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The protective effect of N-acetylcysteine amide against paraquat-induced neurotoxicity

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Abstract: N-acetylcysteine amide (NACA) is a new antioxidant molecule with powerful radical scavenging properties. The aim of this study was to investigate neuroprotective effects of NACA against paraquat (PQ) toxicity in the midbrains of rats by using motor coordination tests and biochemical and histological analysis. Thirty adult Wistar albino rats were divided into three groups: Group 1: control (n = 10), Group 2: PQ (10 mg/kg) (n = 10), and Group 3: PQ (10 mg/kg) + NACA (100 mg/kg) (n = 10). NACA was administrated intraperitoneally 30 min before PQ injection. Performance was measured for a period of 28 days. The rotarod and accelerod tests were performed prior to and after the experimental period. After the experimental period, rats were sacrificed and midbrain tissues were removed. According to biochemical data, malondialdehyde levels exhibited a significant increase ($P < 0.05$) when the PQ group was compared to the control group, whereas the NACA-treated group showed a significant decline ($P < 0.05$). The total glutathione levels ($P < 0.01$) and the glutathione peroxidase and butyrylcholinesterase activities ($P < 0.05$) in the NACA treatment group were significantly raised compared with the PQ group. The main finding in the rotarod and accelerod tests was that the PQ+NACA group had improved motor coordination functions, whereas the PQ group had lost motor coordination ($P < 0.05$). Our histological data were also outstanding and were consistent with biochemical and motor coordination results in terms of the protective role of NACA against PQ-induced neurotoxicity.

Key words: Thiol antioxidant, N-acetylcysteine amide, paraquat, neuroprotective, glutathione

1. Introduction

Free radicals are reported to play a key role in the progression of neurodegenerative disorders. To scavenge free radicals and prevent their damaging effects, new antioxidant molecules were investigated in a large number of studies. Thiol-containing antioxidants have gained special consideration because of their capacity to increase the antioxidant system of cells and the glutathione (GSH) level.[1,2]

N-acetylcysteine amide (NACA) is synthesized by exchanging the carboxyl with an amide group in N-acetylcysteine (Figure 1). It was shown that NACA is more membrane-permeable than NAC and replaces intracellular GSH in red blood cells.[3] GSH, an important intracellular thiol antioxidant molecule, has been considered as an agent to prevent oxidative damage in cells. NACA has also exhibited the ability to scavenge free radicals, chelate metals, and protect red blood cells from free radicals.[4–6] Therefore, NACA has been

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used to treat oxidative stress-related diseases such as retinal degeneration and cataracts and inflammatory lung injury.[7–9]

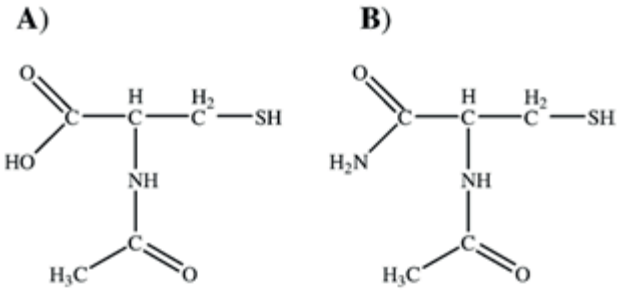

Figure 1. Structures of N-acetylcysteine (NAC) (A) and N-acetylcysteine amide (NACA) (B).

Paraquat (PQ; 1-10-diethyl-40-bipyridylium dichloride) is an effective herbicide and is extensively used worldwide. In addition, PQ effectively induces reactive oxygen species (ROS)[10,11] such as superoxide anions, singlet oxygen, and hydroxyl and peroxyl radicals.[12] PQ can increase the production of ROS with redox cycling, the mitochondrial electron transport chain, and NADPH oxidases (ROS-generating enzymes). It was also shown that neuronal damage was increased by PQ with the activation of glial cells.[11,13] Since ROS stimulates neuronal death through multifactorial actions[13], antioxidant supplementation is very valuable in the treatment of neurodegenerative diseases.

With this background, we hypothesized that NACA may have a neuroprotective effect of crossing the blood/brain barrier due to its strong membrane permeability effect. This study was carried out to determine the neuroprotective potential of NACA against the toxic effects of PQ on midbrain tissues by using biochemical, histological, and motor coordination tests.

