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Research Article

Amantadine's Potential To Mitigate Glutamate-Induced Toxicity In Pyramidal Cells Of The Juvenile Rat Brain Cortex.

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Abstract

Glutamate (Gt) neurotoxicity is implicated in a wide range of neurological conditions, with the loss of glutamate transporters leading to intracellular Gt accumulation. Amantadine (AMn), a non-competitive N-Methyl-D-aspartate (NMDA) antagonist, can partially inhibit Gt transporters and modulate Protein Phosphatase 2A subunit B (PP-2A-B) activity. This study explores the potential of early-life AMn administration to counteract Gt-induced changes in the cerebral cortex using a rat model. Our findings indicate that AMn can reverse Gt-induced structural alterations in the brain cortex and enhance PP-2A activity. Additionally, PP-2A-B activity in the AMn+Gt-treated group was comparable to control levels. Furthermore, AMn administration reduced the apoptotic index in Gt-treated individuals. We propose that the severe histopathological changes observed in the Gt group may be attributed to decreased PP-2A expression, disrupting the balance between phosphatase and protein kinase activities and resulting in a strong positive TUNEL reaction. This study provides a concise overview of the current understanding of PP-2A-B's role in Gt-induced neurotoxicity and AMn treatment while highlighting amantadine's potential as a therapeutic agent.

Keywords : *Amantadine; Glutamate; cerebral cortex; PP-2A.*

INTRODUCTION

Glutamate (Gt) is a key excitatory neurotransmitter in the cerebral cortex, playing a crucial role in synaptic plasticity and development. However, in neurological disorders such as Parkinson's disease, multiple sclerosis, and Alzheimer's disease, Gt transporters may reverse their normal activity, leading to extracellular Gt accumulation. This results in intracellular calcium (Ca2+) buildup and increased leakage through N-methyl-D-aspartate (NMDA) channels, triggering oxidative stress, ischemia, and apoptotic cascades.

Protein phosphatase-2A (PP-2A), a vital enzyme in mammalian cells, regulates metabolism, development, and apoptosis. Its subunit B (PP-2A-B), predominantly expressed in the central nervous system, plays a critical role in neural development. Amantadine (AMn) (Gocovri®), commonly used to manage Parkinson's disease-associated dyskinesia, is a Gt antagonist known to slow neuronal loss by inhibiting Gt transporters through NMDA channel interactions.

This study hypothesizes that early postnatal administration of AMn may disrupt PP-2A-B activity and potentially counteract the pathological changes induced by Gt accumulation in the cerebral cortex.

MATERIALS AND METHODS

Eighty neonatal albino Wistar rats (average age: 6 days) were obtained from Tanta University's animal facility (Tanta, Egypt). The rats were housed with their mothers under controlled conditions (25 °C, 12-hour light/dark cycle) with unrestricted access to food and water.

The rats were randomly assigned into four groups (n = 20):

- Group I (Control): Received an intraperitoneal (i.p.) injection of saline (0.5 ml) daily for seven days.
- Group II (Negative Control): Received amantadine (AMn) (30 mg/kg body weight, i.p.) daily for seven days.
- Group III (Gt Group): Received glutamate (Gt) (15 mg/kg body weight, i.p.) on the seventh day.

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- Group IV (AMn + Gt Group): Received AMn (30 mg/kg body weight, i.p.) daily for seven days, followed by Gt (15 mg/kg body weight, i.p.) on the seventh day.

On the eighth day, all rats were euthanized using Nembutal® Sodium Solution CII (sodium pentobarbital, 60 mg/kg body weight, i.p.). Following decapitation, the brains were dissected: the right hemisphere was fixed in 4% paraformaldehyde for histological analysis, while the left hemisphere was homogenized in phosphate-buffered saline (PBS) and stored at -80 °C for biochemical studies.

Hematoxylin and eosin (H&E) staining was performed following Ahmed's protocol. Brain tissues were cut into 1 cm^3 cubes, dehydrated, and embedded in paraffin. After solidification, 4-µm sections were prepared using a rotary microtome (Microtec Laborgeräte GMBH, Germany). Sections were stained with hematoxylin (10 min) and eosin (10 sec), then dehydrated in graded alcohol. Histopathological assessments were conducted by two independent, blinded pathologists.

