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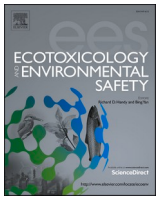

Modulatory mechanisms of copper^{II}-albumin complex toward N-nitrosodiethylamine-induced neurotoxicity in mice via regulating oxidative damage, inflammatory, and apoptotic signaling pathways

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ABSTRACT

N-nitrosodiethylamine (ND) is an extremely toxic unavoidable environmental contaminant. Copper^{II}-albumin (CuAB) complex, a newly developed Cu complex, showed antioxidant and anti-inflammatory potential. Hereby, we explored the plausible neuroprotective role of CuAB complex toward ND-evoked neurotoxicity in mice. Twenty-four male mice were sorted into 4 groups (6 mice each). Control group, mice were administered oral distilled water; and CuAB group, mice received CuAB complex at a dose of 817 μg/kg orally, three times weekly. In ND group, ND was given intraperitoneally (50 mg/kg body weight, once weekly for 6 w). CuAB+ND group, mice were administered a combination of CuAB and ND. The brain was quickly extracted upon completion of the experimental protocol for the evaluation of the oxidative/antioxidative markers, inflammatory cytokines, and histopathological examination. Oxidative stress was induced after ND exposure indicated by a reduction in GSH and SOD1 level, with increased MDA level. In addition, decreased expression of SOD1 proteins, Nrf2, and 5-HT mRNA expression levels were noticed. An apoptotic cascade has also been elicited, evidenced by overexpression of Cyt c, Cl. Casp 3. In addition, increased regulation of proinflammatory genes (TNF-α, IL-6, iNOS, Casp1, and NF-κB (p65/p50); besides, increment of protein expression of P-IKBα and reduced expression of IKBα. Pre-treatment with CuAB complex significantly ameliorated ND neuronal damage. Our results recommend CuAB complex supplementation because it exerts neuroprotective effects against ND-induced toxicity.

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1. Introduction

N-Nitroso compounds are renowned carcinogens that are produced by biochemical interactions amongst nitrites and secondary amines, and pose a major health threat (Siddique and Ali, 2016; Tong et al., 2010). N-nitrosodiethylamine (ND) is one of N-nitroso alkyl compounds ($C_2H_6N_2O$) that are identified in contaminated water (high-nitrates water), foods (such as preserved fruits and juices as well as dairy and meat products) and tobacco smoke. Moreover, some cosmetics and the degradation of some pharmaceutical drugs can also lead to the generation of nitroso compounds (Ali et al., 2016; Cahyani et al., 2021). ND intoxication has mutagenic, carcinogenic, and genotoxic consequences on various organs (Hebels et al., 2009). Besides the common hepatotoxicity of ND, the brain is one of the organs that are significantly affected via either direct neurotoxic injury or hepatotoxicity contributed to ND-induced neurodegeneration (Tong et al., 2010).

The prime mechanism of ND-induced toxicity is attributed to its metabolic biotransformation by cytochrome p450 enzymes into reactive electrophilic ethyl radicals ($CH_3CH_2^+$) which is a deleterious and highly active metabolite (Hebels et al., 2009). These ethyl radicals fasten the cellular macromolecules, enhance oxidative stress, and generate tremendous amounts of reactive oxygen species (ROS) that leads to cellular prejudice including DNA mutations (alkylating guanin's N-7), lipid peroxidation (LPO), damage to proteins, modifications to intracellular signaling cascades, even alterations in gene expression (Ali et al., 2016; Singh et al., 2023; Tong et al., 2010). Hence, oxidative distress is a crucial mechanism underlying ND-induced toxicity (Shaarawy et al., 2009).

Copper (Cu) is a natural element necessary to ensure cell homeostasis in the central nervous system (Mujafarkani et al., 2023; Santamaría et al., 2003). Since the majority of Cu is almost bound entirely to proteins, it functions as a cofactor for various neuroprotective biomolecules and proteins (Duncan and White, 2012) such the copper/zinc-superoxide dismutase (namely Cu/Zn-SOD or SOD1) and ceruloplasmin enzymes, which exemplify prime antioxidant systems in the brain (Alcaraz-Zubeldia et al., 2001; Santamaría et al., 2003). Based on the notion that free Cu ions delivery is hard to regulate, poisoning from excess intake can occur. Organic Cu^{II} complexes are thought to be a promising alternative because they can be chemically altered to regulate their delivery with increased cell-penetrating activity (Kanemaru et al., 2011). Cu complexes are created via bonding of Cu by an inorganic skeleton (Alcaraz-Zubeldia et al., 2001). A cumulative evidence reported that the biologically active Cu complexes revealed several pleiotropic biological effects (Duncan and White, 2012) due to their superb antioxidant, and anti-inflammatory properties (Hegazy et al., 2019).

Previous studies suggested the neuroprotective potential of Cu complex against certain neurotoxicants such as quinolinic acid (Santamaría et al., 2003), and tetrahydropyridine derivatives (Alcaraz-Zubeldia et al., 2001; Rubio-Osornio et al., 2009), and neurodegenerative disorders such as Alzheimer (Donnelly et al., 2008), Parkinsonism (Alcaraz-Zubeldia et al., 2009) and Huntington's disease (Nguyen et al., 2005; Smriti et al., 2023). Cu^{II}-albumin (CuAB) complex is a novel complex, we have previously used; thereby, it has shown promising antioxidant and anti-inflammatory properties against aflatoxin-induced hepatorenal cellular damage (Abo-Hiemad et al., 2022b, 2022a). We exploited the high affinity of Cu to the albumin to create CuAB complex compounds. Besides, the half-life of Cu is altered by binding to albumin, as a transport vehicle in plasma; consequently, albumin is deemed as a promising drug delivery approach (May et al., 2021).

To the best of our knowledge, a literature survey divulges that, to date, there is no study has particularly focused on the potential mitigating effect of novel CuAB complex supplementation to reduce oxidative and inflammatory brain damage induced by ND exposure. Furthermore, information about ND-triggered neurotoxicity (neuronal oxidative stress, inflammation, and apoptosis) is still scarce. Thereby, the current study investigates the neuro-modulatory effects of a novel CuAB complex supplementation on ND-induced neuropathy in mice via regulating the oxidative/antioxidative balance, inflammatory response, and cell death-related signaling pathways.

2. Materials and methods

2.1. Sources of chemicals

ND was obtained from Sigma Aldrich (CAS NO: 62–75–9). The CuAB complex was offered by Professor Ahmed Y. Nassar (Professor of Biochemistry, Faculty of Medicine, Assiut University, Egypt) as a patent coordination treaty (PCT) in the International Bureau of World Intellectual Property Organization (WIPO), Geneva, Switzerland/World Organization (WO) 2008/028497. Briefly, CuAB complex was prepared by adding a mixture of NaCl and Cu salt solution to a pure homogenous mixture of egg white peptides at an adjusted pH. However, ND was freshly prepared in distilled water (1:100 v/v).

2.2. Animals and experimental protocol

Male Wister albino mice weighing 25 g, aged 4–5 w old, were employed for conducting this experiment. Mice were purchased from the Faculty of Veterinary Medicine, Assiut University, Egypt's Centre for Laboratory Animals. Before starting the experiment, mice were confined to comfy standard conditions for two weeks. They were housed in three per cage under the following conditions: temperature (21 ± 2 ℃), humidity (60 ± 5%), and light/dark (12/12 h) cycle. All animals were given a regular baseline ration as well as free access to water. After becoming acclimated, experimental mice were sorted to 4 equal sets (6 mice each). Control group; mice were administered oral distilled water (no treatment was added); CuAB group, animals received CuAB, orally, at a dose of 817 μg/kg body weight, three times a week, starting from the 2nd w till experiment's termination after 13 w (Taha et al., 2022). ND group, which was designated as a positive toxic group, where, mice were treated with ND, given intraperitoneally (50 mg/kg body weight, once weekly for 6 w, beginning the 2nd to the 13th w) (Saber et al., 2018; Younis et al., 2019). In addition, CuAB+ND group, mice were administered both CuAB and ND at the same abovementioned dosage; thereby, CuAB was given 6 h before ND exposure.

