Does N-acetylcysteine have an effect on acetylcholine-induced contractions and histopathological changes on isolated rat ileum?

Emine Koc, PhD, *Sema A. Yavuzer,* MD, *Belgin Can,* MD, *Banu Ocakcıoglu,* MD, *Ahmet Ergun,* MD, *Yuksel Saran,* Dt, PhD.

ABSTRACT

Objectives: To investigate the action of N-acetylcysteine (NAC) on rat isolated ileal contractility, and to determine the effects of NAC on histopathological changes on ileal tissue.

Methods: The study took place at the Faculty of Medicine, Ankara University, Ankara, Turkey, in January 2003. Adult Wistar rats were used in all experiments. Two groups were designed. The experimental group, to which NAC 0.5 g/Kg/day was administered orally by adding to their water for 7 days, and the control group to which only saline was administered. At the end of the experimental periods, one cm pieces of terminal ileum segments were removed for testing ileal contractility, and one cm pieces of ileum segments were removed for histopathological experiments. The acetylcholine (ACh)-induced contraction was recorded, and the ileal tissue examined using light and electron microscopic technics for histopathological changes.

Results: The average peak amplitude of ACh-induced contraction recorded in standard tyrode solution of the experimental group was decreased significantly when compared to the control group in standard and calcium-Free tyrode solution. On histopathological findings, there were swollen mitochondria with disturbed cristae in the ileal muscle.

Conclusion: Our data suggest that the NAC in the present experiment decreased the ACh-induced contractility on rat-isolated ileum.

Saudi Med J 2007; Vol. 28 (8): 1180-1184

From the Departments of Physiology (Koc, Yavuzer, Ocakcıoglu, Ergun), and Histology-Embryology (Can, Saran), Faculty of Medicine, Ankara University, Ankara, Turkey.

Received 27th December 2006. Accepted 14th March 2007.

Address correspondence and reprint request to: Dr. Emine Koc, Department of Physiology, Faculty of Medicine, Ankara University, Tip Fakultesi, Fizyoloji ABD, Sihhiye 06100, Ankara, Turkey. Tel. +90 (312) 3103010. Fax. +90 (312) 3097404. E-mail: ekoc@medicine.ankara.edu.tr

N-acetylcysteine (NAC) is a known thiolic antioxidant. It acts by direct action between its reducing thiol group and radical oxygen species (ROS), and may also protect against oxidative damage in vitro and in vivo.[1,2] It could act as a precursor for the substrate glutathione (GSH).[3] It enhanced interleukin-1beta-induced nitrite production; and this effect was apparently associated with increased iNOS mRNA transcription and protein expression.[4] S-Nitroso-N-acetylcysteine (NACysNO) are nitrosothiols that release nitric oxide (NO) is a nitrosothiol that mimics the effects of endogenous NO, and is reported to cause relaxation in rat aorta.[5] It was also shown that treatment with NAC inhibited the phosphorylation of desmin.[6] N-acetylcysteine inhibits constitutive NF-Kappa Beta family of transcription factor and inhibits growth of vascular SMCs.[7] The NAC also reduced the radical intensity and cytotoxic activity of some antioxidants in cell culture.[8] It was shown that NAC inhibits the growth of various cell types in vitro and in vivo.[9,10] Not much is known on the effects of NAC on gastrointestinal smooth muscle contraction or ileal motility. Therefore, the aim of our study was to investigate the effect of NAC on isolated ileal contractility and to determine the effects of NAC on histopathological changes on ileal tissue.

Methods. The study took place at the Faculty of Medicine, Ankara University, Ankara, Turkey, in January 2003. Healthy, adult Wistar rats (n=14), weighing 180-200 g, and including both genders, were used in these experiments. The animals were obtained from the Animal Care and Research Center of Ankara University, School of Medicine. Before initiating the investigation, ethical approval has been obtained from the ethics committee of Ankara University, Faculty of Medicine. They were kept in pathogen-free cages, under standard environmental conditions. They were fed with normal granulated food and had access to water at all times from a drinking bottle. They were deprived of food, but not water, for 12-16 hours before the experiments.