Immunohistochemical analysis followed Ervolino et al.'s protocol. Tissue sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide for 10 minutes. Primary antibody [Anti-PP-2A (1:200, Sigma-Aldrich, USA)] was applied for 12 hours, followed by SignalStain® DAB (Biocompare, USA). Sections were counterstained with hematoxylin, and four fields per section per animal were analyzed using ImageJ software (v1.24).

Apoptotic cell detection was performed using the TUNEL Assay Kit - HRP-DAB (ab206386) at a 1:1000 dilution, following the manufacturer's protocol. Sections were incubated overnight at 4 °C, washed, and treated with Signal Stain Boost Detection Reagent in a humidified chamber (30 min, room temperature). After DAB staining, sections were counterstained with hematoxylin, and slides were examined by two independent histopathologists.

Brain homogenates were centrifuged (15,000 rpm, 15 min, 4 °C) to collect the supernatant. For the first-dimension isoelectric focusing (IEF), 50 µg of protein was mixed with rehydration buffer and loaded onto ReadyStrip™ IPG strips (Sigma-Aldrich, USA), incubated for 12 hours at 25 °C, and processed using the Ettan IPGphor-3 system (GE Healthcare, Sweden). Strips were stored at −40 °C before equilibration. The second dimension, SDS-PAGE, was conducted using PROTEAN® II xi Cell (Bio-Rad, USA). Protein spots were visualized via potassium hexacyanoferrate staining, digested, and analyzed using MALDI-TOF mass spectrometry.

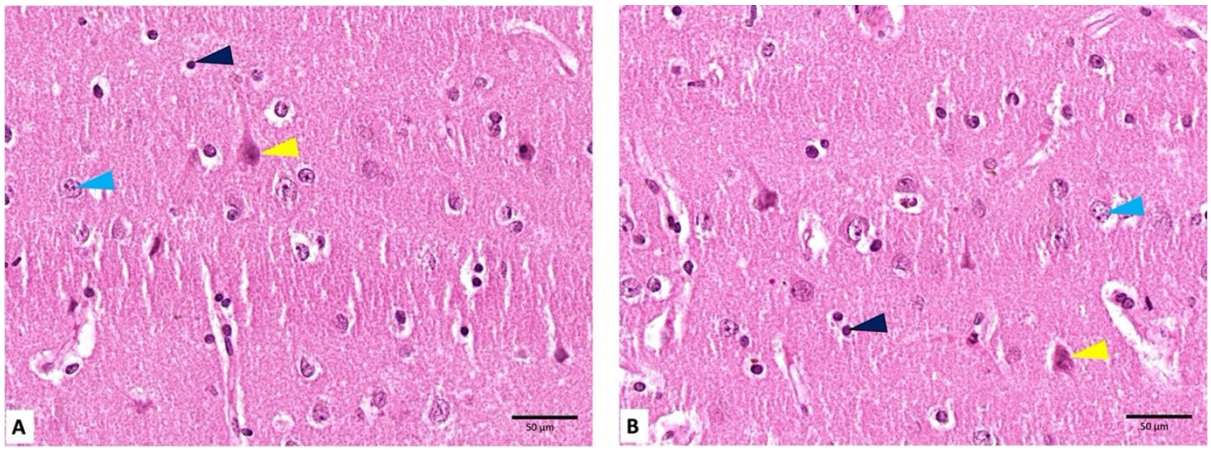
Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS® software (USA). Group comparisons were conducted using one-way ANOVA followed by Tukey-Kramer post hoc tests. A p-value < 0.05 was considered statistically significant.