2.3. Tissue sampling

Upon completion of the experimental protocol, all mice were placed in an induction chamber (open-drop technique) for the conventional inhalation anesthetic protocol; thereby, mice died under prolonged exposure to 4% isoflurane. The brain was quickly extracted, and one part was preserved in formalin for subsequent paraffin embedding and sectioning. While the other fresh parts of fresh brain specimens were utilized for isolation of RNA/protein and preserved at− 80 ℃ or for a subsequent oxidative cascade marker investigation, the remaining fresh tissue pieces were stored at− 20 ℃.

2.4. Animal behavior and locomotor activity investigation

During the experiment, the mice behavior and motor activity were investigated. Mice movements (spontaneous horizontal motor activity, vertical activity, and stereotypic movements) and total travel distance were recorded using the Panlab Infrared (IR) Actimeter machine (Panlab, Barcelona, Spain). Each mouse was positioned in the middle of a clear acrylic cage that possessed an opaque black floor. It was connected to a photoelectric cell and movements were monitored by infrared beams. The actimeter infrared frames were adjusted according to animal size with the lower frame at 3 cm high from the box floor to record horizontal movements and the upper frame at 5 cm high from the box floor to record vertical movements. The activity of each mouse was

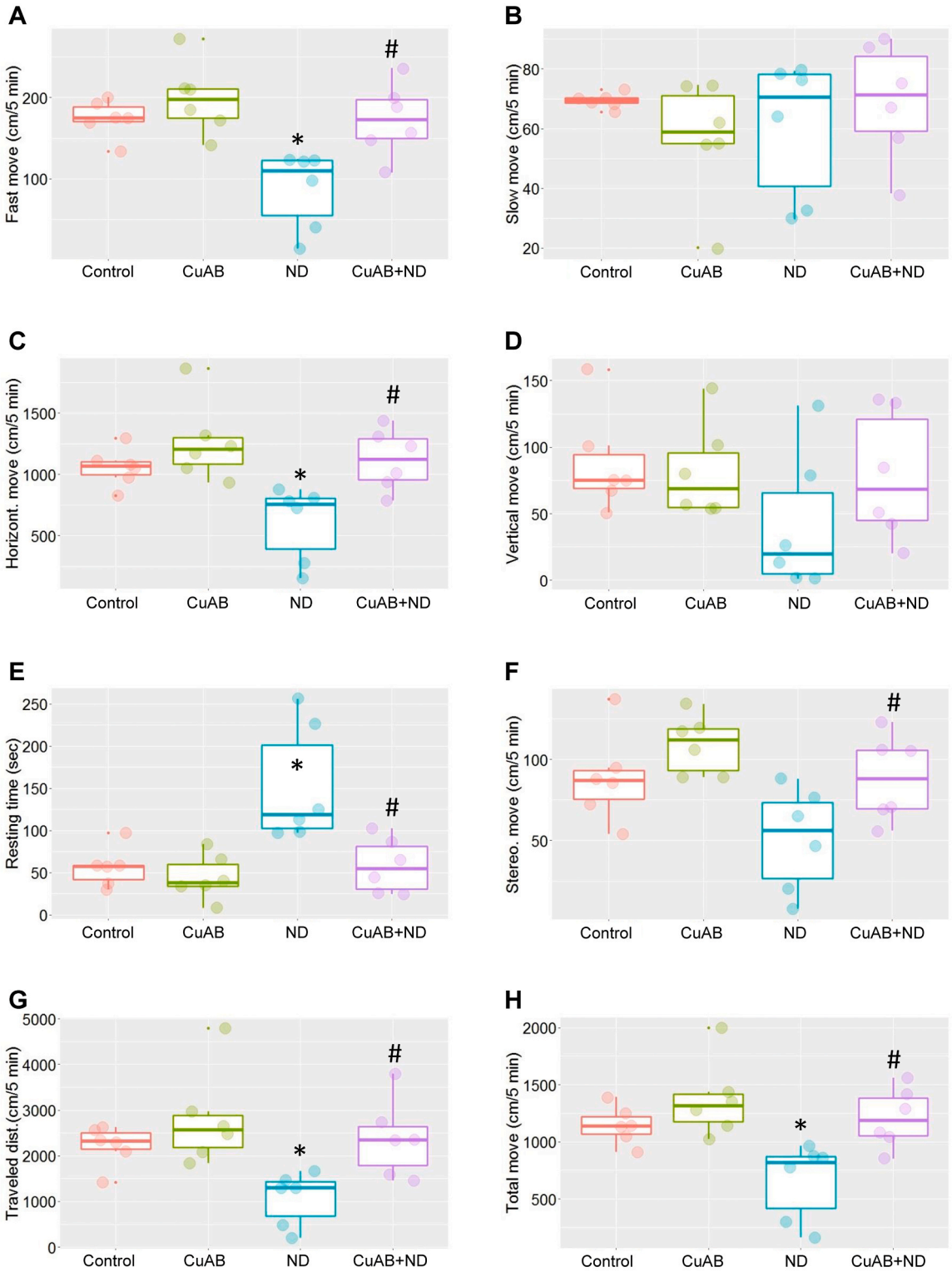


Fig. 1. Box-and-whisker plots of mice behavior and motor activity. (A) fast movement (B) slow movement. The change in horizontal (C) and vertical motor (D) activity, (E) resting time, (F) stereo movement, (G) travel distance, (H) total movement. $P < 0.05$; *ND vs Control; # CuAB+ND vs ND (n = 6).

acquired for 5 min. The animal was resting when the movements were less than 2 times/min, slow when movements were 2–5 times/min, or fast when the animal moved > 5 times per min. All recordings were registered in a dark isolated room (Abedi et al., 2013; Yamaguchi et al., 2017). All actimeter testing was done between 8:30 AM and 2:00 PM in a separate room. The experimental protocol was divided into 2 stages: first, the animals underwent a habituation phase, during which their spontaneous locomotor activity was monitored twice daily for 10 min over the course of 3 days in a row. On the 4th day, during the 2nd 10 min session, just the locomotor activity was recorded, which had been utilized to establish the daily habit, and was employed in data analysis.

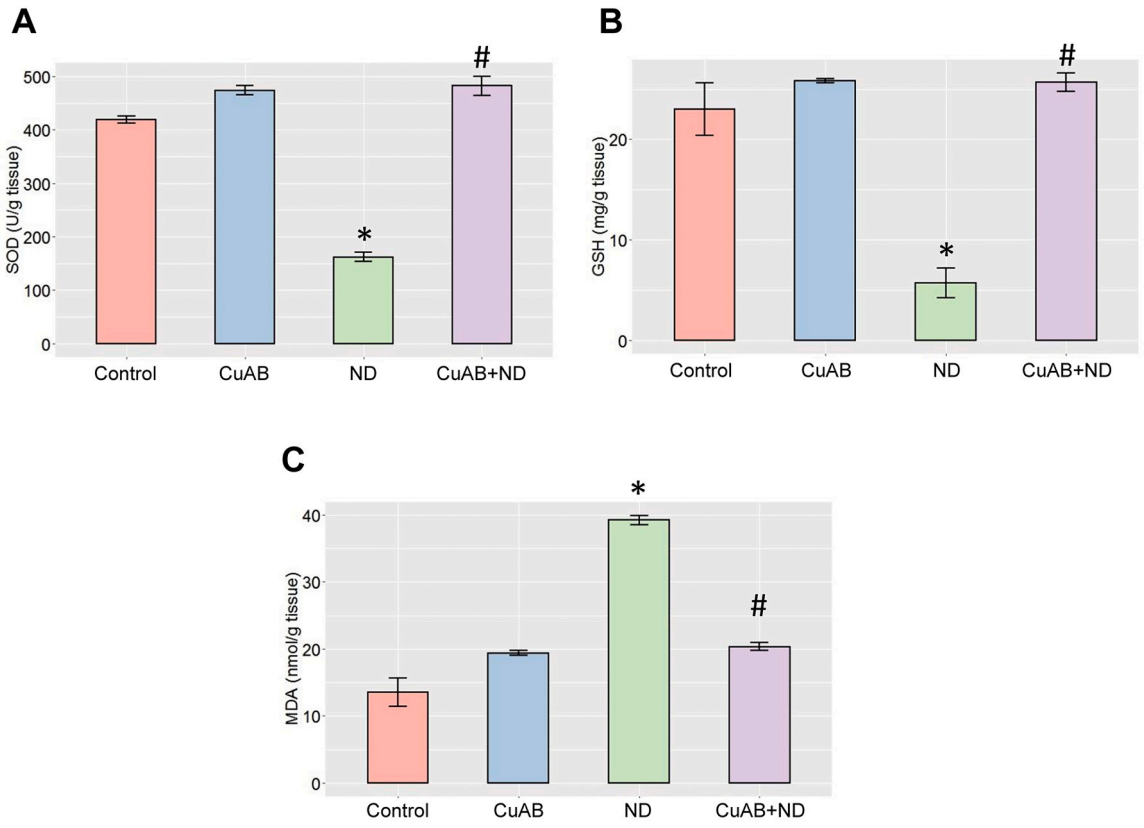


Fig. 2. Bar plot panel of oxidative stress markers in plasma upon ND and/or CuAB treatment. (A) SOD, superoxide dismutase, (B) GSH, reduced glutathione; (C) MDA, malondialdehyde. $P < 0.01$; *ND vs Control; # CuAB+ND vs ND (n = 4).