2. Results and discussion

2.1. Results

Thirty adult Wistar albino rats were divided into three groups: Group 1: control (n = 10), Group 2: PQ (10 mg/kg) (n = 10), and Group 3: PQ (10 mg/kg) + NACA (100 mg/kg) (n = 10). NACA was administrated intraperitoneally 30 min before PQ injection. In this study, the activities of primary antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) and an important enzyme in the communication of neuronal cells, butyrylcholinesterase (BuChE), were determined in midbrain cells (Figure 2). There was not a significant change in CAT enzyme activity (Figure 2A). In the PQ group, SOD activity was significantly increased ($P < 0.05$) when compared to the control group. NACA administration resulted in a significant decrease in SOD activity when compared to the PQ group (Figure 2B). GSH-Px activity did not significantly decrease in the PQ group as compared to the control group. However, the NACA-treated group, when compared to the PQ group, showed a significant rise ($P < 0.05$) (Figure 2C). BuChE activity statistically showed a decline ($P < 0.05$) in the PQ-treatment group as compared with the control group. However, in the NACA-treatment group, BuChE activity was statistically higher ($P < 0.05$) than in both the control and the PQ group (Figure 2D).

Total glutathione (tGSH) levels showed a decline ($P < 0.05$) in the PQ-treatment group as compared with the control group. However, in the NACA-treatment group, tGSH levels were significantly higher than

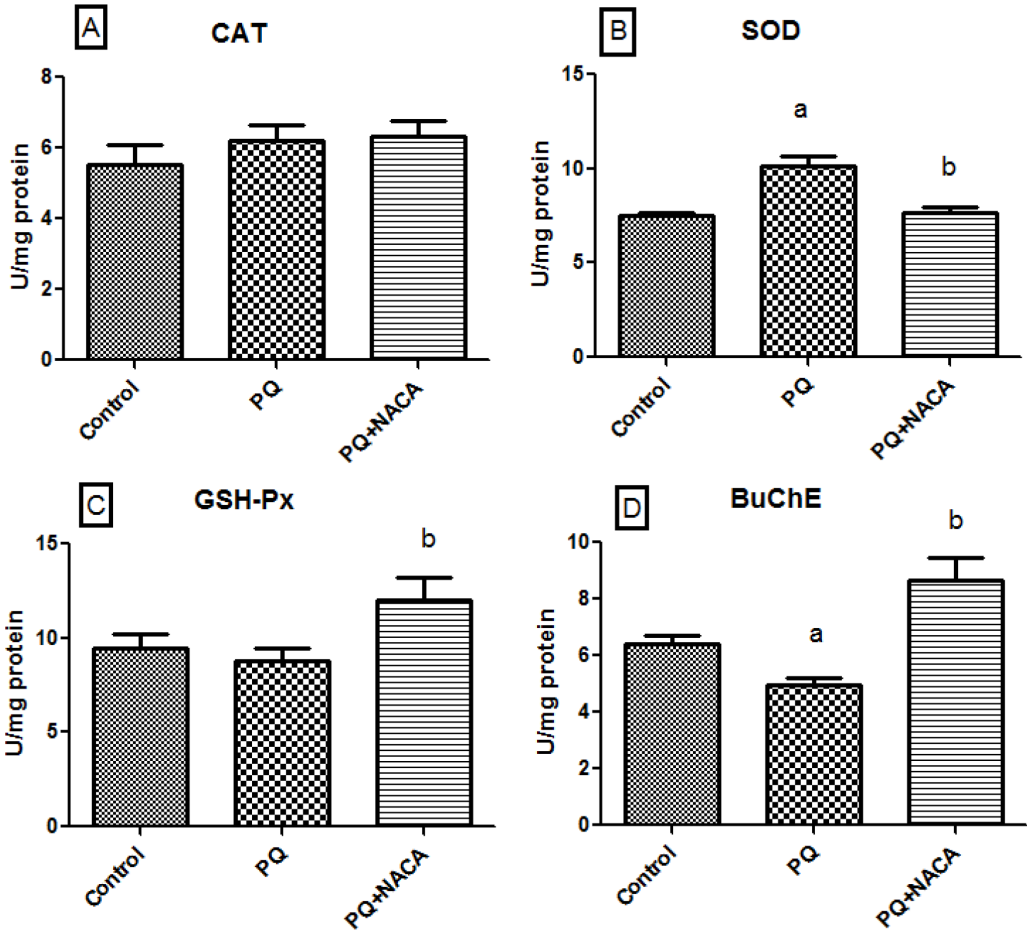

Figure 2. Effect of NACA on alterations of CAT (A), SOD (B), GSH-Px (C), and BuChE (D) enzyme activities in a rat midbrain against paraquat-induced neurotoxicity. Experimental groups were the control, PQ (10 mg/kg), and PQ (10 mg/kg) + NACA (100 mg/kg). Data are expressed as mean ± SD (n = 10), ^{a} significantly different from control group ($P < 0.05$), ^{b} significantly different from PQ group ($P < 0.05$).

those in both the control and the PQ group (Figure 3A).

The mean level of malondialdehyde (MDA) showed a marked increase following PQ administration as compared with the level of the control group ($P < 0.05$). On the other hand, in the NACA-treated group, as compared with the PQ group, MDA levels significantly declined in the midbrain tissues ($P < 0.01$) (Figure 3B).