Two experimental groups were designed. The first group, the control group (n=7), had only saline (0.9% NaCl) added to their water. The second group, the experimental group (n=7) was administrated NAC (Sigma Chem Co.) 0.5 g/Kg/day orally by adding to their water for 7 days. At the end of the experimental periods, under pentobarbital anesthesia (35 mg/Kg intraperitoneal) the abdomens were opened and one cm pieces of terminal ileum segments were removed rapidly for isolated tissue bath experiments, and one cm pieces of ileum were removed for histopathological experiments. To measure the isometric contraction of ileum, tissue segments were suspended in an isolated tissue bath containing 20 ml standard tyrode solution (Tyrode solution in mM: sodium chloride (NaCl) 137, potassium chloride 2.7, magnesium chloride 1.05, Calcium Chloride ($CaCl_2$) 1.8, sodium dihydrogen phosphate 0.42, sodium bicarbonate 11.90, glucose 5.5) and bubbled with 95% oxygen, 5% Carbon Dioxide mixture at 37^0C, pH adjusted at 7.4. To record tension, the samples were mounted vertically between a fixed holder and a force transducer (Ugo Basile isometric transducer No 7003) and the tissues were brought into equilibrium in 30 minutes under an optimal resting tension of 0.3 g. The isometric tension was recorded by an Ugo Basile no: 7050 recorders. Contraction of ileum segments was induced by adding ACh 2.7 x 10^{-7} M into the bath solutions. These procedures were repeated in the calcium-free medium. When calcium-free solutions were used, 2 Methylene-glycol-bis (Beta-aminoethyl-ether)-N, N'-tetraacetic acid (EGTA) 3 mM and equimolar NaCl were added to the tyrode solutions instead of $CaCl_2$. The ACh-induced contraction amplitude was measured in mm from recorded traces, and the calibration was made as 1 g per 10 mm. The ileal tissue was examined using light and electron microscopic technics for histopathological changes. In the light microscopy studies, the ileal tissue was fixed in 10% formaldehyde, dehydrated in alcohol, and embedded in paraffin. Sections were cut in 5-6 microns and stained with Hematoxylin-Eosin (H-E). For electron microscopic examination, the ileal tissue was fixed at 4^0C for a period of 1-2 hours in 2.4% phosphate buffered glutaraldehyde solution, and postfixed in 1% osmium tetroxide. After alcohol dehydration, the samples were embedded in Araldite 6005. Sections were cut on a Leica Ultracut R with a glass knife, stained with uranyl acetate, and lead citrate, and were viewed on a LEO 906 E transmission electron microscope.

Statistical analysis was performed by Mann-Whitney-U and Wilcoxon tests where appropriate, using SPSS software. The results were expressed as means ± SD. Statistical significance was set for $p<0.01$ or $p<0.05$.

Results. The ACh-induced contraction responses of the experimental group and the control group both in the standard tyrode and calcium-free tyrode group are summarized in **Figure 1**. The histopathological changes observed by both light microscope and electron