2.5. Oxidative cascade markers

The levels of Malonaldehyde (MDA; Cat. No. MD2529), SOD1 (Cat. No. SD2521), and reduced glutathione (GSH; Cat. No. GR2511) were analyzed in brain tissue using commercial kits from (Biodiagnostics Co, Giza, Egypt) adhering to the manufacturer's directions.

2.6. RT-qPCR

Brain tissues were used to extract total RNA utilizing QIAzol Lysis Reagent (QIAGEN®, QIAzol™, USA) according to the manufacturer's recommendations. RNA concentrations and qualities (OD260/OD280 ≈ 2.0) were determined. The first strand cDNAs were generated using 1 μg RNA of each sample and oligo (dT) primers (PrimeScript™, TaKaRa Bio Inc, CA, USA) were utilized as templates for RT-PCR analysis based on the manufacturer's protocol. Relative quantification of gene expression was calculated relative to β-actin gene. The PCR using the primers given in Table S1 (purchased from Vivantis Technologies, Malaysia) was carried out according to Ahmed et al. (2022).

2.7. Western blot analysis

The immunoblotting was conducted in refer to our previous report (Shanab et al., 2023). Briefly, protein lysate was retrieved from the brain samples with the addition of a proteinase inhibitor cocktail (PhosStopTM, Roche Diagnostics, USA) was then added. A polyvinylidene difluoride membrane was then used to transfer all the proteins after they had been electrophoretically separated using SDS-poly acrylamide gel electrophoresis and loaded in equal amounts. Then, all membranes were blocked in 1% BSA and incubated in the primary then secondary antibodies (details are shown in Table S2). The Roche Lumi-light Plus kit and the BioRAD chemidoc were employed to identify the bands. Using NIH Image J, band intensities were quantified.

2.8. Histopathology alterations evaluation

Fixed brain tissue samples were dehydrated with progressively increased alcohol concentrations (70%, 80%, 90%, and 100%, respectively). Thereafter, xylene clearance was performed before paraffin embedding. H&E was used to prepare and stain the sections. A camera-integrated digital scanning system (DM300, Leica, Germany) was then used to image the slides. Thereafter, the prominent detected lesions (necrosis, hemorrhage, and inflammatory cell infiltrations) were scored in a range from 0 to 4 according to the degree of injury, with total lesion scores of 12 (Abdeen et al., 2021).

2.9. Statistical analyses

One-way ANOVA and Dunnett t-tests were employed for multiple comparisons. Data were expressed as the mean ± SE at a significance level of $P < 0.05$. RStudio (R version 4.0.2) was employed generate the bar graphs and multivariate analyses (Hierarchical clustering heatmap, principal component analysis (PCA), and variable importance projection (VIP) score).

3. Results

3.1. Locomotor activities and behavioral patterns of mice

As illustrated in Fig. 1, ND intoxication provoked a significant decrease in motor activities of mice as elucidated by diminishing the whole traveled and decreasing several kinds of movements (fast, vertical, horizontal, stereotypic, and total movements) while slow movement and resting time increased confront to the control groups. Contrariwise,

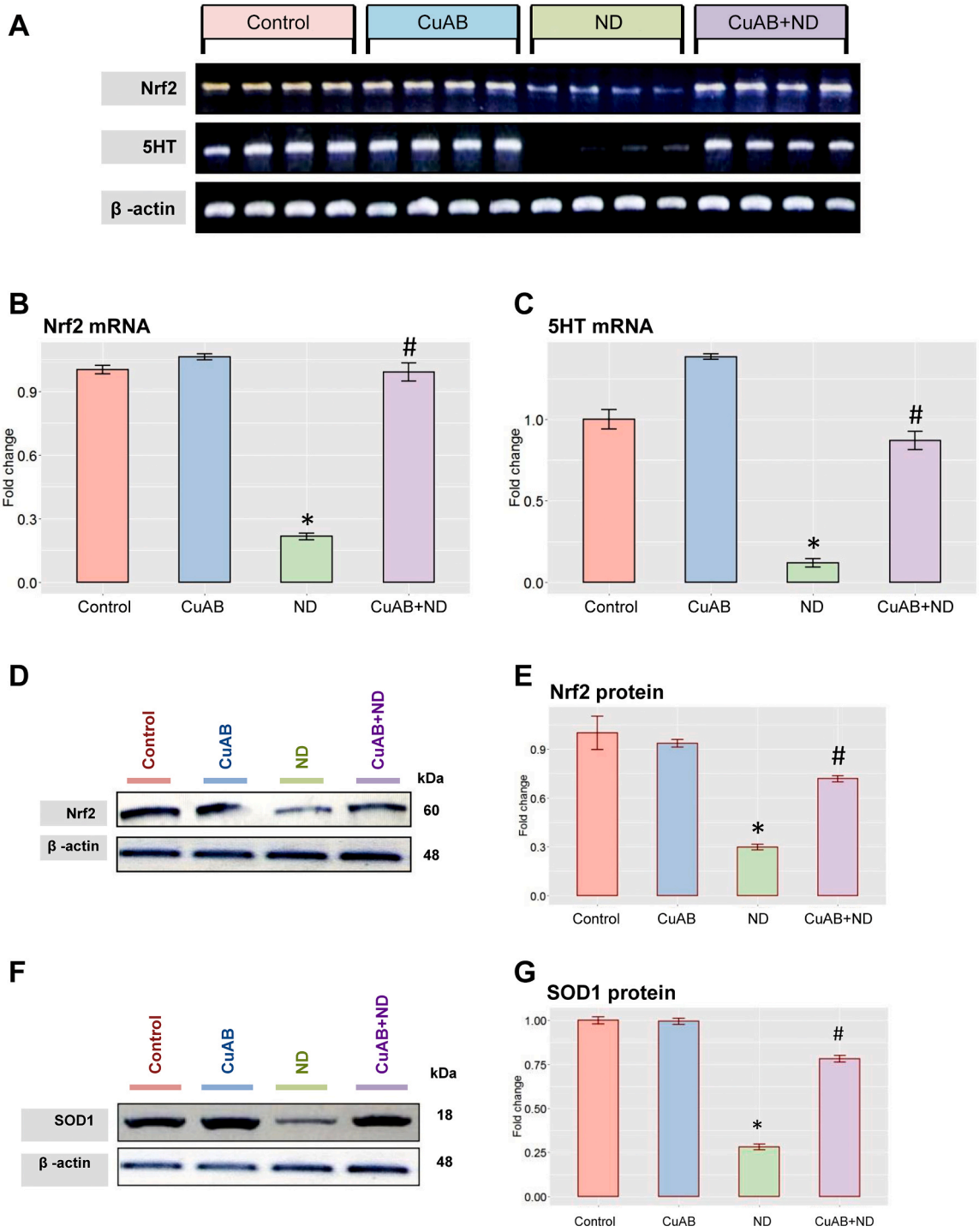


Fig. 3. mRNA and proteins expression of oxidative biomarkers in brain tissue upon ND and/or CuAB treatment. (A) representative bands for Nrf2, 5HT, and β-actin genes; Bar plot panel of mRNA levels of Nrf2 (B) and SOD1 (C). (D) Typical immunoblots for Nrf2, and β-actin proteins; (E) Bar plot panel of Nrf2. (F) Typical immunoblots for SOD1, and β-actin proteins; (G) Bar plot panel of SOD1. All semiquantitative analyses were elaborated after normalization against β-actin. $P < 0.01$; *ND vs Control; # CuAB+ND vs ND (n = 4).

preconditioning with CuAB complex robustly counteracted the deleterious effect of ND on the motor activities of mice.