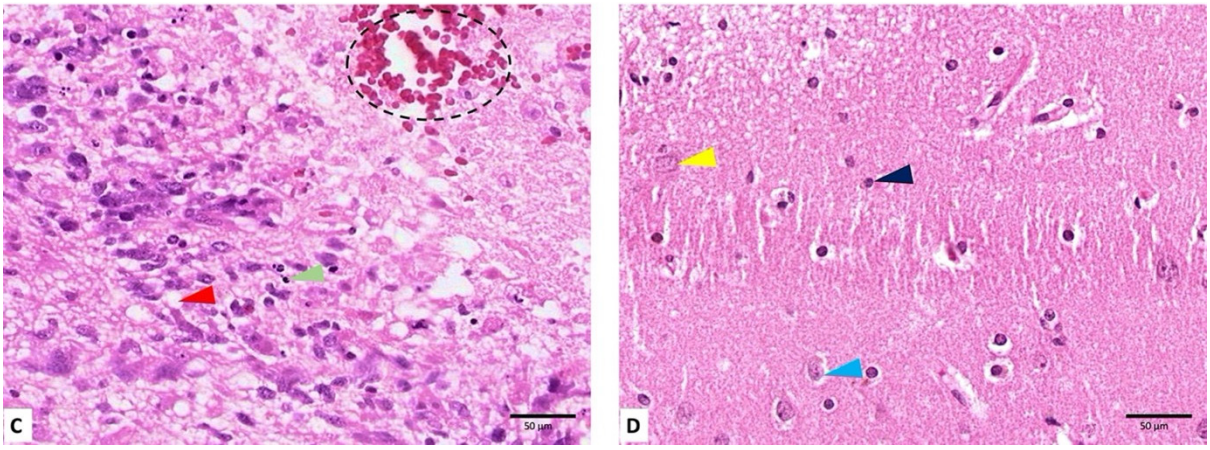
RESULTS

Effect of Amantadine on Histological Architecture and PP-2A Immunoreactivity in the Brain Cortex Following Glutamate-Induced Neurotoxicity in Neonatal Rats

Histological analysis of H&E-stained brain sections revealed that both the Control and AMn groups maintained a well-preserved cerebral cortex architecture, characterized by a distinct six-layered arrangement. The molecular layer appeared as the most superficial, followed by the external granular layer. The third (external pyramidal) and fifth (internal pyramidal) layers were densely populated with pyramidal cells exhibiting large, centrally located nuclei and intact dendrites. The internal granular and polymorphic layers displayed a normal, organized structure. In contrast, the Gt group demonstrated severe histopathological abnormalities, including vacuolated cytoplasm in pyramidal cells, dendritic atrophy, congested blood capillaries, and infiltration of inflammatory cells. However, the AMn + Gt group showed significant restoration of normal cortical architecture, closely resembling the control group (**Figure 1**).

Figure 1. Photomicrographs of brain sections stained with hematoxylin and eosin (X 400, n = 20) were examined.





The Control (A) and AMn (B) groups showed healthy histological architecture of the cerebral cortex, with pyramidal cells exhibiting rounded, large, centrally located nuclei and normal dendrites. In contrast, the Gt group (C) displayed vacuolation of pyramidal cells' cytoplasm, dendritic atrophy, congested blood capillaries, and inflammatory cell infiltration. The AMn + Gt group (D) showed a restoration of normal histological architecture.

Key observations were as follows:

Yellow arrow = pyramidal cells

Black arrow = neuroglia

Blue arrow = granule cell

Dotted circle = congestion and hemorrhage

Red arrow = vacuolation

Green arrow = inflammatory cell infiltration.

PP-2A Immunoreactivity in the Brain Cortex

Immunohistochemical staining with the anti-PP-2A antibody revealed a strong positive reaction in both the Control and AMn groups, whereas the Gt group exhibited a negative reaction. The AMn + Gt group displayed a restored positive reaction (**Figure 2**).

Figure 2. Photomicrographs of brain sections stained with anti-PP-2A antibody (X 400, n = 20) revealed the following: The Control (A) and AMn (B) groups exhibited a strong positive reaction, indicating high PP-2A expression. In contrast, the Gt group (C) showed a negative reaction, suggesting reduced PP-2A expression. The AMn + Gt group (D) displayed a positive reaction, similar to the control group, indicating that AMn treatment restored PP-2A expression in the presence of Gt-induced toxicity.

