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Research Article

Neuroprotective role of n-acetylcysteine (NAC): countering doxorubicin neurotoxicity via TH, Nurr1, and iNOS expression

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ABSTRACT

Chemotherapy is an effective treatment for cancer, but it can cause cognitive disorders broadly referred to as "chemobrain." One of the most commonly used chemotherapeutics, doxorubicin (DOX), has been associated with the potential for brain damage and cognitive dysfunction. N-acetylcysteine (NAC) has been identified as a potential brain protector with antiapoptotic, antioxidant, and anti-inflammatory effects. The objective of this study was to investigate the potential protective effect of NAC against DOX-induced brain damage. Female Wistar albino rats were randomly assigned to one of three groups: control, DOX, or NAC prophylaxis. Brain samples were collected for histopathological and immunohistochemical analyses, with a particular focus on regions that are crucial for cognition and memory. The DOX group exhibited significant histopathological changes, including neuronal shrinkage, degeneration, and necrosis in the striatum, hippocampal region, and cerebral cortex. Immunohistochemical analysis revealed the presence of neuroinflammation and neurodegeneration, with an increase in inducible nitric oxide synthetase (iNOS) immunopositivity. Administration of NAC effectively reduced iNOS immunopositivity, neuronal damage, degeneration, and necrosis in the prophylaxis group. Among the brain regions examined, the prophylaxis group demonstrated the most effective protection in the hippocampal region. Therefore, NAC has the potential to protect against or alleviate DOX-induced cognitive impairments.

INTRODUCTION

Cancer constitutes a significant global health concern, with an estimated 16% of global deaths annually attributed to the disease (Sung et al., 2021). Currently, combinations of surgical intervention, chemotherapy, radiotherapy, targeted therapy, and immunotherapy are used to combat cancer. Among these methods, chemotherapy, either alone or in combination with other treatments, is commonly preferred and considered the most effective (El-Hussein et al., 2020). Doxorubicin (DOX), an anthracycline chemotherapy drug, was originally isolated from *Streptomyces peucetius* in the 1970s. Since then, it has become a standard treatment for a range of cancers, including ovarian, breast, stomach, lung, non-Hodgkin and Hodgkin lymphomas, sarcoma, multiple myeloma, and pediatric cancers (Rau, 1992). DOX is frequently utilized in the management of mammary tumors (Hernandez-Aya and Gonzalez-Angulo, 2013). However, despite its effectiveness, some studies have indicated that patients receiving DOX therapy may develop cognitive problems, commonly referred to as "chemobrain" (Raffa et al., 2006; Andryszak et al., 2017). DOX can cause neurotoxicity by crossing the blood-brain barrier directly or by triggering neuroinflammation (Du et al., 2021). It also inhibits DNA topoisomerase II, which releases free radicals, including superoxide and hydrogen peroxide, resulting in tissue damage through oxidative stress (Cheruku et al., 2017).

In contrast, N-acetylcysteine (NAC) is capable of traversing the blood-brain barrier and augmenting the concentration of glutathione (GSH), rendering it an efficacious antioxidant as it diminishes the levels of free oxygen radicals (Dean et al., 2011; Prakash et al., 2014). NAC has been utilized in research related to neurodegenerative disorders, such as Parkinson disease, and in cases where neurotoxic agents are used in chemotherapy to reduce or prevent neuronal damage (Gil-Martínez et al., 2018; Abdel-Wahab et al., 2019; Kitamura et al., 2021).

The dopaminergic system is associated with learning, motor movements, memory, and the reward mechanism in the mammalian brain (Mehta and Riedel, 2006). Nuclear receptor-related protein 1 (Nurr1) and tyrosine hydroxylase (TH) are key players in the development and functionality of dopaminergic neurons (Perlmann and Wallén-Mackenzie, 2004).

The objective of this study is to investigate the histopathological and immunohistochemical effects of NAC on brain damage induced by DOX in key regions involved in cognitive and memory processes, namely the cerebral cortex, hippocampus, substantia nigra (SN), striatum, and ventral tegmental area (VTA). This study also aims to investigate the potential prophylactic effect of NAC. An immunohistochemical evaluation was conducted to assess the expression levels of Nurr1, a crucial factor in dopaminergic neuron development and function, and TH, which is involved in dopamine synthesis

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by dopaminergic neurons. The presence and expression levels of inducible nitric oxide synthetase (iNOS), which is associated with neuroinflammation and neurodegeneration, were also evaluated.