3.2. Oxidative/antioxidative status

Results of endogenous antioxidant redox (SOD1 and GSH) together with the levels of MDA, a LPO biomarker, are displayed in Fig. 2. The levels of MDA were remarkably enhanced together with drastic reduction in SOD1 levels and GSH activities following ND exposure that spotted brain tissue oxidative damage. In addition, oxidative stress was further assured by decreased protein levels of Nrf2 and SOD1 as well as dramatic downregulation of mRNA expression levels of Nrf2 and 5-HT in brain tissue (Fig. 3). ND-triggered oxidative damage was significantly attenuated by CuAB complex supplementation.

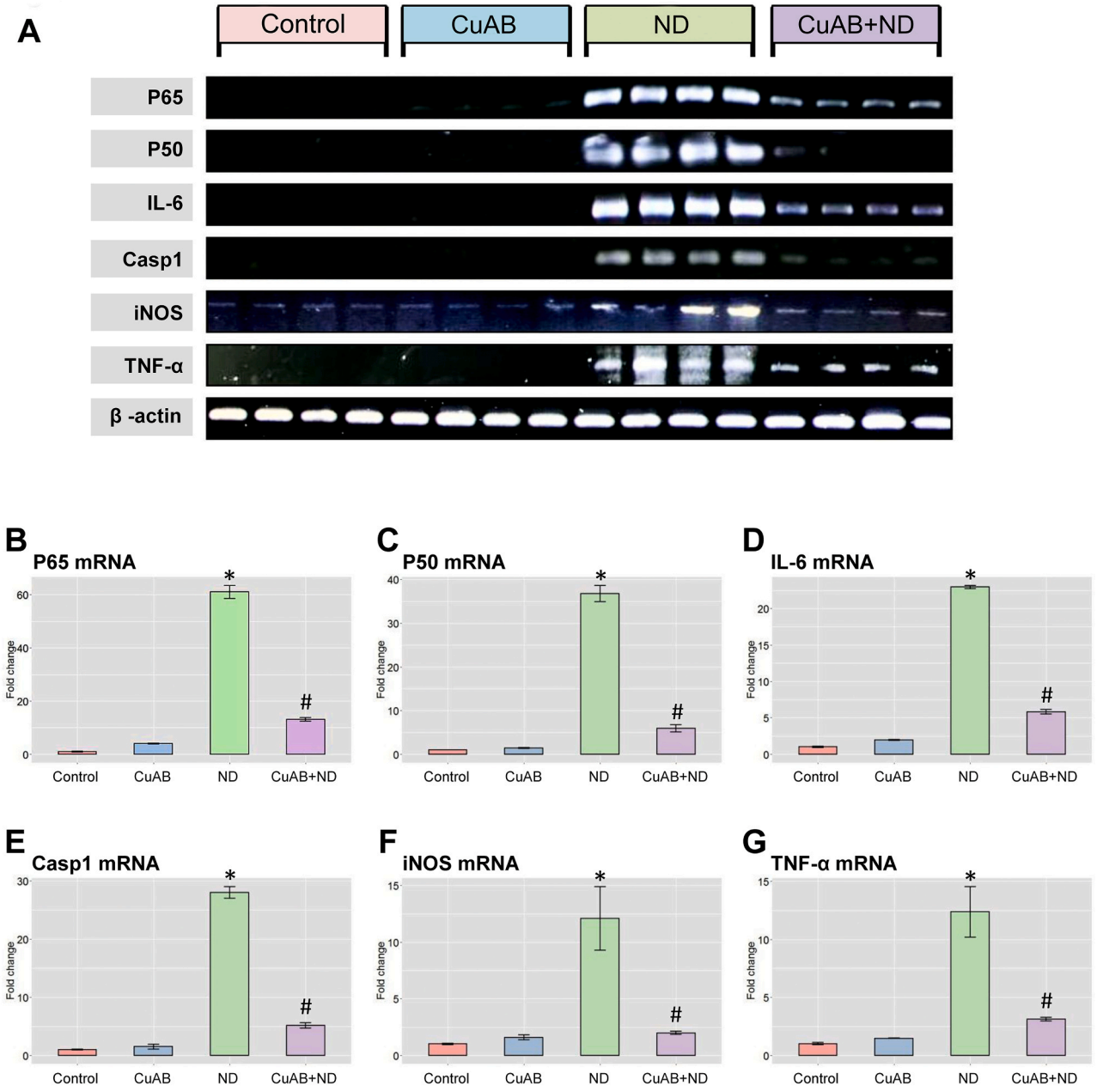


Fig. 4. mRNA expression of proinflammatory cytokines in brain upon ND and/or CuAB treatment (A) representative bands for NF-κB/p65, NF-κB/p50, IL-6, Casp1, iNOS, TNF-α, and (B-G) Semiquantitative analysis of proinflammatory cytokines after normalization against β-actin gene. $P < 0.01$; *ND vs Control; # CuAB+ND vs ND (n = 4).

3.3. Inflammatory pathway

As shown in Figs. 4 and 5, the ND-treated mice exhibited brain inflammation. This was indicated by the upregulation of mRNA levels of several inflammation-related genes such as NF-κB/p65, NF-κB/p50, IL-6, Casp1, iNOS, and TNF-α in the ND-exposed group compared to other groups. Further, the levels of protein expression of NF-κB/p65, NF-κB/p50, IL-6, and P-IKBα were dramatically increased, whereas IKBα substantially decreased following ND-exposure. Remarkably, pretreatment with the CuAB complex leads to noteworthy betterment of ND-induced neuroinflammation.

3.4. Apoptotic pathway

Alterations in protein and mRNA expression of apoptotic biomarkers in relation to ND and/or CuAB complex administrations are presented in Fig. 6. ND exposure considerably induces overexpression of the Cyt c protein, its cascade proteins, Cl. Casp3 (Casp3–17 and 19). Altogether, this suggests activation of the apoptotic pathway contrarily to the control group. In contradiction, we observed well-regulated modulation of the expression of these proteins when ND-intoxicated mice pretreated with CuAB complex. These findings corroborate credence to the assumption that Cu had a significantly inhibitory effect on ND-insulted apoptosis in brain tissue.

3.5. Hierarchical clustering heatmap, PCA, and VIP Score

PCA was employed to analyze the associations between different treatment groups and variables. Accordingly, parameters were categorized into three principal coordinate components (PC1, PC2, and PC3),