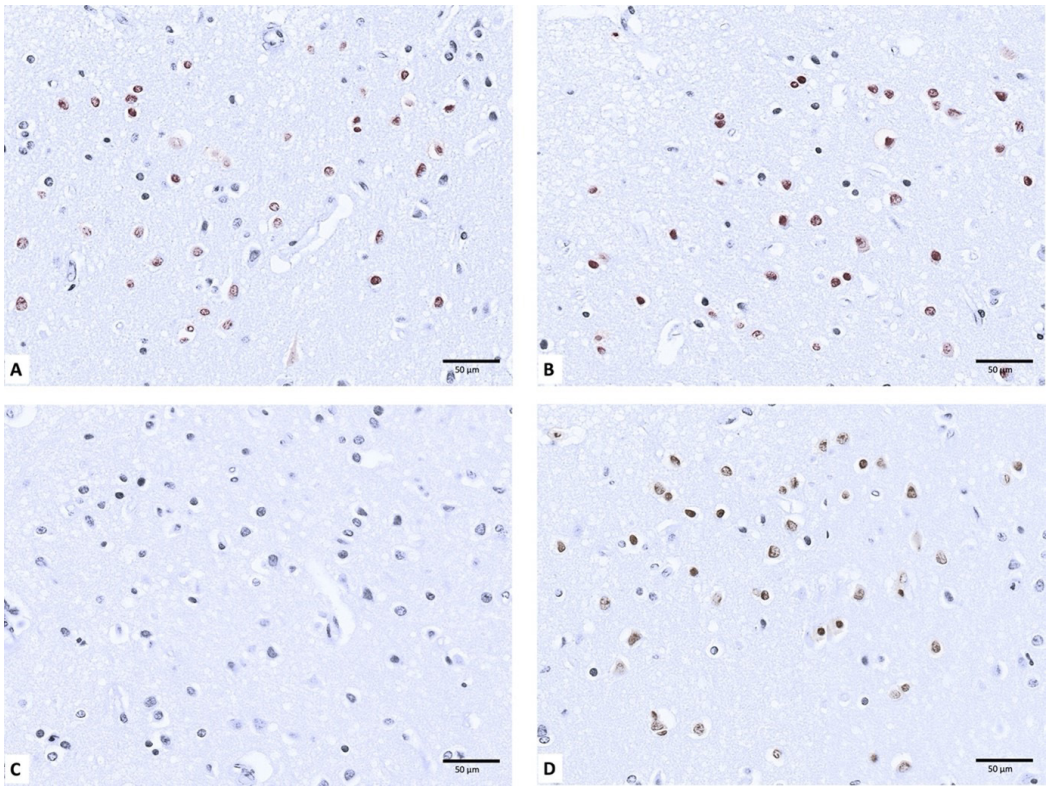
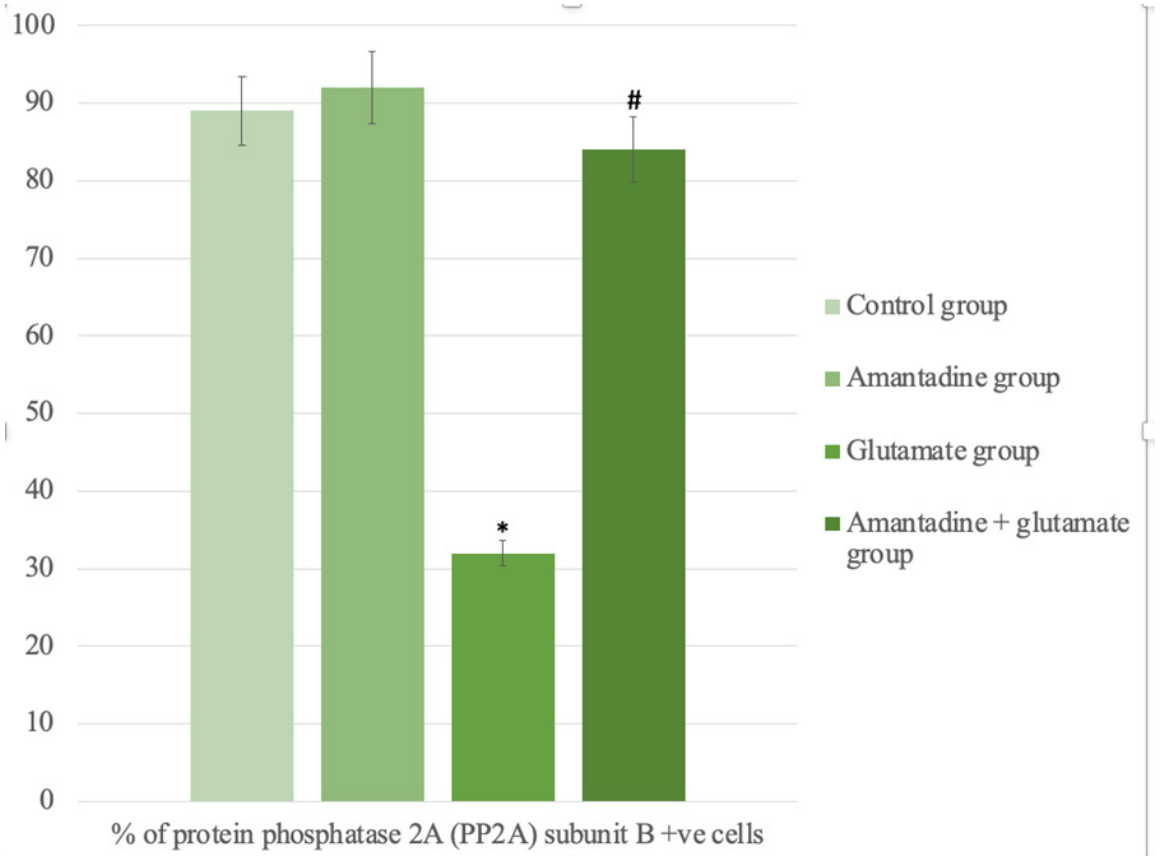


