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ORIGINAL ARTICLE

Neuroprotective Effects of Amifostine in Mouse Model of Alzheimer's Disease

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SUMMARY

Background: **Alzheimer's disease is a progressive neurodegenerative disease that causes an irreversible decline in the functional, cognitive, and behavioral activities of affected individuals. Amifostine is a cytoprotective drug with well-documented pleiotropic effects such as anti-inflammatory, antioxidant, and anti-apoptotic effects. The study was carried out to investigate the neuroprotective effect of amifostine in a mouse model of Alzheimer's disease.**
Methods: **Swiss Webster albino mice were divided into four groups (n = 10): (I) control, (II) scopolamine (1 mg/kg i.p. once daily for 7 days), and two treatment groups. The treatment groups received the test drugs prophylactically for 2 weeks, followed by induction with scopolamine and the test drug at the same doses for one week, followed by (III) donepezil (5 mg/kg daily, i.p. for three weeks) or (IV) amifostine (200 mg/kg daily, i.p. for three weeks). After the treatments, behavioral tests were conducted using the spontaneous Y maze test and the novel object recognition test (NORT). The brain tissue homogenates of the experimental mice were processed for biological analysis. The levels of inflammatory (TNF-α, IL-6, and IL-1β), and oxidative stress (SOD and MDA) markers, as well as acetyl cholinesterase, were determined.**
Results: **Scopolamine intraperitoneal administration resulted in impairment of cognitive performance and neurotoxicity. Amifostine significantly attenuated scopolamine-induced injury, as observed in improved spatial working memory. Moreover, amifostine significantly reduced lipid peroxidation, increased SOD level, and reduced the proinflammatory markers and acetyl cholinesterase activity in brain tissue homogenates.**
Conclusion: **Preconditioning with amifostine had a neuroprotective effect, maintained cognitive function, and enhanced cholinergic activity in the scopolamine-induced mouse model of Alzheimer's disease.**
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KEYWORDS

amifostine, oxidative stress, neuroinflammation, Alzheimer's disease, NOR, Y maze

LIST OF ABBREVIATIONS

AD - Alzheimer's disease
ROS - Reactive oxygen species
TNF-α - Tumor necrosis factor-alpha
IL-1β - Interleukin-1β
iNOS - Inducible nitric oxide synthase
NORT - Novel object recognition test
PBS - Phosphate buffered saline
ELISA - Enzyme-linked immunosorbent assay

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SOD - Super oxide dismutase
MDA – Malondialdehyde
AChE - Acetylcholine esterase
NLRP3 - NLR family pyrin domain containing 3

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative illness that causes an irreversible decline in the functional, cognitive, and behavioral activities of the affected individuals. The incidence of AD is currently estimated to be over 50 million individuals worldwide, and it is anticipated to reach 139 million by the year 2050 [1]. The disease pathology is very complex and intertwined as the activation of one pathway triggers a vicious cycle of several other pathways, including oxidative stress and neuro-inflammation [2]. Oxidative stress plays a key role in initiating pathological alterations even in the disease's earliest symptom-free stages [3]. The development of oxidative damage in the brain can be attributed to many factors, including amyloid plaque deposition, impaired mitochondrial energy metabolism [4], neuroinflammation, lipid peroxidation, and the excessive accumulation of metals like aluminum, mercury, copper, zinc, and iron [5]. Once elevated, reactive oxygen species (ROS) can interact and damage cellular components such as lipids, proteins, carbohydrates, and nucleic acids [2]. Amifostine, also known as WR-2721, is a cytoprotective drug that effectively safeguards normal tissues from the adverse effects of chemotherapy and radiation while preserving the tumor response [6]. The primary mechanism of amifostine´s protective activity is generally considered to be free radical scavenging. In addition, other protective mechanisms have been reported, including chemical repair via hydrogen donation [7], deactivation of cytotoxic agents by direct binding, DNA stabilization, and repair [8]. The anti-inflammatory effect of amifostine was established by its capacity to reduce the expression of major inflammatory markers such as TNF-α and IL-1β [9].

Barbosa et al. [10] suggested its potential use in the treatment of oral mucositis. *In vitro* investigations demonstrated that amifostine decreased the neurotoxicity caused by paclitaxel [11] and cyclophosphamide [12]. In addition to its antioxidant effect, amifostine improved hippocampal neurogenesis and brain recognition memory [13]. More importantly, amifostine had shown effectiveness in reducing ischemic injury in a previous study, in which preconditioning with amifostine prevented heart ischemic/reperfusion (I/R) damage, owing to its antioxidant properties and anti-apoptotic effect [14].

The current study aimed to investigate the neuroprotective effect of amifostine on a mouse model of Alzheimer's disease by conducting behavioral tests and assessing acetyl cholinesterase enzyme activity, oxidative stress, and inflammatory cytokines in the hippocampus tissue homogenates.

MATERIALS AND METHODS

Study locations

The present study was carried out in the Department of Pharmacology, College of Medicine, Al-Nahrain University and Alrazy Center for Research and Diagnostic Kits, Baghdad, Iraq, from January 2022 to November 2022.

Sources of the experimental animals

Swiss Webster, male albino mice were obtained from the Alrazy Center for Research and Diagnostic Kits, Baghdad, Iraq. The animal weight ranged from 25 to 30 grams and the age from 2.5 to 3 months. The mice were maintained in sterilized cages (10 mice per cage), were kept in a standardized environment with controlled temperature and lighting, and were then left without disturbance for one week for acclimatization.