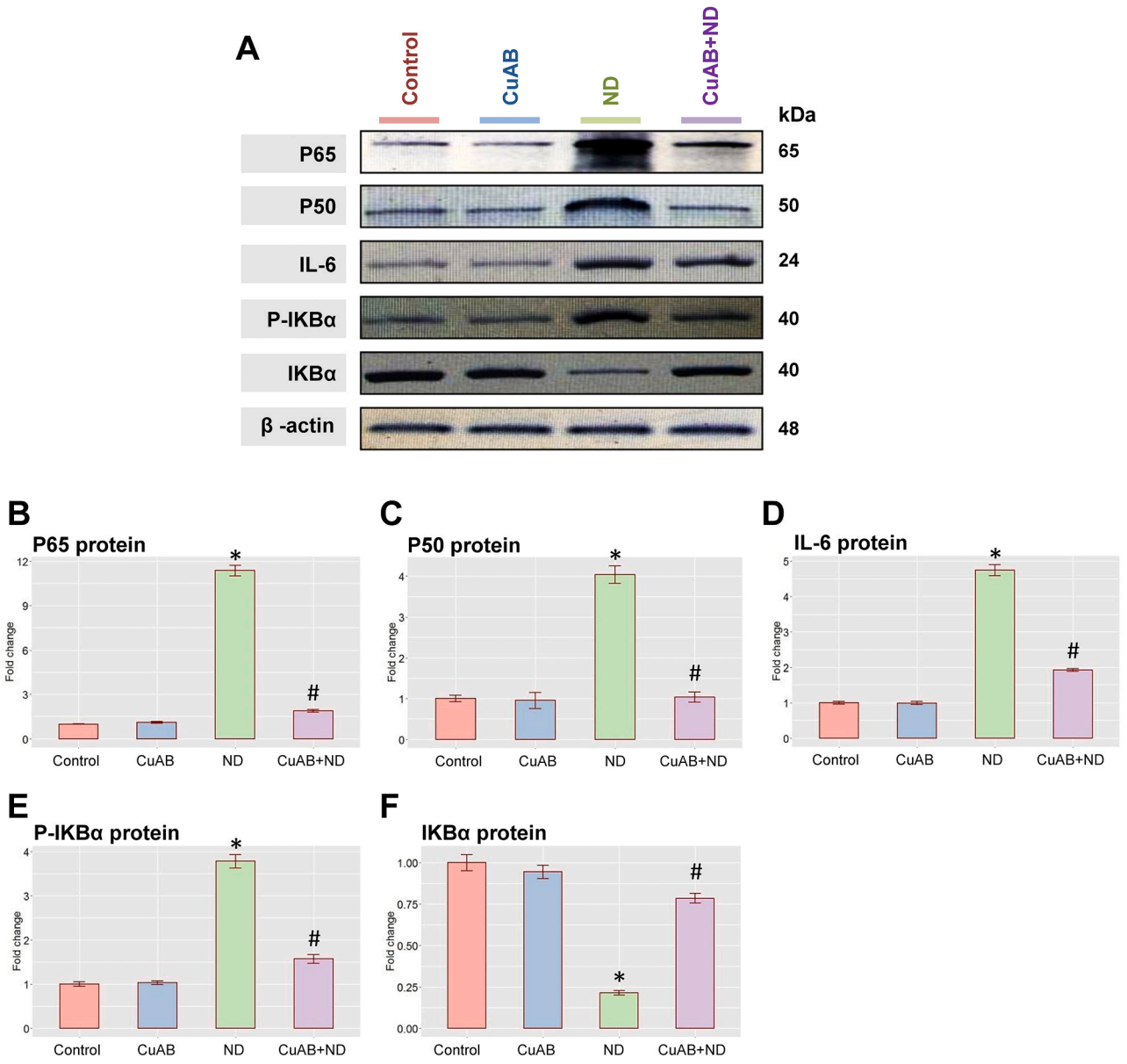


Fig. 5. Protein expression of proinflammatory cytokines in brain upon ND and/or CuAB treatment. (A) Typical immunoblots for NF-κB/p65, NF-κB/p50, IL-6, P-IKB-α, IKB-α, and β-actin genes; (B-F) Semiquantitative data after normalization against β-actin. $P < 0.01$; *ND vs Control; # CuAB+ND vs ND (n = 4).

that together accounted for 93.5% of the variance. Most of the evaluated variables were differentiated by PC1 and represented the greater proportion of variance (72%), whilst PC2 (17.3%) and PC3 (4.2%) reflected the lower proportion of variance. The PCA divulged that the Control, CuAB, and CuAB+ND groups were aggregated on the left side together and secluded from those exposed with ND (mostly along the PC1; Fig. 7A).

The clustering heatmap displayed in Fig. 7B exemplifies an instinctive visual representation of all sets of data, which sums up the significant disparity between the levels of all studied parameters in relation to ND toxicity contrasted to the other groups. These results posit that the animals exposed to ND exhibited greater injury than animals in the other groups.

Additionally, the VIP showed that the mRNA expression of NF-κB/p65, IL-6, Casp1, NF-κB/p50, TNF-α, and resting time, respectively, were the most influential variables that discerning ND-exposed animals from the rest (Fig. 7C).

3.6. Histopathological scanning of brain

To verify, biochemical and molecular observations, histological changes in the brain tissues after ND treatment were investigated and depicted in Fig. 8. As considered in the Control group, normal feature of the cerebral cortex's histological architecture (normal cells of the brain include neurons as well as endothelial and perivascular cells) was found. The brain tissues of the ND-treated group showed severe hemorrhagic changes with mononuclear cell infiltration and focal microglia cell aggregations. In addition, edema of neuronal cells with pyknotic nuclei. Clear zones were surrounded by degenerated and necrotic neurons. Variable thinning of the granular and Purkinje cell layers was also noticed (Fig. 8B, C and D). However, the pretreatment with CuAB complex significantly restored these alterations toward normal architecture as shown by apparently normal brain tissue with mild degrees of necrosis and degeneration in the neurons (Fig. 8F). These findings were statistically assured by scorning data as present in Fig. 8G, where, ND exerted a significant damaging effect on neuronal cells when compared to controls. However, rat-treated with both ND and CuAB showed

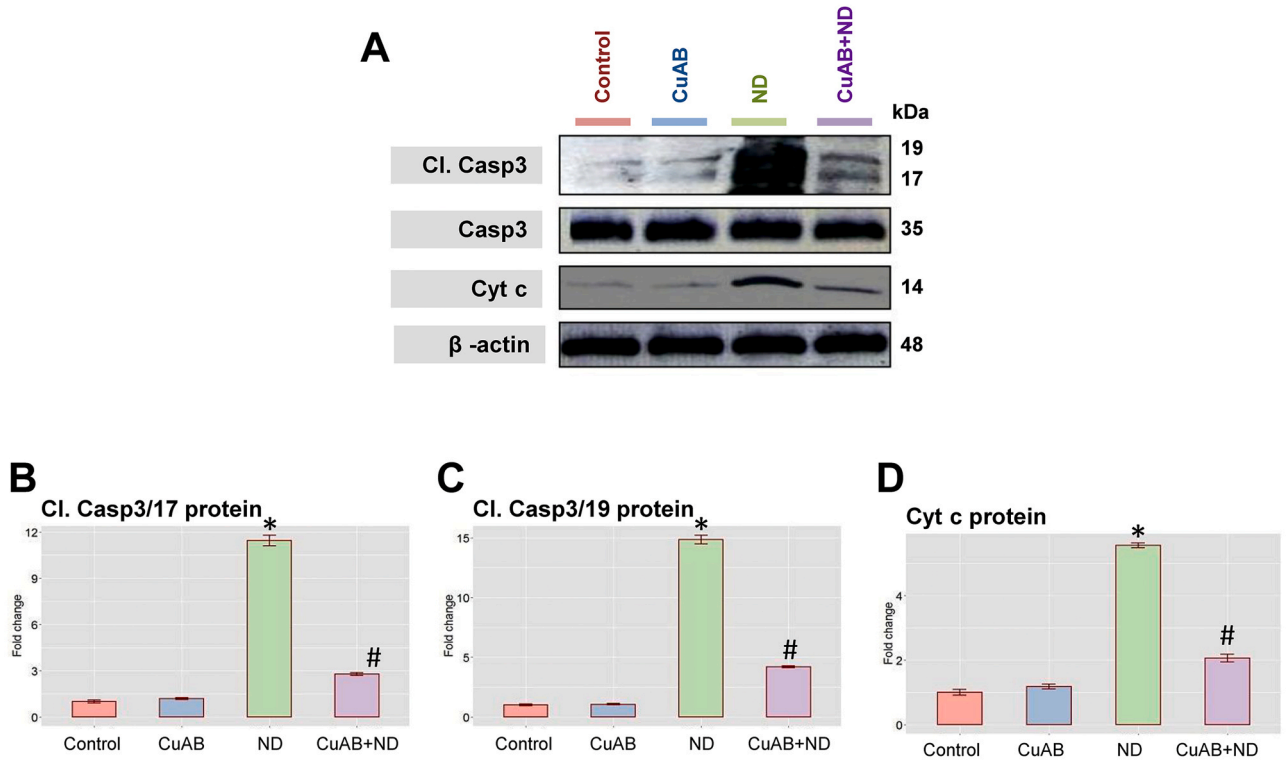


Fig. 6. Protein expression of apoptotic proteins in brain upon ND and/or CuAB treatment. (A) Typical immunoblots for Cyt c, Cl. Casp3- 17/19, Casp3, and β-actin proteins; (B-D) Semiquantitative data after normalization against β-actin. $P < 0.01$; *ND vs Control; # CuAB+ND vs ND (n = 4).

marked improvements than those treated with ND only.

