicity in fetal midbrain cultures (26). It was shown that NAC dose-dependently attenuated the cytokine-induced activation of the iNOS promoter and hence NO production, indicating that the drug exerted its inhibitory effects at the transcriptional level (27). Bergamini et al. demonstrated that NAC inhibits in vivo NO production during lipopolysaccharide (LPS)-induced shock (28). NO, when produced in excess, is able to lead to neuronal cell death. Under these conditions, NAC may protect from the damage caused by NO overproduction.

On the other hand, it has been reported that NO and NO donors can suppress hydroxyl radical generation both in vivo and in vitro. NO seems to inhibit superoxide anion radical production by activated neutrophils, either by decreasing NADPH activity or assembly (29). It has been stated that the treatment with NO donors or a precursor for the synthesis of NO causes reduction of infarct size and lipid peroxidation after hypoxia-ischemia (30). Neurodegenerative changes in the axotomised dorsal root ganglion neuron have been reported to be due to the inhibition of nNOS (31). The present study has shown that a cholesterol-rich diet did not cause overproduction of NO in brain tissue. Likewise, high dose NAC administration caused a decrease NO level in brain tissue and did not provide enough protection against lipid peroxidation. These observations suggest that inhibition of nitric oxide production results in enhanced lipid peroxidation at high dose NAC administration.

In conclusion, the present study confirms the effect of cholesterol-enriched diet to produce a state of oxidative stress with biochemical and biological characteristics of

hypercholesterolemia. In addition to, we have demonstrated that NAC can have a dual effect on brain lipid peroxidation which was induced by a high-cholesterol diet. The effects of NAC depend on the administration dose. A low dose of NAC can prevent lipid peroxidation. However, a high dose of NAC might cause enhanced lipid peroxidation. If the NAC dose is carefully selected, NAC may have a neuroprotective effect against oxidative stress and hypercholesterolemia. In order to show this neuroprotective effect, further studies should be carried out by using different concentrations of NAC.

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