MATERIALS and METHODS

Animals

All experimental procedures and applications were approved by Kirikkale University Huseyin Aytemiz Experimental Research and Application Center, Experiments Ethics Committee (report number: 23/06/25). The experiments involved the use of 24 female Wistar albino rats with a weight range of 200-250 g. The rats had unrestricted access to water and were fed a standard chow diet. The rats were housed in a facility with a 12-hour light/dark cycle and controlled temperature (25±2 °C) and relative humidity (42±5%).

The 24 rats were randomly allocated to three groups, each comprising eight animals. The control group was administered saline intraperitoneally. The second group received a single intraperitoneal dose of 10 mg/kg DOX (Adriamycin, Deva®, Turkey) after 20 days. The third group received daily intraperitoneal administration of 100 mg/kg NAC for 20 days as prophylaxis, followed by a single dose of 10 mg/kg DOX on day 20.

Tissue Collection and Histopathological Analysis

On the 21st day of the study, all groups were euthanized using a combination of 10 mg/kg xylazine (Xylazinbio 2%, Bioveta®, Czech Republic) and 200 mg/kg ketamine (Ketasol 10%, RichterPharma®, Austria). Following the removal of brain samples, they were fixed in a 10% buffered formaldehyde solution for 72 hours to facilitate histopathological and immunohistochemical examinations. Once the tissue collection was completed, a routine pathological examination was conducted and the tissues were trimmed and embedded in paraffin wax. After obtaining paraffin blocks, serial sections with a thickness of 4-5 µm were taken for both immunoperoxidase tests and histopathological examinations. Hematoxylin and eosin staining was applied to all tissue sections, and subsequent observations were made using a light microscope. Photomicrographs were acquired using an Olympus BX51 microscope (Japan).

Immunohistochemical Examination

Serial tissue sections were obtained and they were labeled using commercial primary antibodies against Nurr1 (Santa Cruz (N-20)/1/100, USA), TH (Santa Cruz (H-196)/1/200, USA), and iNOS (Sigma Aldrich/1/200, USA). Immunohistochemical staining was conducted using a commercial immunoperoxidase kit (Thermo, USA) in accordance with the manufacturer's instructions. Chromogen visualization was achieved using 3-amino-9-ethylcarbazole (AEC), while Mayer's hematoxylin was used as the counterstain. Negative controls were also included, following the same staining procedure, with nonimmunized rat serum replacing the primary antibody.

Following the placement of tissue sections on electrostatic adhesive slides, dewaxing was performed using xylene, followed by hydration using graded alcohols. The sections underwent antigen retrieval with a 30-min microwaving process in citrate buffer (pH 6.0). To prevent endogenous peroxidase activity, a solution of 3% H_2O_2 in methanol was applied for 15 min. Furthermore, nonspecific labeling was prevented by preincubation with normal goat serum for 10 min. The slides underwent a series of treatments with the appropriate primary antibody (Nurr1, TH, or iNOS), followed by the secondary antibody and then streptavidin. Overnight incubation at 4 °C was employed for the primary antibody. This was followed by a 30-min application of the secondary antibody and an additional 30-min incubation with streptavidin. Subsequently, AEC was utilized as the chromogen and the slides were sealed with an aqueous mounting medium. Microphotographs were captured and examined using an Olympus BX51 microscope (Japan).

Data and Statistical Analysis

Utilizing ImageJ (USA) image analysis software, positive staining density was quantified with the aid of a 40× objective. The measurement process involved capturing the integrated optical density of all immunopositive stains, and the subsequent calculation allowed the determination of the average area of Nurr1, TH, or iNOS immunopositivity relative to the total area, employing ImageJ software.

Data were shown as means ± standard deviations (SDs), and comparisons between groups for histopathological and immunohistochemical analyses were performed with the Mann-Whitney U test. GraphPad Prism version 9.0 (GraphPad Software, USA) was employed for all statistical analyses and graph preparations with the significance level set at $p<0.05$.