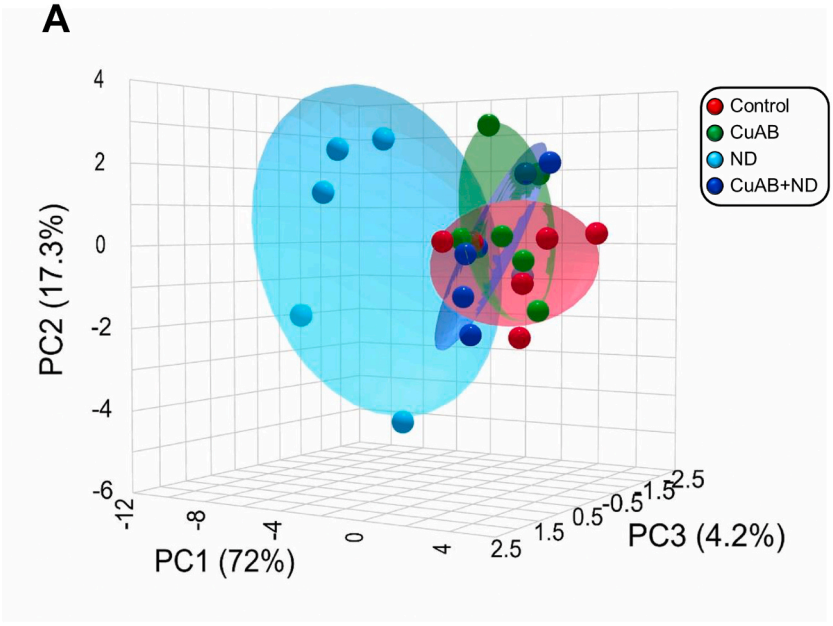
4. Discussion

ND is an extremely toxic unavoidable environmental contaminant, that poses a serious health hazard after repeated exposure (Lai et al., 2023; Shaarawy et al., 2009; Shreevastava et al., 2015). Credible evidence robustly proposed that LPO, increased oxidant creation, and cellular antioxidant insufficiency are considered the fundamental mechanisms thought to be involved in its pathogenesis (Kabir et al., 2020; Siddique and Ali, 2016; Tong et al., 2010). The brain is one of the vital organs particularly vulnerable to ND-induced oxidative damage as it has an abundance of metabolically active neurons that consume nearly 20% of the total body's oxygen. Furthermore, the brain's antioxidant defenses are relatively poor, and its free radical-scavenging enzymes are lacking, rendering it less capable of dealing with free ROS (Altyar et al., 2023; Lan et al., 2016).

Consequently, the current results expound noteworthy oxidative damage upon ND intoxication, evidenced by a substantial decline in GSH activity, and SOD1 level along with a marked rise in MDA concentration in mice brain tissues. Additionally, the protein and gene expressions of Nrf2, SOD1, and 5-HT were downregulated. The Nrf2, 5-HT, SOD1, and GSH play important roles against oxidative damage and can attenuate cellular injury in neuronal tissues. Intriguingly, Nrf2 signaling trajectory is a major modulatory mechanism of detoxification that contributes to boosting the cellular antioxidant capacity (Srivastava et al., 2016). While 5-HT promotes mitochondrial function, increasing cellular respiration and ATP, thus protecting the neurons when challenged by stress (Fanibunda et al., 2019). Furthermore, the antioxidant defense enzyme, SOD1 catalyzes the dismutation of superoxide anion radical into oxygen and innocuous hydrogen peroxide, which is subsequently scavenged by GSH (Wispriyono et al., 2021). However, when antioxidants are exhausted, Fenton's reaction takes place and generates a significant amount of $OH^{\bullet}$, which destructively assaults the membrane lipids, resulting in LPO generation and thereby, accumulation of MDA (Abdelnaby et al., 2022; Aboubakr et al., 2021). Thus, the brain abundance of easily oxidizable fatty acids makes it more prone to LPO damage (Lan et al., 2016) which is expounded by worthy raises in MDA levels in our results. Our histopathological screening further confirmed the neuronal membrane damage evidenced by cerebral cell degradation, with thinning of the granular and Purkinje cell layers. These findings appeared to align with those of antecedent reports that demonstrated neural tissue's antioxidant incompetency upon ND exposure (Tong et al., 2010). In addition, Rajawat (2022) has corroborated the neurotoxic oxidative and architectural damage in ND-intoxicated mice.

Accordingly, the present investigation exhibited significant behavioral and motor deficits upon ND exposure. This is probably due to the lipophilic properties of ND making it likely to cross the blood-brain barrier causing structural, biochemical, and molecular aberrations in the brain (Tong et al., 2009). ND impaired the gene expressions related to glial and neuronal constitutes and induced sustained disruptions in cholinergic neurotransmitter homeostasis (Tong et al., 2009). In addition, decreased neuromuscular strength could be caused by insulin and insulin-like growth factor signaling impairment, which has a negative impact on brain function following ND exposure. It might have modified the expression of genes related to myelin formation, maturation, integrity, and synaptic plasticity (Rajawat, 2022). Our findings appeared to be in concordance with those attained by previous reports that exhibited neurodegeneration after ND administration (Shreevastava et al., 2015). In the same vein, Rajawat (2022) revealed aberrant behavioral parameters caused by ND-induced neurotoxicity.

Congruence to mounting evidence, inflammation and oxidative stress are robustly associated. Thus, we proposed that inflammation is a potential mechanism involved in NDAE-prompted neuropathy. It was noted that the Nrf2 expression knockdown boosted the NF-κB pathway and enhanced ROS, and iNOS levels (Srivastava et al., 2016). The NF-κB (NF-κB/p50, NF-κB/p65), a key transcriptional inducer of the inflammatory processes expressed in both neurons and glia, is sequestered in



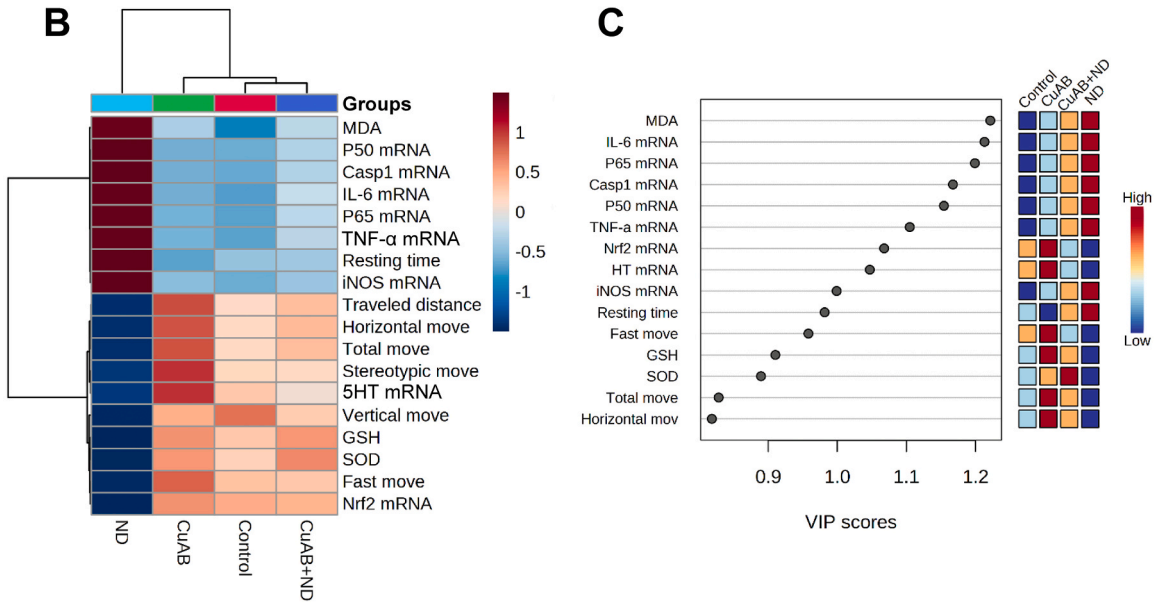


Fig. 7. Multivariate analyses of ND and/or CuAB treatment. (A) 3D score plot of PCA for identifying the four experimental groups (Control, CuAB, ND, and CuAB+ND); Percentage values specified on the axes indicate the contribution rate of the variable along the PC1 (72%), PC2 (17.3%), and PC3 (4.2%). (B) Clustering heatmap exhibits an intuitive visualization of all data sets. (C) Variable importance in projection (VIP) scores.