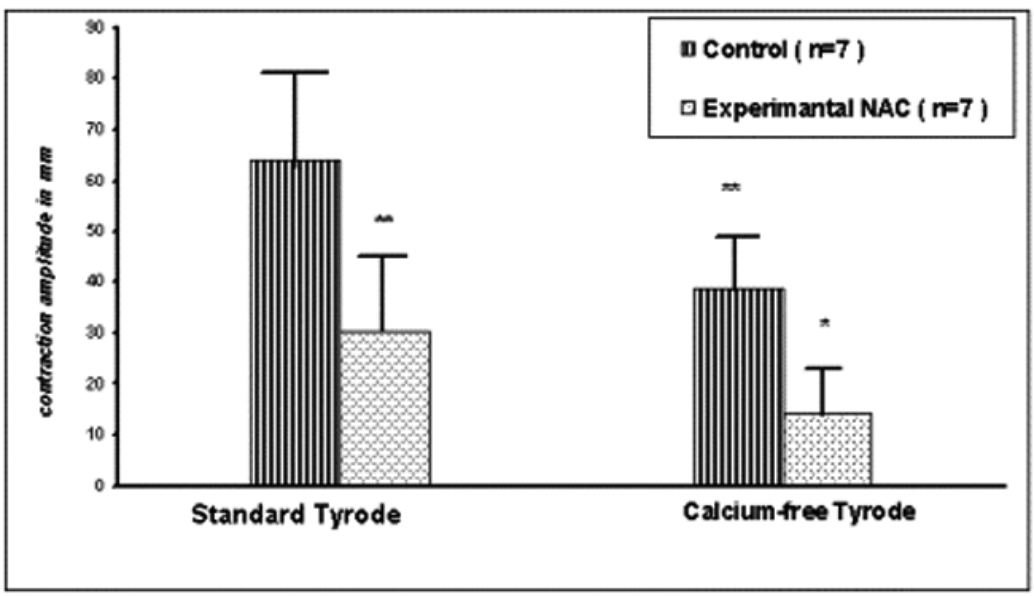

Figure 1 - The changes of contraction amplitude as millimeters measured in standard and calcium-free tyrode solution in control group and N-acetylcysteine (NAC) administrated group. Data are given as mean ± SD, *P*-significant level for the changes experimental period, **p*<0.05. ***p*<0.01, n=number of animals. The calibration is 1 g per 10 mm in our setup.

microscope are shown in **Figures 2-4**. As shown in **Figure 1**, the average peak amplitude of ACh-induced contraction recorded in standard tyrode solutions of the experimental group was significantly lower when compared to the control group ($p<0.05$). Furthermore, contractile response to the ACh was lower in the experimental and control groups as compared to the standard tyrode group when calcium-free tyrode solution was used ($p<0.05$ and $p<0.01$). The histopathological results showed that the smooth muscle cells of the control ileum were normal, cells were fusiform with homogenous cytoplasm and centrally placed elongated nuclei and several nucleoli (**Figure 2**). In the longitudinal sections of the smooth cells of the experimental animals, the nuclei of cells appeared shorter and broader, and were frequently folded. There was thickness of the cytoplasm and staining with greater intensity than the rest of the fibrils, reflecting the abundance of myofilaments characteristic in contracted cells (**Figure 3**). Ultrastructurally, an irregular outline due to infolding of the nucleus was prominent (**Figure 4**). A larger number of mitochondria, related to energy requirement were present. Some of the mitochondria appeared slightly swollen and had disturbed cristae. The majority of sarcoplasm was filled with myofilaments, which were oriented in relatively larger bundles or irregular cross bands, considered to be contraction bands. Free ribosomes were abundant. Granular endoplasmic reticulum vesicles surrounding individual mitochondria in cells engaged in active protein synthesis were seen.

Discussion. In the present study, our results showed that the administration of NAC to rats (one week, 0.5 g/kg/day) significantly decreased ACh-induced contractions in isolated ileum in both standard and calcium-free tyrode solution medium as compared to the control groups.

It is well known that NAC acts as a precursor of GSH synthesis. Bridgeman et al[11] reported that plasma GSH concentration increased significantly in patients taking NAC 600 mg/Kg per day for 5 days. Also, Hashimoto[12] showed that intracellular GSH levels increased NAC-treated cells. The NAC has been shown to exert effects on GSH metabolism in animals and in humans,[13] both in vitro[14] and in vivo, in patients with congenital GSH synthetase deficiency. Oral supplementation of NAC increased plasma GSH levels in leucocytes.[15] With the administration of 600 mg NAC per day for 14 days in healthy individuals, the superoxide anion production by neutrophiles was decreased. Satoh and Sakagami[8] showed that cysteine, N-acetyl cysteine, and GSH significantly reduced the radical intensity and cytotoxic activity of these oxidants in cell culture medium. They thought that all these compounds have a

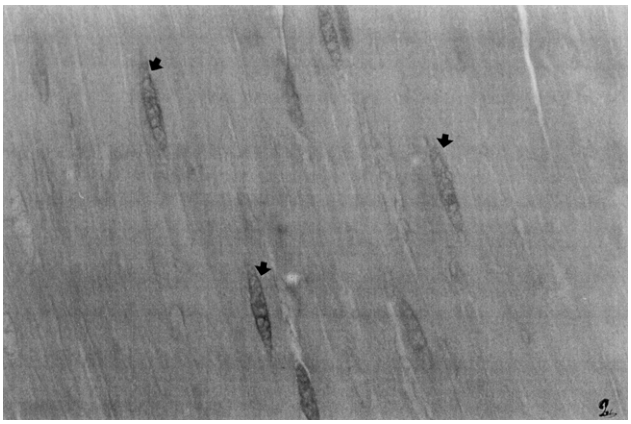

Figure 2 - Light microscopic photograph of control group. Smooth muscle cells from the tunica muscularis of ileum. Elongated nuclei with their prominent nucleoli are seen (arrows). Hematoxylin-Eosin x 250.