According to histological results, molecular layers and large pyramidal neurons were observed in the H&E-stained sections of the control group (Figure 4A). In the toluidine blue method, the large pyramidal neurons in the pyramidal internal layer were found to have pyramid-shaped soma and extensions (Figure 4B). In addition, the Nissl bodies in the cytoplasm of the neurons were stained purple (Figure 4C).

When the PQ-administered group was compared with the control group, cerebral injury was statistically significant ($P < 0.003$), showing an increase (3.6 ± 1.2) as depicted in Table 1. In the PQ group, cerebral cortex neuronal loss (Figure 5A), changes in neuronal morphology (Figure 5B), an increase in microglial cells (Figure 5C), and perivascular (Figure 5D) and perinuclear edema (Figure 5E) were observed. When the NACA-

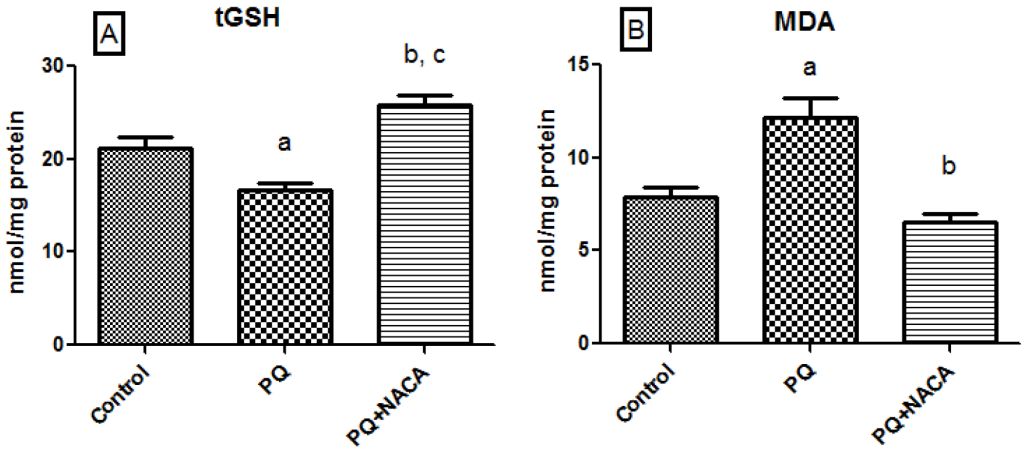

Figure 3. Effect of NACA on alterations of tGSH (A) and MDA (B) levels in a rat midbrain against paraquat-induced neurotoxicity. Experimental groups were the control, PQ (10 mg/kg), and PQ (10 mg/kg) + NACA (100 mg/kg). Data are expressed as mean ± SD (n = 10), [a] significantly different from control group ($P < 0.05$). [b] significantly different from PQ group ($P < 0.01$), [c] significantly different from PQ+NACA vs. control group ($P < 0.05$).

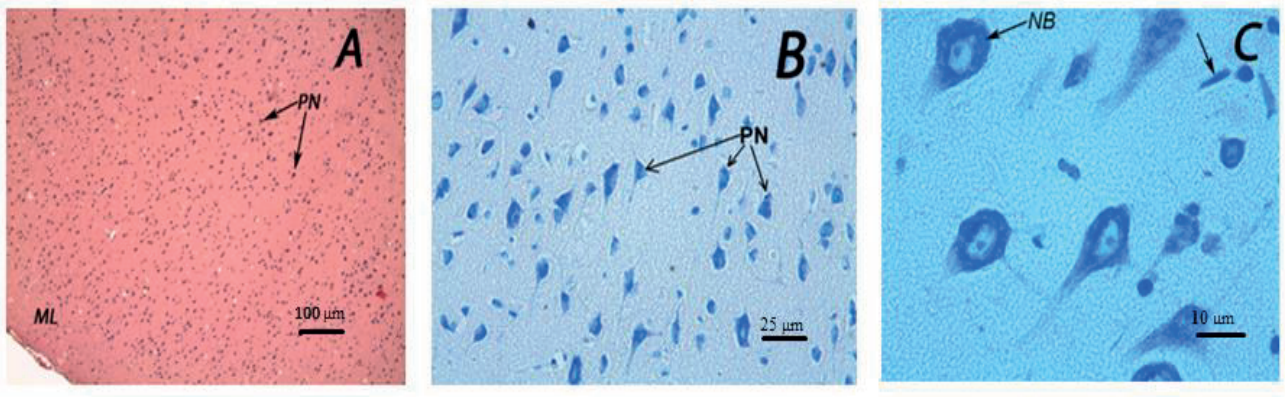

Figure 4. Control group: (A) cerebral cortex view, ML: molecular layer, PN: pyramidal neurons, H&E; (B) pyramidal neurons in pyramidal interna, TM; (C) purple granular style Nissl bodies (NB) followed in cytoplasm of pyramidal neurons and flat-fusiform nuclei in monitored microglia cells (arrow), TB.

treatment group was compared with the PQ group, the histological score (2.3 ± 0.5) had significantly decreased ($P < 0.01$), as shown in Table 1. In the PQ+NACA group, morphological properties of pyramidal neurons were similar to those of the control group (Figure 6A). Perivascular and perinuclear edema decreased in intensity as compared to the PQ group (Figure 6B). However, the increase in microglial cells was still observed in the NACA-treatment group (Figure 6C).