Image analysis further demonstrated that the percentage of PP-2A immunopositive cells was 89% in the Control group and 93% in the AMn group. In contrast, the Gt group showed a significant reduction to 33.4% compared to the Control. Notably, the AMn + Gt group exhibited a 234% increase in PP-2A immunopositivity relative to the Gt group, indicating a substantial restoration of PP-2A expression (**Figure 3**).

Figure 3. The effect of AMn administration on PP-2A immunoreactivity after Gt-induced neurotoxicity in NBr rats was examined.



The percentage of PP-2A immunopositive cells was 89% in the control group and 93% in the AMn group, showing a significant ($p < 0.05$) decrease in the Gt group to 33.4% compared to the control group. In the AMn + Gt group, there was a significant ($p < 0.05$) increase of 234% in PP-2A immunopositive cells compared to the Gt group.
* Significant ($p < 0.05$) difference compared to control group
# Significant ($p < 0.05$) difference compared to Gt group
Data are presented as mean ± SD, (n=20).

Effect of Amantadine on Cellular Apoptosis and PP-2A Protein Intensity in the Brain Cortex Following Glutamate-Induced Neurotoxicity in Neonatal Rats

TUNEL staining revealed a strong positive apoptotic reaction in the Gt group compared to the Control group, which exhibited a weak negative reaction. In contrast, the TUNEL reactivity in the AMn + Gt and AMn groups was comparable to that of the Control group (**Figure 4**). Image analysis demonstrated a 14-fold increase in the apoptotic index in the Gt group compared to the Control. However, the AMn + Gt group showed a 9-fold reduction in apoptosis compared to the Gt group, indicating a protective effect of AMn (**Figure 5**).

Two-dimensional gel electrophoresis analysis of PP-2A protein intensity further revealed a 33% reduction in PP-2A-B expression in the Gt group relative to the Control. Notably, PP-2A-B expression in the AMn + Gt group was comparable to that of the Control, suggesting that AMn effectively restored PP-2A expression levels (**Figure 6**).

Figure 4. Photomicrographs of brain sections showing TUNEL reactions (X 400, n = 20) revealed the following: The Gt group (C) exhibited a strong positive TUNEL reaction, indicating significant apoptosis, while the Control group (A) showed a weak negative reaction, suggesting minimal apoptosis. The AMn + Gt group (D) and AMn group (B) displayed TUNEL reactions comparable to the control group, indicating that AMn treatment effectively reduced apoptosis, even in the presence of Gt-induced toxicity.