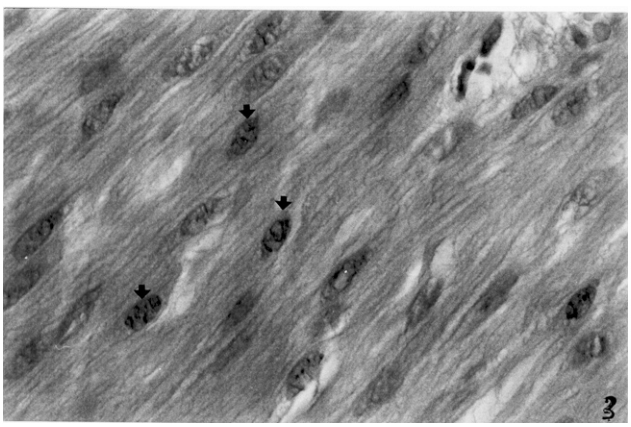

Figure 3 - Light microscopic photograph of experimental group. Nuclei of smooth muscle cells are shorter and broader (arrows). Hematoxylin-Eosin x 250.

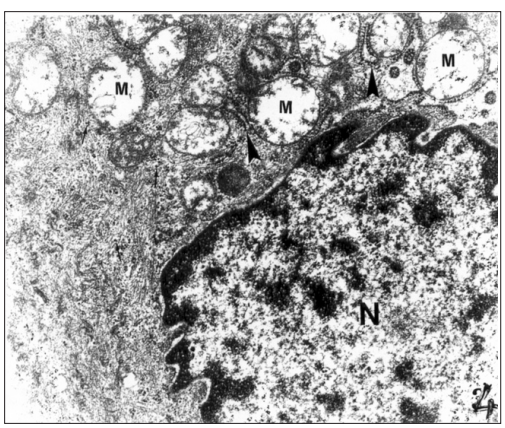

Figure 4 - Electron microscopic photograph of experimental group. A smooth muscle cell nucleus (n), swollen mitochondria (m), granular endoplasmic reticulum (arrow head), free ribosomes (arrows). Uranyl acetate-lead citrate x 16700.

free SH group, and cysteine (which has disulfide bonds) has no radical scavenging activity, therefore the free SH group might play a significant role in protecting the cells from oxidant-induced cytotoxicity. Thus, NAC might directly or indirectly, via its metabolites, act as a scavenger of ROS.[16]

We showed that NAC decreased the ACh-induced ileal contraction, and therefore the ileal contractility. When we designed the study, our hypothesis was that NAC may increase the ileal contractility by its antioxidant effects. It was interesting to find decreased contractility. So, therefore, we thought that the dosage of NAC that we used was not enough to increase GSH or, as Meister's data indicates, it may be toxic at these doses.[17] However, there are some reports that are compatible with our findings. Lida et al[18] thought that, in glioblastoma and glioma cell lines, intracellular GSH concentration was not changed by NAC; thus, the effect of NAC may depend on its radical scavenging effect instead of its GSH modulating effect. Also, Alton Meister reported that administration of cysteine is not an ideal therapy because it may be followed by toxic reactions.[17] In addition, Kharazmi et al[19] showed that high concentrations of NAC has a strong cytotoxic effect on neutrophils. Vina et al[20] demonstrated that intraperitoneal injection of cysteine or NAC causes depletion of GSH in rat brain.[20]

Our findings suggested that 0.5 g/kg/day NAC administration (although we cannot determine the ROS activity) may cause an increase in ROS production, which affects ACh-induced contraction. Our histopathological findings supported the physiological data. As shown in **Figures 3 & 4**, there are large numbers of mitochondria, and some of the mitochondria appeared slightly swollen, and had disturbed cristae. Mitochondria degeneration may be due to the presence of abnormal collections of mitochondria since high-energy requirements of contracting muscle cell cannot be metabolized. It was shown that mitochondrial lesions can diverse cellular bioenergetic capacity and also increase the generation of ROS.[21] Martensson et al[15] found that chronic depletion of mucosal GSH in mice causes severe degeneration of the epithelial cells of the jejenum and colon. Lash et al[22] demonstrated that GSH concentration diminishes 60% in isolated intestinal epithelial cells after one hour of incubation in reduction free of GSH or precursor amino acid. Also, Cuzzocrea et al[23] showed that NAC attenuated the ileum injury (histology). Haznedaroglu et al[24] found that NAC did nor prevent intestinal reperfusion injury by means of histopathological findings and malondialdehyde levels.