The rotarod and accelerod results are given in Table 2. In brief, there was no difference among the groups in the basal rotarod and accelerod performance measurements. The time that the animals remained on the rod decreased in the PQ group as compared with the control group at speeds higher than 10 rpm in the rotarod test ($P < 0.05$). The length of time that the animals could remain on the rod was found to decrease in the PQ group at 20, 25, 30, and 35 rpm. In this group, the results of the accelerod test were in accordance with the rotarod results at both 4 and 10 min when compared to the control group. The main finding in these results was that the PQ group had lost motor coordination in light of the rotarod and accelerod results whereas the

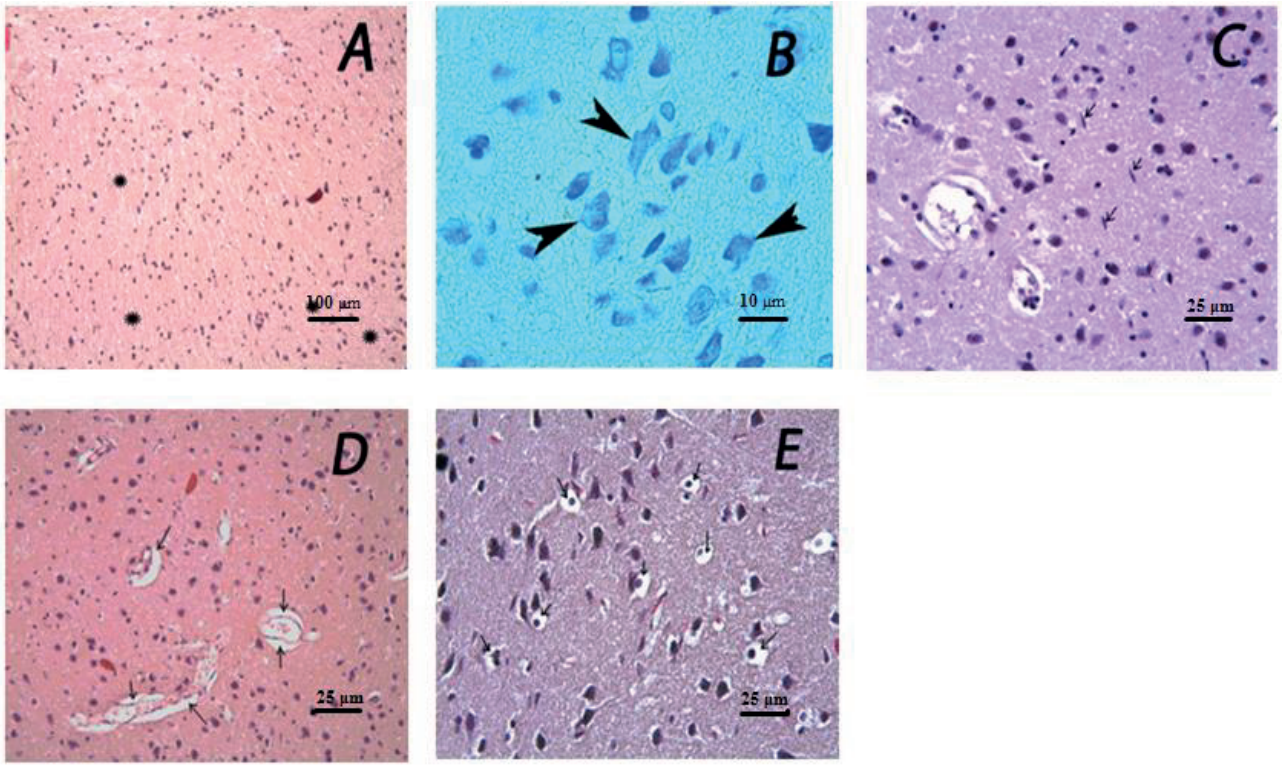

Figure 5. PQ group (10 mg/kg): (A) cerebral cortex neurons in some areas are seen as sparse (*), H&E; (B) neurons altered morphologically and in their cytoplasm unmonitored Nissl bodies (arrow), TM; (C) appearance of microglial cells, H&E; (D) perivascular edema viewed as significant (arrows), H&E; (E) perinuclear edema (arrows), H&E.