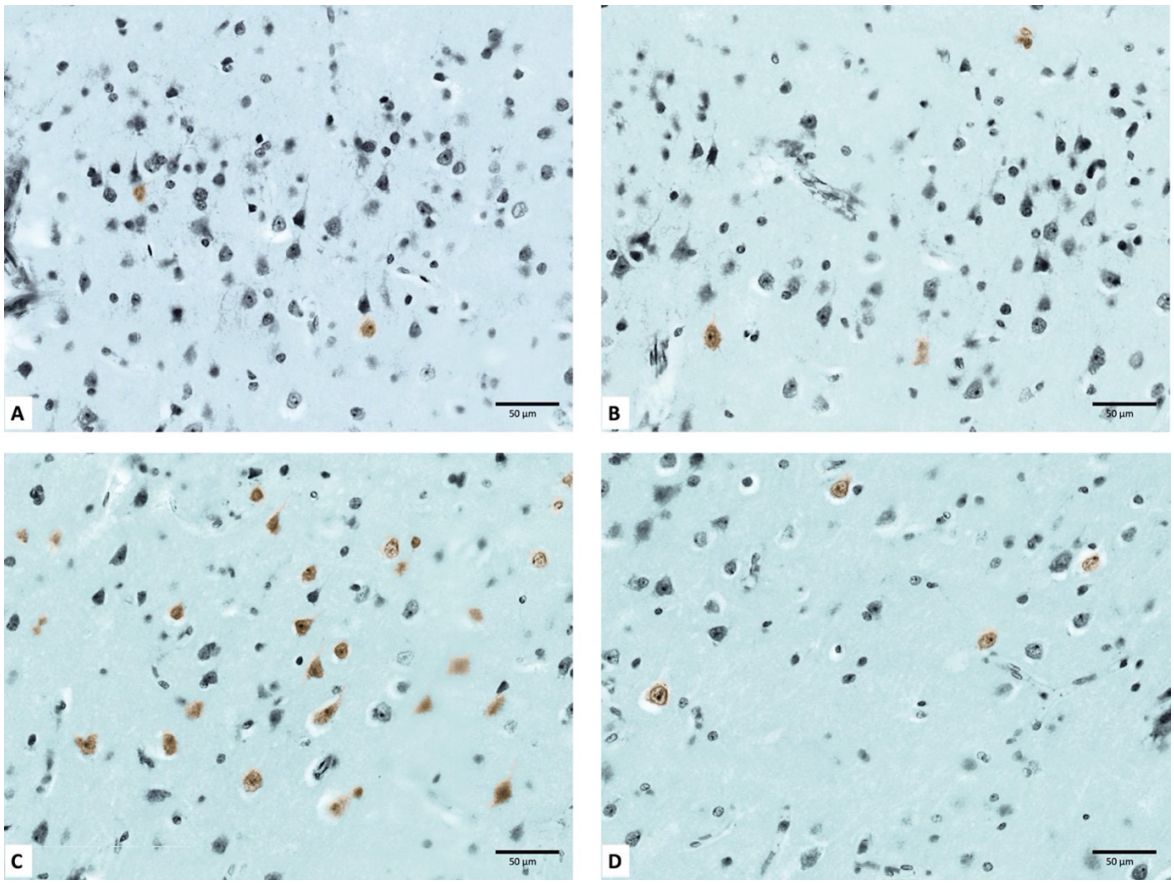
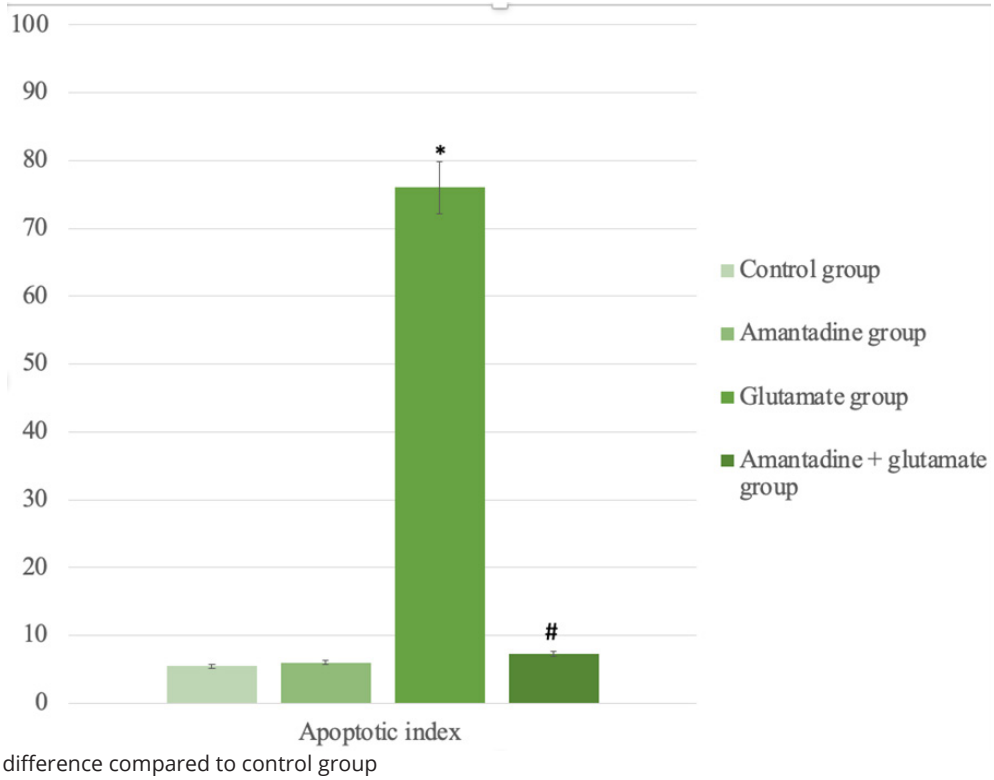