When we omitted calcium from the perfusion medium, we showed that the contractile response decreased significantly in the control and experimental groups ($p<0.05$, $p<0.01$). This finding is not interesting in terms of the control group because of decreased calcium entry in calcium-free medium. However, contractile response was extremely reduced in the experimental and control groups in calcium-free medium. Therefore, we thought that NAC would act as a toxic substance with these doses, and these effects would cause a disturbed energy metabolism as we showed in the histological data. N-acetylcysteine seems to be able to protect against apoptosis through mechanisms that maintain mitochondrial integrity.[25] Our histological findings showed that, when 0.5 g/kg/day NAC was administrated, mitochondria were swollen and cristae were disturbed. In contrast with our present results, previous results showed that NAC administration had beneficial effects on synaptic mitochondria, at least partially by its direct antioxidant action. The NAC would act potentially on mitochondrial oxidative phosphorylation complexes by possibly protecting oxidative damage by maintaining the protein sulfhydryl groups.[26,27] The NAC could reduce the lipid peroxides in mitochondrial membrane that can impair mitochondrial complex activities.[28,29] In contrast with these studies showing a beneficial effect of NAC on mitochondria,[26-29] our histological data show a swollen mitochondria and disturbed cristae, in which the inner mitochondrial membrane (cristae) is responsible for the mitochondrial production of ATP in aerobic metabolism. The possibility that the dose of NAC in the present experiment was insufficient to modify complex specific activities on cells that would increase GSH in cytosol and complex antioxidant activities in mitochondria exists. Our results agree with the report that, some antioxidants, cysteine, NAC, and GSH, significantly reduced radical intensity and cytotoxic activity and these antioxidants induced cytotoxicity via their pro-oxidants action.[8]

In conclusion, we showed that NAC decreased the ACh-induced contractions on isolated ileal strips by yet undefined mechanisms. In view of our data, and since no other study seems to have reported the effect of NAC on isolated ileal contractility, we can say that, although we did not examine the ileal levels of ROS or GSH, the dietary supplementation of NAC decreases ileal contractility, probably by increasing ROS activity in mitochondria.

References

1. Aruoma OI, Hallıwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide and hypochlorous acid. ***Free Radic Biol Med*** 1989; 6: 593-597.
2. De Flora S, Izzotti A, D'agostini F, Cesarone CF. Antioxidant activity and other mechanisms of thiols involved in chemoprevention of mutation and cancer. ***Am J Med*** 1991; 91: S122-S130.