the cytosol in an inactive state via its association with the IKBα complex (Albensi, 2019; Duan et al., 2014). Upon oxidative stress, IKBα is phosphorylated (P-IKBα) leading to the release of the p50/p65 dimer to the nucleus and fastening the DNA to activate the transcription of proinflammatory genes, resulting in cytokine storms (Shih et al., 2015). NF-κB/p50 and NF-κB/p65 together with Casp1 (inflammatory mediated caspases) pathway, activate the outrage of proinflammatory cytokines and as well as adhesion molecules that boost inflammatory response and stimulate the recruitment of inflammatory cells in brain tissues (Lindbergh et al., 2020; Molla et al., 2020). In line with this assertion, our trial spotted noteworthy upregulation of mRNA expression of the proinflammatory genes, NF-κB/p65, NF-κB/p50, IL-6, Casp1, iNOS, and TNF-α in neuronal tissue. In addition, their protein levels are also increased while reducing the IκBα protein expression, indicating neuroinflammation. This data is congruent with our histological investigation, where noteworthy inflammatory cell incursions in brain tissue were demonstrated. Notably, following ND exposure, upregulation of the expression of these inflammatory mediators was formerly noted in various studies (Lindbergh et al., 2020). Sadeeshkumar et al. (2017) found that ND exposure evoked inflammation in the brain as indicated by the prominent immune reaction of NF-κB in neurons. Additionally, Radovits et al. (2008) and Abdeen et al. (2021) reported that enhanced iNOS expression is correlated with inflammation and tissue damage.

Apoptosis is a hallmark in several neurodegenerative disorders (Saleem, 2021). ND has been shown to cause irreversible mitochondrial membrane disintegration, releasing the cytochrome c (Cyt c) to the cytosol (Pinto et al., 2019); as a consequence, the Casp3 is activated (Glushakov et al., 2018) with its downstream apoptotic genes triggering the apoptotic pathway (Shaarawy et al., 2009). Furthermore, the well-known apoptosis inhibitor Nrf2 (Srivastava et al., 2016), is decreased upon ND exposure. The current study divulged a significantly increased expression of apoptotic proteins (cleaved Casp3–17,

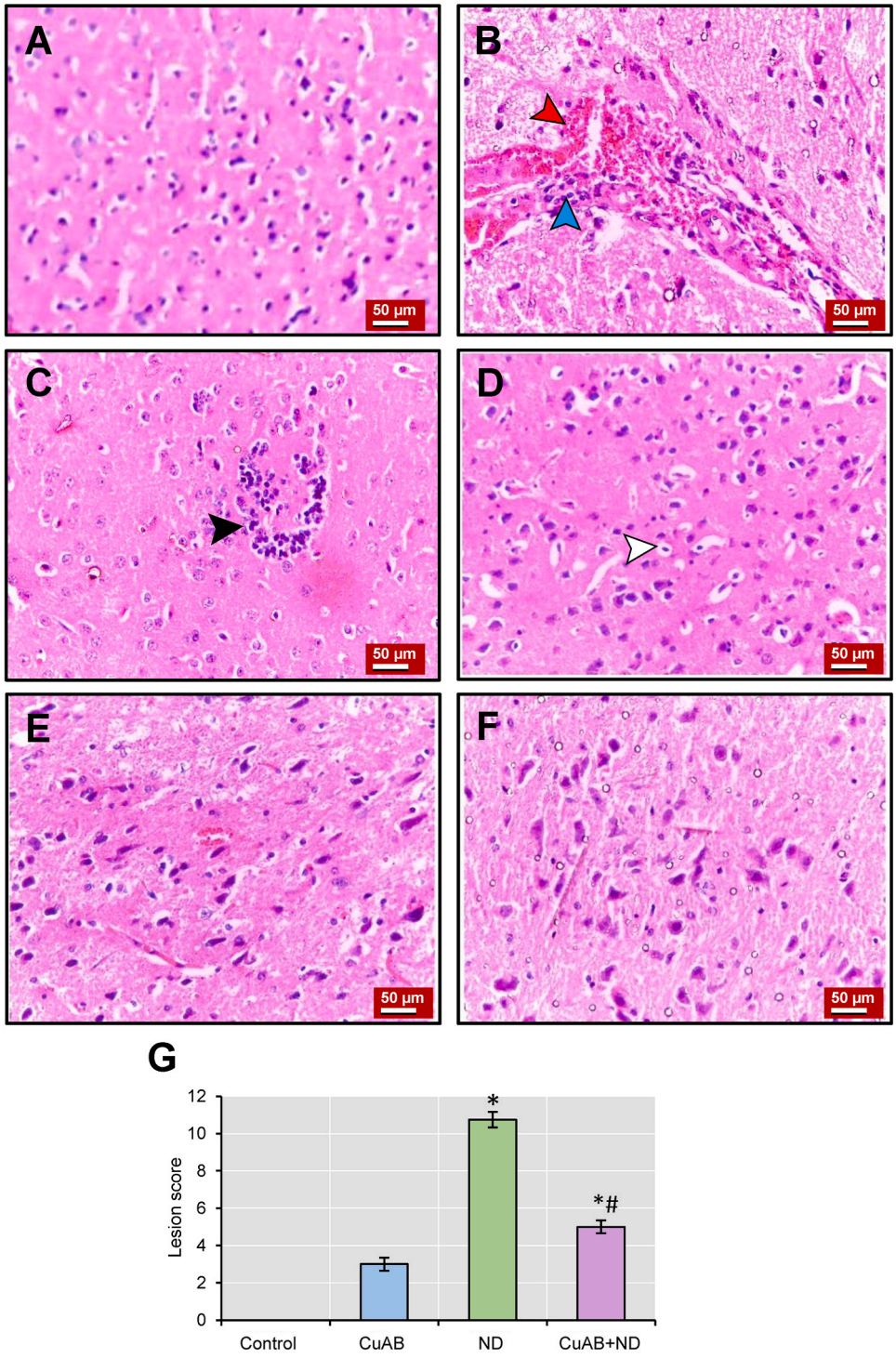


Fig. 8. Effects of the CuAB treatment with ND-intoxication on mice brain tissue. Control rats (A) showed normal architecture of the brain. (B); severe hemorrhage (red arrow) with mononuclear cell infiltration (blue arrow) and (C) focal microglia cell aggregations (black arrow) besides (D) cerebral edema with clear zones around the degenerated and necrosed neurons with pyknotic nuclei (white arrow) were seen in NEDA treated group. (E) CuAB-treated rats showed normal neurons. (F) Protective group is apparently normal. (G) Total lesion scores. $P < 0.01$; *ND vs Control; # CuAB+ND vs ND (n = 4).

Casp3–19), and Cyt c confirming neuro apoptosis of ND-exposed mice. Hence, in consonance with earlier reports, our findings obviously propose the embodiment of apoptotic pathways in ND-induced brain injury (De La Monte et al., 2009). These data are in the same vein as study of Mohammed et al. (2019) who found over-expression of caspases in mice hepatocytes following ND exposure.