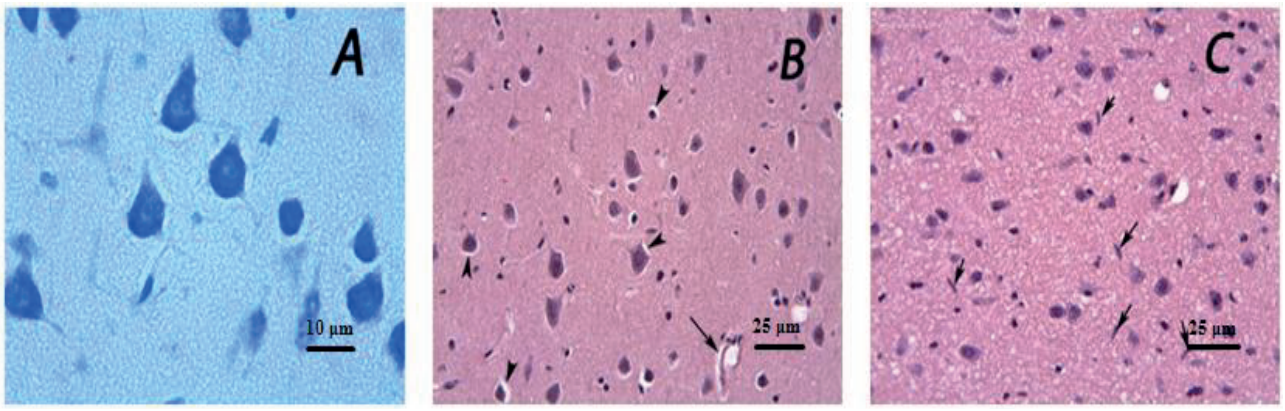

Figure 6. PQ (10 mg/kg) + NACA (100 mg/kg) group: (A) appearance of pyramidal neurons, TM; (B) perivascular (arrow) and perinuclear edema (arrow head); (C) microglial cells, H&E.

PQ+NACA group had improved motor coordination functions ($P < 0.05$).

2.2. Discussion

As a widely used herbicide, PQ has been reported to have initiated the oxidative stress process by disrupting the mitochondrial energy system in brain tissues. Neutral amino acid transporters translocate PQ into the

Table 1. The results of histological evaluation of the groups. Cerebral injury was statistically increased in the PQ group compared with the control group. NACA treatment had an ameliorative effect against PQ damage.

Group	Histological score
Control	0.83 ± 0.4
PQ	3.6 ± 1.2 [a]
PQ+NACA	2.3 ± 0.5 [b]

Data are expressed as mean ± SD. [a] Significantly different from control group ($P < 0.003$). [b] Significantly different from PQ group ($P < 0.01$).

Table 2. The results of motor coordination tests of the groups before and after the experimental period. It is clear that the PQ group had lost motor coordination in light of the rotarod and accelerod data. However, the PQ+NACA group had enhanced motor coordination functions ($P < 0.05$).

Variable	Group [median (min–max)]					
	Control		PQ		PQ+NACA	
	Before	After	Before	After	Before	After
5 rpm	300 (240–300)^{d}	300 (300–300)	300 (290–300)^{f}	300 (272–300)	300 (276–300)^{f}	300 (255–300)
10 rpm	300 (290–300)^{d}	300 (260–300)^{b,c}	300 (175–300)^{a,f}	154 (16–300)^{c}	300 (292–300)^{a,h}	290 (232–300)
15 rpm	300 (204–300)^{d}	300 (200–300)	300 (150–300)^{f}	115.5 (8–192)	300 (110–300)^{a,f}	217 (70–300)
20 rpm	300 (76–300)^{b,d}	286 (64–300)^{b,c}	129 (58–300)^{a,d}	32.5 (6–106)^{c}	179 (76–300)^{e}	149 (121–300)
25 rpm	225 (80–300)^{b,e}	230 (98–300)^{b,c}	101 (40–250)^{a,e}	55 (14–231)^{c}	140 (45–300)^{a,e}	100 (30–210)
30 rpm	30 (15–300)^{g}	83 (14–300)^{b}	64 (19–103)^{a,g}	18 (9–99)^{c}	85.5 (23–300)^{a,g}	64 (17–120)
35 rpm	112 (12–300)^{g}	93 (9–300)^{b}	32.5 (11–85)^{a}	12 (6–82)^{c}	57.5 (15–100)	64 (17–90)
40 rpm	16 (7–110)	28 (9–90)	22 (9–67)^{a}	12 (8–68)^{c}	35.5 (11–96)	37 (15–91)
ACC_4	110 (90–151)	102 (56–143)^{b}	104 (86–141)^{a}	28.5 (11–89)^{c}	96 (72–139)	93 (70–106)
ACC_{10}	153 (106–197)	143 (103–160)^{b,c}	135 (101–182)^{a}	19 (16–70)^{c}	123.5 (68–195)^{a}	107 (57–131)