3. Lauterburg BH, Corcoran GB, Mitchell JR. Mechanism of action of N-acetylcysteine in the protection against the hepatotoxicity of actaminophen in rats in vivo. ***J Clin Invest*** 1983; 71: 980-991.
4. Jiang B, Haverty M, Brecher P. N-acetyl-L-cysteine enhances interleukin-1beta-induced nitric oxide synthase expression. ***Hypertension*** 1999; 34: 574-579.
5. Ceron PI, Cremonez DC, Bendhack LM, Tedesco AC. The relaxation induced by S-nitroso-glutathione and nitroso-N-acetylcysteine in rat aorta is not related to nitric oxide production. ***Pharmacol Exp Ther*** 2001; 298: 686-694.
6. Tate Y, Kawasaki K, Ishibashi S, Ikeda O, Shimada K. Effects of N-acetylcysteine on nitroglycerin-induced relaxation and protein phosphorylation of porcine coronary arteries. ***Heart Vessels*** 1998; 13: 263-258.
7. Lee JS, Kypreos KE, Sonenshein GE. Synchronization of cultured vascular smooth muscle cells following reversal of quiescence induced by treatment with the antioxidant N-acetylcysteine. ***Exp Cell Res*** 1998; 239: 447-453.
8. Satoh K, Sakagami H. Effect of cysteine, N-acetyl-L-cysteine and glutathione on cytotoxic activity of antioxidant. ***Anticancer Res*** 1997; 17: 2175-2180.
9. Sundaresan M, Yu Z, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. ***Science*** 1995; 270: 296-299.
10. Bellas RE, Lee JS, Sonenshein GG. Expression of a constitutive NF-cappa B-like activity is essential for proliferation of cultural bovine vascular smooth muscle cells. ***J Clin Invest*** 1995; 96: 2521-2527.
11. Bridgeman MM, Marsden M, Selby C, Morrison D, Dahee W. Effect of N-acetylcysteine on the concentration of thiols in plasma, bronchoalveolar lavage fluid and lung tissue. ***Thorax*** 1994; 49: 670-675.
12. Hashimoto S, Gon Y, Matsumoto K, Takeshıta I, Horie T. N-acetylcysteine attenuates TNF-alpha-induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells. ***Br J Pharmacol*** 2001; 132: 270-276.
13. Bridgeman MM, Marsden M, MacNee W, Flenley DC, Ryle AP. Cysteine and glutathione concentrations in plasma and bronchoalveolar lavage fluid after treatment with N-acetylcysteine. ***Thorax*** 1991; 46: 39-42 .
14. Sjodin K, Nilsson E, Hallber A, Tunek A. Metabolism of N-acetylcysteine. ***Biochem Pharmacol*** 1989; 38: 3981-3985,
15. Martensson J, Jain A, Meister A. Glutathione is required for intestinal function. ***Proc Natl Acad Sci USA*** 1990; 87: 1715-1719.
16. Urban T, Akerlund B, Jarstrand C, Lindeke B. Neutrophil function and glutathion-peroxide (GSH-px) activity in healthy individuals after treatment with N-acetyl-L-cysteine. ***Biomed Pharmacother*** 1997; 51: 388-390.
17. Meister A. New aspect of glutathione biochemistry and transport: selective alteration of glutathione metabolism. ***Fed Pro*** 1984; 43: 3031-3042,
18. Iida M, Sunaga S, Hirota N, Kuribayashi N, Sakagami H, Takeda M, et al. Effects of glutathion-modulating compounds on hydrogen-peroxide induced cytotoxicity in human glioblastoma and glioma cell lines. ***J Cancer Res Clin Oncol*** 1997; 123: 619-622.
19. Kharazmi A, Nielson H, Schiatz PO. N-acetylcysteine inhibits human neutrophil and monocyte chemotaxis and oxidative metabolism. ***Int J Immunopharmacol*** 1988; 10: 39-46.
20. Vina J, Romeo FS, Saez GT, Pollardo FV. Effects of cysteine and N-acetylcysteine on GSH content of brain adult rats. ***Experientia*** 1983; 39: 164-165.
21. Martinez M, Hernandez AI, Martinez N. N-acetylcysteine delays age-associated memory impairment in mice: role in synaptic mitochondria. ***Brain Res*** 2000; 855: 100-106.
22. Lash LH, Hagen TM, Jones DP. Exogenes glutathione protects intestinal epithelial cells from oxidative injury. ***Proc Natl Acad Sci USA*** 1986; 83: 4641-4645.
23. Cuzzocrea S, Mazzon E, Costantino G, Serraino l, De Sarro A, Caputi AP. Effects of N-acetylcysteine in rat model of ischemia and reperfusion injury. ***Cadiovasc Res*** 2000; 18: 537-548.
24. Hazinedaroglu SM, Dulger F, Kayaoglu HA, Pehlivan M, Serinoz E, Canbolat O, et al. N-acetylcysteine in intestinal reperfusion injury: an experimental study in rats. ***ANZ J Sur*** 2004; 74: 676-678.
25. Cossarizza A, Franceschi C, Monti D, Salvioli S, Bellesia E, Rivabene R, et al. Protective effect of N-acetylcysteine in tumor necrosis factor-a-induced apoptosis in U937 cells: The role of mitochondria. ***Exp Cell Res*** 1995; 220: 232-240.
26. Haugaard N, Lee NH, Kostrzewa R, Horn RS, Haugaard ES. The role of sulfhydryl groups in oxidative phosphorylation and transport by rat liver mitochondria. ***Biochim Biophys Acta*** 1969; 172: 198-204.
27. Martinez M, Ferrandiz ML, De Juan E, Miquel S. Age related changes in glutathione and lipid peroxide content in Mouse synaptic mitochondria relationship to cytochrome c oxidase decline. ***Neurosci Lett*** 1994; 170: 121-124.
28. Wharton DC, Griffith DE. Studies of the transport system. Assay of cytochrome oxidase. Effect of phospholipids and other factors. ***Arch Biochem Biophys*** 1995; 96: 103-114.
29. Zhang Y, Marcillat O, Giulivi C, Ernster L, Davies KJ. The oxidative inactivation of mitochondrial electron transport chain components and ATPase. ***J Biol Chem*** 1990; 265: 16330-16336.