RESULTS

Histopathology

Comparing the DOX-induced group with the control group, significant histopathological changes were observed. Particularly in the hippocampal region, shrinkage, hyperchromasia, degeneration, and necrosis were found in pyramidal neurons in the dentate gyrus (DG) and cornu ammonis-3 (CA3) regions of the hippocampus, neurons in the frontal region of the cerebral cortex, and neurons in the nucleus accumbens (ACB) and caudoputamen (CP) in the striatum. The nuclei of necrotic neurons were generally pyknotic and the Nissl bodies in the neuronal cytoplasm were lost (Figures 1A, B, and C). In contrast, the control group showed neurons with nuclei located in the center, prominent nucleoli in the nuclei and Nissl granules in the cytoplasm, and basophilic-stained nuclei and eosinophilic-stained cytoplasm (Figures 1D, E, and F). The DOX-induced group exhibited perineuronal edema and satellitosis around the affected neurons (Figures 1A, B and C). Congestion was observed in the blood vessels in the cerebral cortex, striatum, thalamus, and midbrain (Figures 1B and 1C).

The observations indicated that neurons in the hippocampal region exhibited less damage in the NAC prophylaxis group compared to the group induced with DOX. In addition, the pathological changes of DOX toxicity were reduced in the NAC prophylaxis group, including the reduction of neuronal degeneration and necrosis and even the disappearance

of perineuronal edema (Figures 1G, H, and I). In contrast, in the NAC prophylaxis group, vascular changes were similar to those observed in the DOX-induced group and there was no reduction in vascular congestion, although other findings more closely resembled those of the control group.

Immunohistochemistry

Comparing the DOX-induced group to the other groups, it was observed that the iNOS immunopositivity in the DOX-induced group was statistically significant compared to that of the others ($p<0.0001$) (Figures 2A, D, and H; Table 1). iNOS immunopositivity was mainly detected in selected hippocampal regions, such as CA1, CA3, and DG, as well as in the striatum, thalamus, midbrain, SN, and VTA and in glial cells and astrocytes. Furthermore, a notable disparity was identified between the control group and the prophylaxis group ($p<0.05$) (Figures 2A, E, H, and I; Table 1). The prophylaxis group exhibited iNOS immunopositivity closest to that of the control group and no statistically significant difference was noted between these two groups (Figures 2E, H, and I). In the prophylaxis group, iNOS immunopositivity was not prominent in the neurons, especially in the hippocampal neurons; rather, it was observed mainly in glial cells in the cerebral cortex, striatum, SN, and VTA.

The proportion of Nurr1-immunopositive cells was consistently high in both the control and prophylaxis groups. In contrast, it was significantly reduced in the DOX-induced group in comparison to the other two groups ($p<0.001$) (Figures 2B, F, J, and L; Table 1). Nurr1 immunopositivity was observed in neurons and glial cells to varying degrees, mainly in the SN, VTA, striatum, and hippocampus, with neuronal immunopositivity being more prominent (Figure 2B, F, J, and L).

The TH immunopositivity results were found to be quite similar to those for Nurr1 immunopositivity. Accordingly, the expression of TH was markedly decreased in the DOX-induced group compared to both the prophylaxis and control groups ($p<0.001$) (Figures 2C, D, G, and K; Table 1). The intense immunopositivity observed in the control and prophylaxis groups was notable in neurons in the SN, VTA, and striatum.