a: Significantly different from after (Wilcoxon signed ranks test, $P < 0.05$); b: significantly different from group PQ (Conover test after significant Kruskal–Wallis H test, $P < 0.05$); c: significantly different from group PQ+NACA (Conover test after significant Kruskal–Wallis H test, $P < 0.05$); d: significantly different from 25, 30, 35, and 40 rpm (Conover test after significant Friedman test, $P < 0.05$); e: significantly different from 30, 35, and 40 rpm (Conover test after significant Friedman test, $P < 0.05$); f: significantly different from 20, 25, 30, 35, and 40 rpm (Conover test after significant Friedman test, $P < 0.05$); g: significantly different from 35 and 40 rpm (Conover test after significant Friedman test, $P < 0.05$); h: significantly different from 15, 20, 25, 30, 35, and 40 rpm (Conover test after significant Friedman test, $P < 0.05$); values are given as median (min–max).

blood/brain barrier and then it is taken up into the brain tissues by dopamine transporters. PQ causes dysfunction of the cellular mitochondrial function by increasing the ROS in the brain tissue.[14]

Cells have developed some defense mechanisms to neutralize ROS from the intracellular area.[15] First, molecules known as antioxidants directly scavenge free radicals.[16] Second, cells widely use CAT, SOD, GSH, GSH-Px, and other antioxidants against ROS.[17] GSH, the most prevalent three-peptide thiol in the cells, plays a vital role in the antioxidant defense system of cells.[18] Mitochondrial GSH plays an especially important role in cell protection.[19]

NACA is a designed form of N-acetylcysteine that possibly exchanges the carboxyl group for its passage through cell membranes. Recent studies have reported evidence that NACA has some properties such as

crossing the blood/brain barrier, scavenging free radicals, chelating copper, and protecting red blood cells from oxidative stress.[4–6] It was also demonstrated that NACA can be used as a novel, potentially effective treatment for traumatic brain injury.[20] The aim of this study was to investigate and determine the neuroprotective effects of NACA through its powerful antioxidant properties against PQ toxicity in rat midbrains. In this context, the activities of primary antioxidant enzymes CAT (Figure 2A), SOD (Figure 2B), and GSH-Px(Figure 2C) and an important enzyme in the communication of neuronal cells (BuChE; Figure 2D) were identified in midbrain cells.

In parallel with the literature, in the PQ group, SOD activity was significantly increased ($P < 0.05$) when compared to the control group.[21] NACA administration resulted in a decrease in SOD activity when compared to the PQ group. This result may be explained by the direct $^{\bullet}O_2^-$ radical scavenging properties of GSH, which were increased ($P < 0.05$) by NACA treatment.

GSH-Px activity did not significantly decrease in the PQ group as compared to the control group. However, the NACA-treated group, when compared to the PQ group, showed a rise ($P < 0.05$). This result was parallel with the change in tGSH levels linked to GSH-Px activity. There was not a significant change in CAT enzyme activity. These results demonstrated that GSH-Px enzyme activity, a second way of metabolizing hydrogen peroxide, may be more effective than CAT enzyme activity.

BuChE catalyzes the hydrolysis of the neurotransmitter acetylcholine, which has a vital role in the regulation of the cholinergic system, neuronal proliferation, and differentiation.[22] In addition, some evidence shows that BuChE and monoamine oxidase are involved in the pathological processes of neurodegenerative diseases, including Alzheimer disease.[23,24] In the present study, BuChE activity statistically showed a decline ($P < 0.05$) in the PQ-treatment groups as compared with the control group. However, in the NACA-treatment group, BuChE activity was higher ($P < 0.05$) than that in both the control and the PQ group. Our results are in agreement with other studies that found that this enzyme can be inhibited in chronically stressed animals.[25]

Cells themselves have antioxidant mechanisms that detoxify ROS production under a pathological process. GSH is the vital antioxidant component of cells due to its ability to directly scavenge ROS. However, GSH cannot be moved directly to cells. Therefore, cells need molecules that can easily pass into cells and increase the GSH level. GSH levels are generally used for a better picture of redox status at a biochemical scale.[26] In this study, tGSH levels showed a decline ($P < 0.05$) in the PQ-treatment group as compared with the control group. Fukushima et al. also reported that the tGSH level was significantly reduced by PQ administration.[27] However, in the NACA-treatment group, tGSH levels were significantly higher than those in both the control and the PQ group (Figure 3A). In cells, it was indicated that tGSH level exactly reflects the reduced GSH level.[20] We have suggested that NACA probably increased GSH biosynthesis by supplying the sulfhydryl groups and increasing the activity of g-glutamyl-cysteine synthetase.[28]