Cu is a crucial trace mineral in the brain tissue in which it abundantly accumulates playing a key role in various neurophysiological processes (An et al., 2022; Gromadzka et al., 2020). It is expelled from nerve terminals influencing neuronal transmission via interacting with synaptic proteins and neurotransmitter receptors (An et al., 2022). Furthermore, it is involved in the myelination of neurons and catecholamine biosynthesis (Rubio-Osornio et al., 2009). Based on this, the pretreatment with CuAB complex significantly counteracted the deleterious effect of ND on the locomotor function and behavioral activities of mice. Furthermore, the current histopathological examination

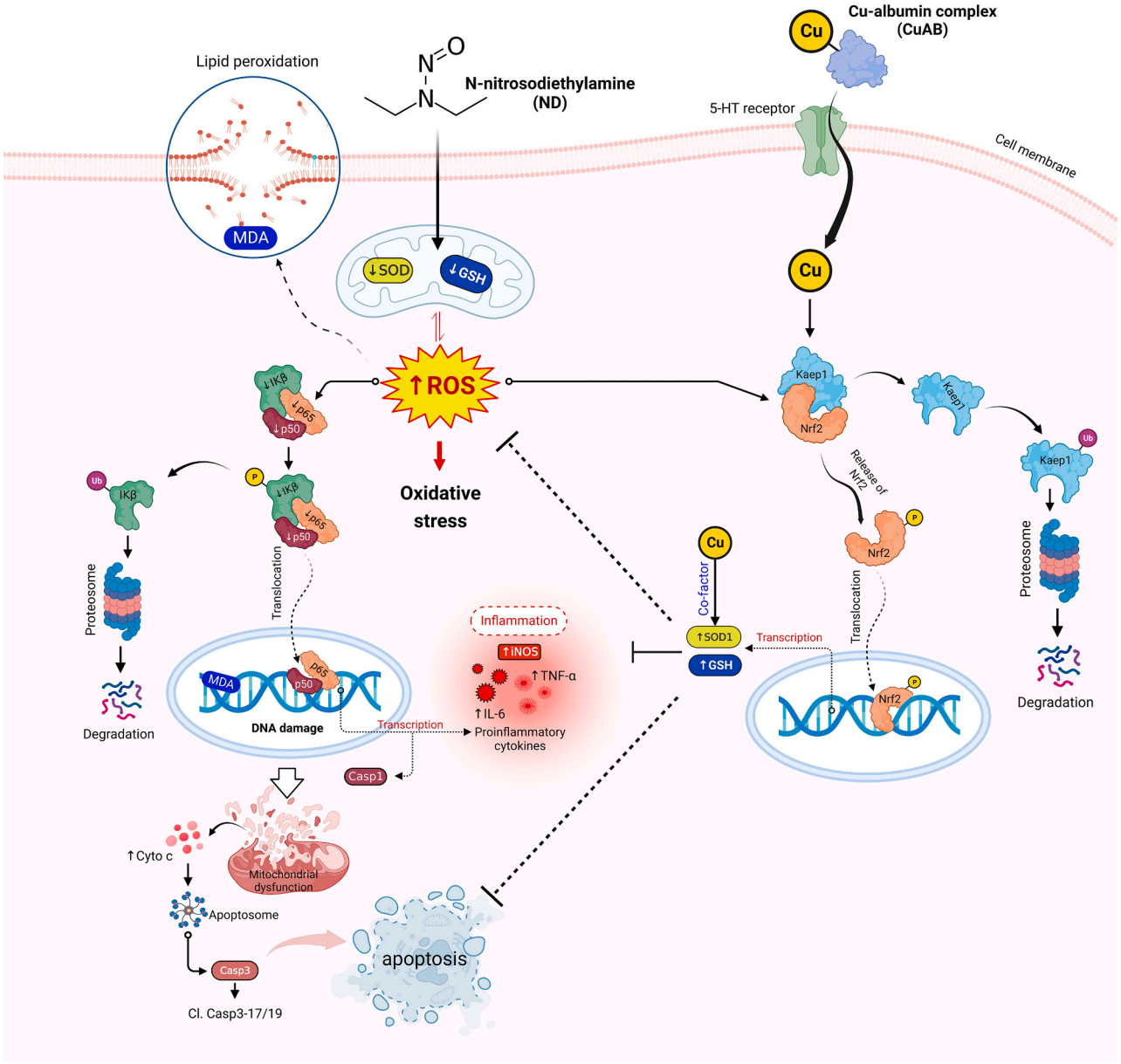


Fig. 9. The molecular underpinnings for CuAB's protective effects after ND-induced neurotoxicity.

conferred noteworthy protection, demonstrated by considerable improvement of brain architecture with Cu treatment. Our findings are congruent with those obtained by Desai and Kaler (2008) who reported that administration of Cu complexes revealed neuroprotection against neurotoxic compounds-induced motor incoordination.

Recently, there is an evidence corroborating that CuAB complex consumption enhances the endogenous antioxidant enzymes activity via upregulating the expression of oxidative stress genes, especially Nrf2-dependent antioxidant defenses which in turn prevent neuronal death (Abo-Hiemad et al., 2022b; Fujie et al., 2016). Besides, Cu act as a cofactor for SOD1 enzyme enhancing its activity (Duncan and White, 2012; Nevitt et al., 2012). Moreover, if combined with albumin, Cu^{II} ions have been found to block ROS generation (Abo-Hiemad et al., 2022b). As confirmed by prior research, copper complexes show prophylactic effect against a variety of diseases and are able to decrease oxidative stress and increase the levels of antioxidant enzymes in the body (Elgazzar et al., 2012; Shatat et al., 2013; Taha et al., 2022). By these mechanisms, in the current research, CuAB complex was capable of suppressing LPO by restoring the GSH, and SOD1 nearly to normal levels. That beneficial impact was further confirmed by a remarkable increase in brain protein and mRNA expression of Nrf2, SOD1, and 5-HT, which affords protection against oxidative stress. Our findings agree with the former reports of Abo-Hiemad et al., (2022b) who reported that CuAB complex could preserve the hepatic cells against oxidative damage caused by aflatoxicosis.

In addition, Cu^{II}-complexes have also exhibited profound anti-inflammatory properties through blocking the NF-κB activation by suppressing the upstream signal-regulating IkBα phosphorylation (Abo-Hiemad et al., 2022a; Elgazzar et al., 2012). Moreover, the putative inhibition of NF-κB by Nrf2 reduces the inflammatory reaction and promotes mitigation of neurotoxicity (Gao et al., 2022). In addition, Cu suppresses iNOS mRNA and protein expression (Radovits et al., 2008). In the same vein, our investigation emphasizes the inhibitory effect of CuAB complex on proinflammatory mediators' expression (NF-κB/p65, NF-κB/p50, IL-6, Casp1, iNOS, and TNF-α). Also, reflected in our histology by reduced inflammatory cell infiltration and cell damage when Cu was provided to ND-intoxicated animals. This result confirms the potential anti-inflammatory properties of this novel Cu complex which

was also confirmed by Abo-Hiemad et al. (2022b) and Abo-Hiemad et al. (2022a) study.

According to our results, CuAB complex supplementation exhibited a potent protective effect against ND-elicited increment of apoptotic proteins, Cl Caspa3, Cyt c protein levels in brain tissues. In conformity with our results, a previous investigation demonstrated the protective activity of Cu against liver apoptosis (Abo-Hiemad et al., 2022b).

We conducted a multivariate statistical analysis using PCA to assemble the multiple contributions from diverse interventions on the brain tissue. Each treatment was primarily distinguished on the PC1 axis (72%). ND-exposed animals might be discriminated substantially from the other treatments as evidenced by their gathering on the left side of the gel, apart from other treatments. The CuAB+ND group, on the other hand, was closely clustered with the Control and CuAB groups. The clustering heatmap revealed observable variations in all variable concentrations with respect to ND exposure contrasted to the other groups. Thus, these results robustly corroborate the proposed protective effect of CuAB complex against ND toxicity. Fig. 9 highlights the molecular mechanisms that underlie the protective effect of CuAB upon ND-intoxication. Based on these findings, we propose that pre-treatment with CuAB complex could alleviate the ND-induced neuronal damage.

5. Conclusions

All data revealed that ND could pass to brain tissues and induce neuronal damage via several pathways, including oxidative stress, apoptosis, inflammation, and structural changes in brain tissues. CuAB complex supplementation could abrogate ND-inflicted neuronal damage. This neuroprotective effect of CuAB complex is credited to its antioxidant, anti-apoptotic and anti-inflammatory properties alongside its ability to restore the normal architecture of the injured brain tissues. We, therefore, suggest the supplementation of CuAB complex could be an efficient neuroprotective strategy against diseases or toxins associated with neurodegeneration.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Institutional review board statement

The use of research animals and the study’s design were endorsed by the Ethics Committee for the Control and Prevention of Cruelty to Experimental Animals of the Faculty of Veterinary Medicine at South Valley University (Approval no. 32).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115841.

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