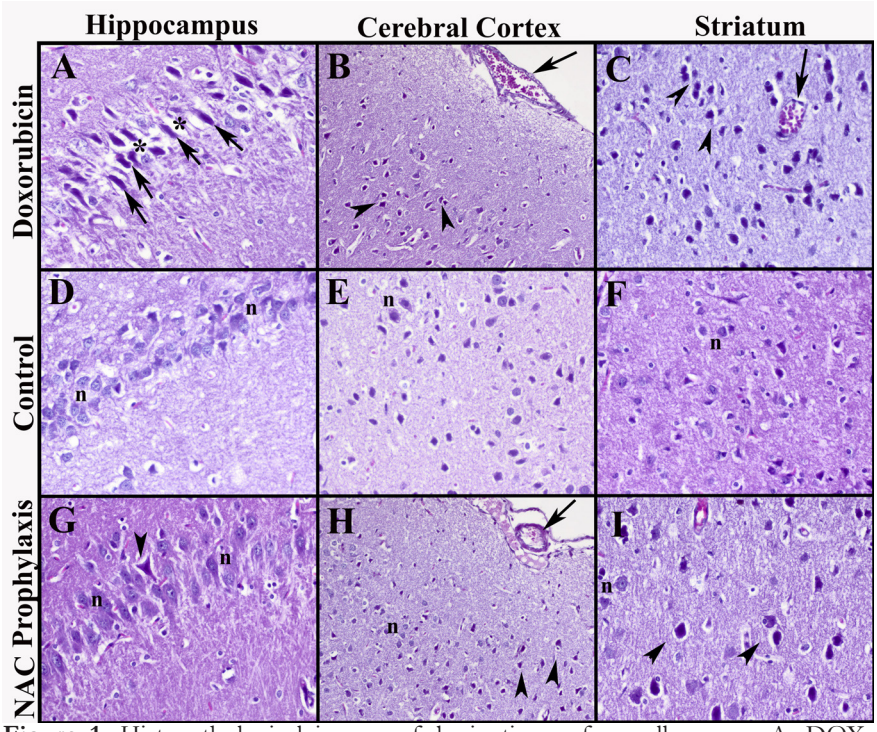

Figure 1. Histopathological images of brain tissues from all groups. A. DOX-induced group. Degeneration and hyperchromasia (arrow) in pyramidal neurons in the hippocampal CA3 region and perineuronal edema (asterisk). Hematoxylin and eosin (H&E) staining; 400× magnification. B-C. DOX-induced group. Shrunken and degenerative neurons (arrowhead) and congested blood vessels (arrow) in the cerebral cortex and striatum, respectively. H&E staining; 100× and 200× magnification, respectively. D-E-F. Control group. Normal brain structure characterized by healthy neurons (n) with central nucleus and normal nuclei and cytoplasmic Nissl bodies in the hippocampal CA3 region, cerebral cortex, and striatum. H&E staining; 400×, 100×, and 200× magnification, respectively. G. NAC prophylaxis group. Normal pyramidal neurons (n) and degenerative, hyperchromatic neurons (arrowhead) in the hippocampal CA3 region. H&E staining; 400× magnification. H-I. NAC prophylaxis group. Degenerative, shrunken neurons (arrowhead) and normal neurons (n) together with congested blood vessels (arrow) in the cerebral cortex and striatum. H&E staining; 100× and 200× magnification, respectively.

Table 1. Statistical data on the immunopositivity of iNOS, Nurr1, and TH

	iNOS		Nurr1		TH		p
	n	Mean±SD	n	Mean±SD	n	Mean±SD	
Control	8	0.089±0.073 ^{A,a}	8	3.191±0.282 ^{B,b}	8	4.042±0.151C ^{C,b}	<0.001
DOX	8	5.437±1.018 ^{c}	8	1.005±0.310 ^{A,a}	8	2.569±0.595 ^{B,a}	<0.001
Prophylaxis	8	1.082±0.208 ^{A,b}	8	3.389±0.209 ^{B,b}	8	3.986±0.112 ^{C,b}	<0.001
p		<0.001		<0.001		<0.001	

A, B, C: There is a statistical difference between groups with different letters in the rows. a, b, c: There is a statistical difference between groups with different letters in the columns.