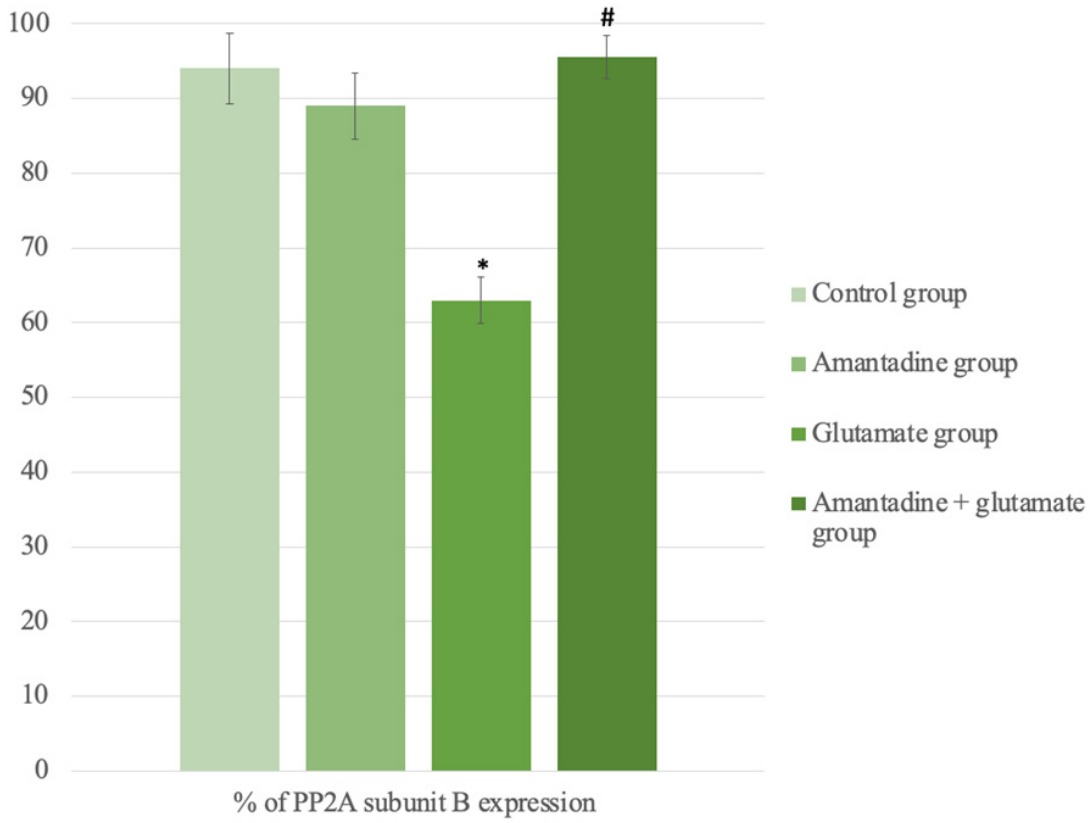
Figure 5. The effect of AMn administration on cellular apoptosis after Gt-induced neurotoxicity in NBr rats was evaluated. The apoptotic index increased significantly ($p < 0.05$) by 14-fold in the Gt group compared to the control group. In contrast, the AMn + Gt group showed a significant ($p < 0.05$) reduction in apoptotic index, decreasing by 9-fold compared to the Gt group.