MDA is an important indicator of the level of lipid peroxidation in a cell. The MDA level increases in a cell at the same degree as the oxidative stress increases. The mean level of MDA showed a marked increase following PQ administration as compared with the level of the control group ($P < 0.05$). On the other hand, in the NACA-treated group, as compared with the PQ group, MDA levels decreased in midbrain tissue ($P < 0.01$) (Figure 3B). Palmeira et al. indicated that PQ-induced damage of brain tissue might be related to uncoupled oxidative phosphorylation by lipid peroxidation. In addition, this study also showed that PQ-induced mitochondrial injury was possibly the most important reason for neurocyte death.[29]

Our histological results also confirmed the toxic effect of systemic exposure to PQ. When the PQ-

administrated group was compared with the control group in terms of histological scores, cerebral injuries were observed to have significantly increased ($P < 0.003$) (Figures 4 and 5). However, when the NACA-treatment group was compared with the PQ group, the histological scores had significantly decreased ($P < 0.003$) (Figure 6). In parallel to our results, PQ was shown to induce neuronal oxidative stress damages through the activation of microglial cells.[11] Ossowska et al. also reported that long-term PQ exposure caused about 37% loss of dopamine neurons and cerebral damage in rats.[30]

The rotarod and accelerod tests have been widely adopted and provide a simple drug-free measurement of overall motor insufficiencies in animal models of disease, such as Parkinsonism,[31] and may offer a useful quantitative test to evaluate the efficacy of therapeutic processes.[32] The main findings of the current study, based on the rotarod and accelerod results, were that the PQ group had lost motor coordination whereas the PQ+NACA group had recovered motor coordination functions. The remaining time of animals on the rod in the rotarod test was found to have decreased in the PQ group at speeds higher than 10 rpm when compared to the control group. NACA administration caused amelioration of motor coordination functions. The main important finding of the present study is that NACA-treated rats exhibited an increased length of the time that each animal was able to stay on the rod relative to the PQ group during the rotarod and accelerod tests (Table 2). Similarly, other studies have shown that this technique can provide a very useful test of posture and stepping in brain-damaged rats.[31,32]

Thiol-containing molecules are a class of antioxidants that are especially related to intracellular GSH levels. NACA, a designed form of N-acetylcysteine, has an exchanging carboxyl group that allows passage through cell membranes. In our study, NACA significantly reduced midbrain damage that may have occurred after PQ exposure caused oxidative stress. We believe that increasing the levels of GSH plays an important role in NACA's healing effects. We also think that NACA is a novel and effective antioxidant that is a good candidate for treatment of oxidative stress-related diseases.

3. Experimental

3.1. Chemicals

NACA was provided by Dr Glenn Goldstein (David Pharmaceuticals, New York, NY, USA). Other chemicals were obtained from Sigma (St. Louis, MO, USA) and Fisher Scientific (Fair Lawn, NJ, USA).

3.2. Animals and experimental groups

Thirty adult male Wistar rats, weighing 250–350 g, were provided by the İnönü University Laboratory Animals Research Center. Animals were fed with standard rat chow and tap water ad libitum for 10 days and were randomly divided into three groups of ten each, as follows; control (n = 10), PQ (10 mg/kg) (n = 10), and PQ (10 mg/kg) + NACA (100 mg/kg) (n = 10). The rotarod and accelerod tests were performed 24 h prior to the experiment. NACA was administrated once a day intraperitoneally and 30 min before PQ injection. PQ administration was carried out by subcutaneous injection twice a week (Figure 7). The experiment continued for a period of 28 days. At the end of this period, the rotarod and accelerod tests were performed again. Twenty-four hours after the last NACA injection, the rats were sacrificed under ketamine and xylazine anesthesia (1.2–1.4 g/kg) and the midbrain tissues were quickly removed.