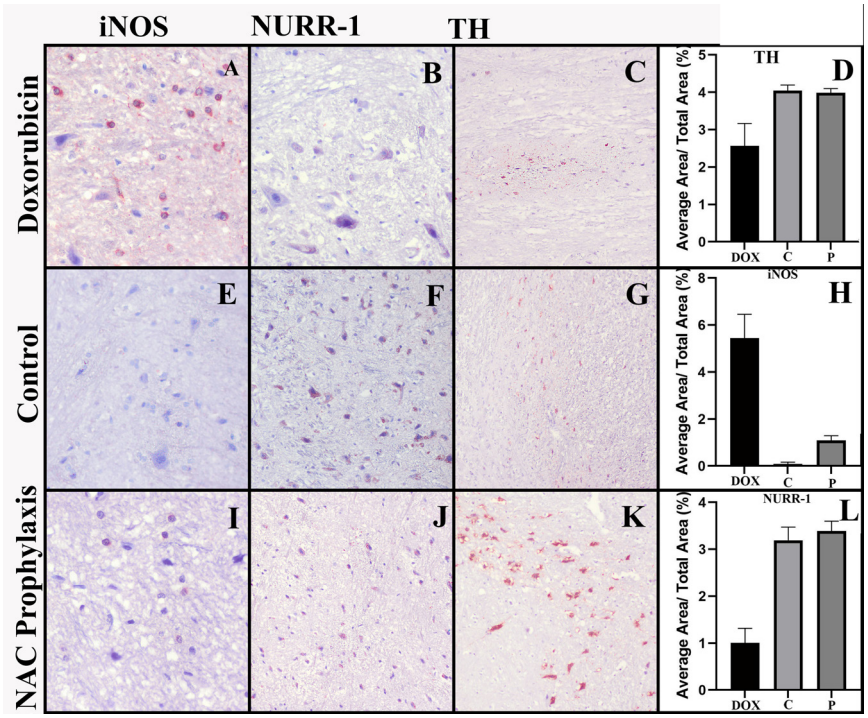

Figure 2. Immunoreactivities of anti-iNOS, anti-Nurr1, and anti-TH in all groups. A-B-C. DOX-induced group. Intense anti-iNOS immunoreactivity in the striatum and mild Nurr1 and TH immunopositivity in the SN and VTA, respectively. IHC. AEC chromogen with hematoxylin counterstain; magnification of 200×, 200×, and 100×, respectively. D. Comparative graph of anti-TH immunoreactivity in all groups. x: Average area of immunopositivity of TH/Total area percentages; y: Groups. E-F-G. Control group. Very mild anti-iNOS immunoreactivity in the striatum and strong Nurr1 and TH immunopositivity in the SN and VTA, respectively. IHC. AEC chromogen with hematoxylin counterstain; magnification of 200×, 200×, and 100×, respectively. H. Comparative graph of anti-iNOS immunoreactivity in all groups. x: Average area of immunopositivity of iNOS/Total area percentages; y: Groups. I-J-K. NAC prophylaxis group. Mild to moderate anti-iNOS immunoreactivity in the striatum and significantly strong Nurr1 and TH immunopositivity in the SN and VTA, respectively. IHC. AEC chromogen with hematoxylin counterstain; magnification of 200×, 200×, and 100×, respectively. L. Comparative graph of anti-Nurr1 immunoreactivity in all groups. x: Average area of immunopositivity of Nurr1/Total area percentages; y: Groups.

DISCUSSION

DOX-induced neurotoxicity arises from a number of mechanisms, including DNA damage, apoptosis induction, reactive oxygen species (ROS) production, neurotransmitter downregulation, and synaptic dysplasia (Tangpong et al., 2011; Kitamura et al., 2014; Habbas et al., 2015; Lim et al., 2016; Keeney et al., 2018). In particular, it has been shown that DOX causes impairment of the mitochondria in the hippocampus, which in turn results in elevated levels of reactive ROS (Park et al., 2018). Furthermore, Kwatra et al. (2016) found a marked reduction in serotonin and dopamine levels in rats treated with DOX, as quantified spectrophotometrically following homogenization

of the hippocampal region. The results of the histopathological analysis revealed significant alterations in the brain tissues of the DOX-induced group in comparison to the control group. These changes were observed to encompass shrinkage, hyperchromasia, degeneration, and necrosis in diverse brain areas, including the striatum, cerebral cortex, and hippocampus. In contrast, the histopathologic changes, and particularly those in the hippocampal region, were less pronounced in the NAC prophylaxis group. The DOX-induced group also exhibited perineuronal edema and vascular congestion and showed significant immunopositivity for iNOS, indicating the presence of neuroinflammation and neurodegeneration.