* Significant ($p < 0.05$) difference compared to control group

Significant ($p < 0.05$) difference compared to Gt group
Data are presented as mean ± SD, (n=20).

Figure 6. The effect of AMn administration on PP-2A protein intensity in the brain cortex after Gt-induced neurotoxicity in NBr rats was examined. PP-2A-B expression was significantly ($p < 0.05$) reduced by 33% in the Gt group compared to the control group, while its expression in the AMn + Gt group was comparable to the control group.



* Significant ($p < 0.05$) difference compared to control group
# Significant ($p < 0.05$) difference compared to Gt group
Data are presented as mean ± SD, (n=20).

DISCUSSION

Glutamate (Gt) is a key excitatory neurotransmitter in the cerebral cortex, playing a crucial role in brain development (20). However, excessive Gt exposure can trigger oxidative stress pathways, leading to neuronal apoptosis (21). In this study, we hypothesized that Gt administration during early postnatal life disrupts PP-2A subunit B (PP-2A-B), contributing to pathological alterations in the cerebral cortex. PP-2A-B is involved in various cellular processes, including metabolism, division, axonal development, and apoptosis regulation (10). Previous studies have reported a decline in PP-2A-B levels following ischemic stroke (22) and demonstrated its neuroprotective role, with agents like ferulic acid mitigating ischemic damage via PP-2A-B upregulation (23). Our findings revealed severe histopathological abnormalities in the Gt group, while the AMn + Gt group exhibited restoration of normal cortical structure. Additionally, PP-2A immunopositive cell counts significantly declined in the Gt group but increased by 234% in the AMn + Gt group compared to the Gt group.

The Gt group also displayed a strong positive TUNEL reaction and a 33% reduction in PP-2A-B expression relative to controls. Given the link between PP-2A-B dysregulation and various neurodegenerative diseases (24), its downregulation may contribute to conditions such as Parkinson's disease (24), Alzheimer's disease (25), and tauopathy-related neurofibrillary tangle formation (26,27). Moreover, Gt-induced neurotoxicity has been associated with oxidative stress and apoptosis in cultured neuronal cells (28). The severe histopathological changes observed in the Gt group may be attributed to PP-2A downregulation, leading to an imbalance between phosphatase and protein kinase activity, ultimately resulting in enhanced apoptosis. The AMn + Gt group's similarity to the control group suggests a protective role of AMn against Gt-induced toxicity.

Our study focused on the cerebral cortex, but further research is needed to determine whether similar neuroprotective effects of AMn extend to other CNS regions or during intrauterine development. Additionally, exploring the molecular mechanisms

underlying AMn's protective role in Gt-induced neurotoxicity could provide valuable insights for potential therapeutic applications. In the Gt group, a decrease in PP-2A and PP-2A-B activity was observed, suggesting an imbalance between phosphatase and protein kinase activities.

Conversely, AMn administration increased PP-2A and PP-2A-B activity in the Gt+AMn group, restoring PP-2A-B levels to those observed in the control group. Furthermore, AMn significantly reduced the apoptotic index in Gt-treated individuals and effectively reversed Gt-induced histopathological changes in the cerebral cortex. PP-2A and PP-2A-B dysregulation is implicated in various neurological disorders, including Alzheimer's disease and stroke. While our findings suggest a protective role of AMn against Gt-induced neurotoxicity, further research is needed to assess its impact on other CNS regions, its effects during the intrauterine period, and the underlying molecular mechanisms of its neuroprotective action.

Statement Of Ethics

The study protocol was approved by Research and Ethics Committee, Quality Assurance Unit, Immam Abdulrahman university, KSA

Conflict Of Interest

Author has declared that no competing interests exist.

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The research is self-funded. No funds were received

Author Contribution

Ahmed S. Ahmed : Data collection, data analysis, discussion of results, and paper editing.

Mark M. Rohn : Data collection, Discussion of results.

Asim S. Khan : Data collection, data analysis, paper editing

Data Availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request

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