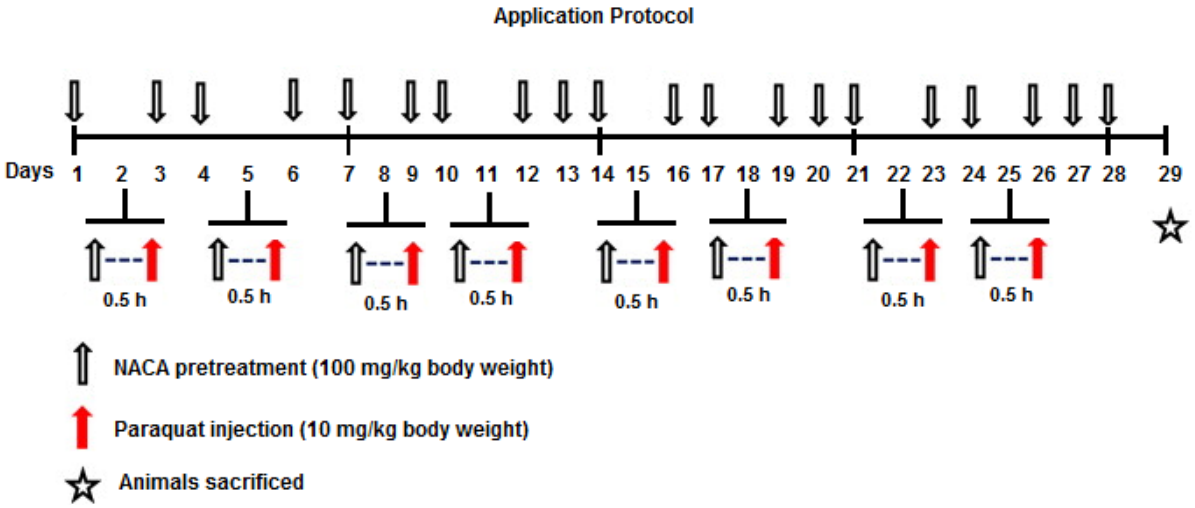

Figure 7. Schematic diagram of experimental application protocol.

3.2.1. Rotarod and the accelerod test

A Rotamex 4/8 system apparatus (Columbus Instruments, Columbus, OH, USA) was used for the rotarod and accelerod tests. First tests were conducted 24 h before the experiment to select the test group. The animals that survived on the rod with the two previous trials were selected for drug testing. The results were expressed as percentage of successful animals remaining on the rod until the shear time (60 s) was reached. The total time spent on the rotarod as well as the fall time and all setup parameters were recorded. The rats were placed on a rotating rod and rotated at different speeds (no more than 5 min for each speed), starting at the slowest speed (5 rpm) and gradually increasing by 5 rpm for 40 rpm. The animal could stay on the rod at a certain spin rate. A test was established with an acceleration of 1 to 79 rpm in 4 to 10 min, respectively. Since several rats were generally tested in the same session, each rat was allowed to rest for about 5 min between different speed tests. This helped to reduce stress and fatigue.

3.2.2. Homogenization

The tissues were homogenized and subjected to ultrasonication (30 s pause for 20 s) in 5 volumes of ice-cold PBS (pH 7.4) in three cycles. The supernatant was obtained after centrifugation (15,000 $\times g$, 10 min, 4 °C) and immediately subjected to an enzyme assay. In addition, for lipid peroxidation analysis, the tissue was homogenized in 1.15% KCl.[33]

3.2.2.1. Determination of enzyme activities

CAT activity was measured at 37 °C following the disappearance of H_2O_2 at 240 nm and was expressed as U/mg protein in the tissue.[34] SOD activity was measured by the xanthine oxidase/cytochrome c method and a unit of activity (U) was the amount of SOD required to cause half maximal inhibition of cytochrome c degradation.[35] BuChE activity was determined by the method of Muller et al.[36] One unit of butyrylcholinesterase (U) was defined as the amount of 1 μmol substrate per minute hydrolyzed. GSH-Px activity was described in an assay coupled with glutathione reductase and by measuring the ratio of NADPH oxidation and was given as μmol of NADPH disappearing per minute per mg of protein.[37]

3.2.2.2. Total glutathione (tGSH) and MDA assay

The tGSH level in samples was determined according to Theodorus et al.,[38] and the amount of tGSH was given as nmol/mg protein. MDA level was measured as described by Buege and Aust,[39] and MDA results were defined as nmol/mg protein in the homogenate.

3.2.2.3. Protein assay

The Bradford method was used in the determination of protein levels of the tissue samples.[40]

3.3. Histological examination

Midbrain tissue sections were fixed in 10% formalin solution. Following routine tissue processing, paraffin-embedded specimens were cut into 5-μm-thick sections for staining with hematoxylin and eosin. A Leica DFC 280 light microscope and the Leica Q Win Plus Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK) were used for morphometric analysis. The obtained preparations were analyzed according to perinuclear and perivascular edema, microglia increase, neuronal loss, morphological changes, and loss of Nissl bodies. The following semiquantitative scoring was used: 0 = no cerebral damage, 1 = $<$10% of the cerebral region was damaged, 2 = 10%–25% of the cerebral region was damaged, 3 = 25%–50% of the cerebral region was damaged, 4 = 50%–75% of the cerebral region was damaged, and 5 = $>$75% of the cerebral region was damaged.

3.4. Statistical analysis

Biochemical data were expressed as the mean $\pm$ SD by using the GraphPad Prism 5.0 statistical package program. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to determine the significance between the control and experimental groups. Histological results were statistically analyzed by the Kruskal–Wallis H test and were considered to be significant when $P < 0.05$. The Mann–Whitney U test was also used for cooperation of the groups. The statistical analyses of histological results and rotarod and accelerod test data were performed using SPSS 12.0.

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