Chemotherapeutic drugs, especially DOX, can lead to cognitive dysfunction ranging from mild to severe, affecting approximately 75% of cancer patients (Ahles and Saykin, 2007). Such cognitive dysfunction may include memory and attention problems, difficulties in multitasking, and emotional and behavioral changes (Seigers and Fardell, 2011). These clinical findings suggest that regions of the brain, particularly those with dopaminergic neurons, may be affected. In our study, both histopathological and immunohistochemical analyses demonstrated the involvement of pyramidal neurons located in the hippocampal regions of CA1, CA3, and DG, as well as neurons in the striatum, frontal cortex, VTA, and SN in DOX-induced rats.

Cognitive impairment forms the basis of many disorders, including neurodegenerative diseases, mood disorders, anxiety, chronic pain, and psychosis. Studies have directly associated oxidative stress with cognitive impairments (Skvarc et al., 2017). However, it is believed that NAC can reverse cognitive impairments by increasing GSH levels (Cao et al., 2012). In addition, NAC has been demonstrated to modulate a number of biological processes, including oxidative stress, apoptosis, mitochondrial dysfunction, and neurotransmitters such as glutamate and dopamine. These effects have been observed both directly and indirectly (Frye et al., 2018). In this study, NAC administered prior to DOX administration was shown to have no effect on vascular changes in the DOX-treated group. However, it prevented neuronal damage at a high rate. Immunohistochemical analysis further confirmed the protective effects of NAC. Conversely, NAC administration reduced iNOS immunopositivity, particularly in glial cells, suggesting its anti-inflammatory properties. The prophylaxis group had the immunopositivity levels closest to those of the control group, indicating the potential preventive effects of NAC against DOX-induced neuroinflammation.

While studies have investigated the effects of DOX on the nervous system, research on the prophylactic effect of NAC against this damage remains limited. Previous studies were limited to histopathology, the damage was assessed by blood parameters and serum biochemistry, and the damaged dopaminergic neurons and mechanisms of action were not shown in the damaged tissue (Mohammed et al., 2019).

Research has demonstrated that Nurr1 exhibits anti-inflammatory properties and promotes the development of dopaminergic neurons from neural stem cells (Chen et al., 2018). It is also known that the most commonly used markers to demonstrate the presence of dopaminergic neurons immunohistochemically are TH and DAT, and TH is involved in dopamine synthesis (Huot et al., 2007). Furthermore, studies have revealed that NAC plays dual roles in the hippocampal CA1 and DG regions, acting as both an anti-inflammatory and an antiapoptotic agent (Song et al., 2019; Fan et al., 2020). In the present study, the DOX-induced group showed significantly lower immunopositivity for both Nurr1 and TH compared to the control group. Furthermore, the administration of NAC effectively preserved the expression levels of Nurr1 and TH, suggesting its potential in preserving dopaminergic neurons and neurotransmitter synthesis, especially in the VTA, SN, and striatum. Additionally, this study has demonstrated that NAC has prophylactic properties and that its effects are not limited to the hippocampal region but also extend to the striatum, VTA, and SN.

NAC administration also decreased iNOS immunoreactivity, demonstrating its anti-inflammatory effect, and resulted in the dopaminergic system approaching levels similar to those of the control group.

CONCLUSION

The study has demonstrated the favorable impact of NAC on DOX-induced brain damage. NAC administration reduced neuronal damage, degeneration, and necrosis in regions of the brain crucial for cognitive and memory processes. Furthermore, NAC exhibited anti-inflammatory properties, as evidenced by decreased iNOS immunopositivity, and preserved the expression levels of Nurr1 and TH, which are crucial for dopaminergic neuron function. These findings indicate that NAC may have a prophylactic effect in preventing the cognitive impairments associated with DOX treatment. Furthermore, the utilisation of varying doses of DOX in forthcoming studies may prove advantageous in terms of comparative assessment.

DECLARATIONS

Ethics Approval

Kirikkale University Huseyin Aytemiz Experimental Research and Application Center, Experiments Ethics Committee 23/06/25 Number Ethics Committee Decision

Conflict of Interest

The authors declare that they have no competing interests

Consent for Publication

Not applicable

Author contribution

Idea, concept and design: TA, MÇ

Data collection and analysis: GY, SBK, ÖD, RK

Drafting of the manuscript: TA

Critical review: MÇ, GY, SBK, ÖD, RK

Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to [reasons such as privacy

concerns]. Data are available from the corresponding author upon reasonable request.

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