Experimental design

After acclimatization, any mouse with abnormal behavior or any ill-looking mousewas excluded. The remaining mice were randomly divided into 5 groups, of 10 mice each. Group 1 (control) included mice that received intraperitoneal saline solution for 21 days; group 2 (induction) mice received only 1 mg/kg scopolamine (i.p.) for seven days to induce Alzheimer's disease model. The treatment groups received the test drugs prophylactically for two weeks, and then induction with scopolamine started in the third week, together with the administration of the test drugs. Group 3 (donepezil) mice were administered donepezil (5 mg/kg, i.p.) once daily; and group 4 (amifostine) mice received 200 mg/kg amifostinei, p., once daily.

Behavioral tests

The following tests were conducted starting from the seventh day of induction with scopolamine.

Spontaneous Y maze test

A Y-shaped maze that was custom-designed to have three arms that were oriented at 120 degrees from each other was used for testing. The test was conducted by allowing the mice to move spontaneously through the maze and by estimating spontaneous alterations that reflect spatial working memory. Mice with unaltered working memory can recall the recently visited arms of the maze and explore new arms. Mice were placed in a specific location in the maze and left to freely move in the maze for 10 minutes. Sequential inputs into the three arms constitute an alternation. The tests were video recorded, and arm entries and total arm alterations were counted to calculate the spatial alternation for which a high percentage implied that the mouse retained memory for the arms it had previously visited, whereas a low percentage indicated defective spatial memory (hippocampal dysfunction) [15,16]. Spontaneous alteration (%) was calculated according to the following equation:

$$Alteration\ \% = \frac{Alteration\ Number}{(Total\ Arms\ Entries - 2)}\ x\ 100 \quad [18]$$

Novel object recognition test

The basic experimental design involved three sessions. In the first session (habituation), the mice were placed in a sterilized empty area for 10 minutes. The second session (training) was 24 hours later; the mice were returned to the area and allowed to explore two identical objects for 10minutes. The third (testing) session was carried out 4 hours after the end of the second session; the mice were placed back in the area but one of the previous objects was substituted with a novelone, after which the mice were allowed to explore for 10 minutes. Rodents have an innate attraction for novelty; therefore, a mouse that recognizes a familiar item will spend more time examining the novel object. After each test, the area and objects were sterilized and cleaned with 70% alcohol [16,17]. The tests were video recorded and the time spent investigating each object was recorded.The recognition index was calculated according to the equation below:

$$Recognition\ Index = \frac{T_1}{T_1 + T_2}\ X\ 100 \quad [18]$$

T_1 is the time for exploring familiar objects; T_2 is the time for exploring the novel object.

Collection and preparation of brain tissue samples

Diethyl ether was used to induce anesthesia. The animals were sacrificed, and their brains were extracted and rinsed thoroughly with phosphate-buffered saline (PBS) to eliminate any residual blood and were then weighed by using an electrical sensitive balance. After rinsing in PBS [tissue weight (g)/PBS volume (mL) = 1:9], the animal brains were homogenized. Brain tissues were homogenized by using an electrical homogenizer while placing the sample on ice. The resulting homogenate was subjected to centrifugation in a cold centrifuge device at 3,000 rpm for 20 minutes. The desired supernatant layers were carefully collected for biological analysis [18].

Determination of inflammatory and oxidative stress markers

Inflammatory markers (such as tissue tumor necrosis factor-alpha [TNF-α], interleukin-1 beta [IL-1β], and interleukin-6 [IL-6]) and oxidative stress markers (such as tissue superoxide dismutase [SOD] and malondialdehyde [MDA]) [12] were measured by utilizing the commercially available enzyme-linked immunosorbent-assay (ELISA) kits (Bioassay Technology Laboratory, China). The analysis was performed by following the manufacturer's instructions.

Determination of acetylcholinesterase

The measurement of acetylcholinesterase (AChE) was achieved through the utilization of a readily available ELISA kit (Sunlong Biotech., China). The testing technique utilizes a biotin double antibody sandwich. The analysis was conducted by following the manufacturer's instructions.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS; version 26) was used to analyze the collected data. The difference between the groups was determined by using the analysis of variance (ANOVA) and post hoc–LSD test. The significance level of $p < 0.05$ was considered significant, while a level of $p < 0.001$ was considered as a highly significant difference. Explanatory bar charts were constructed by using Microsoft Word Excel (2010) software.

RESULTS

Effects of amifostine on the spatial working memory of mice according to the behavioral tests

One week of intra-peritoneal administration of 1 mg/kg scopolamine caused a significant ($p < 0.05$) impairment in spatial working memory (51.75 ± 7.78 %) compared to the control group (64.97 ± 11.53%). Both donepezil and amifostine treatments showed comparable effects with % alteration values (66.29 ± 8.86 and 62.28 ± 7.62%, respectively), which was significantly ($p < 0.05$) higher than in the induction group and with no significant difference from the healthy control (Figure 1A). In the recognition index test, the results (Figure 1B) showed that induction with scopolamine was able to reduce the recognition index to a significant degree (50.86 ± 11.48%) from the healthy control group (67.90 ± 9.16%) ($p < 0.05$). Treatment with donepezil significantly ($p < 0.05$) attenuated the scopolamine effect on the recognition index (66.23 ± 9.37%), and the difference was non-significant for the healthy control group. Amifostine treatment resulted in a comparable effect to donepezil treatment on the recognition index (64.56 ± 8.41%). However, the difference was not statistically significant from the induction group or control group.

Effects of amifostine on the choline esterase enzyme activity of the experimental mice

The current research showed that induction of AD with scopolamine (1 mg/kg) caused a highly significant ($p < 0.05$) increase (2.31 ± 0.55 ng/mL) in choline esterase enzyme activity compared with the healthy control (1.420 ± 0.10 ng/mL). Meanwhile, the group treated with standard donepezil drug (5 mg/kg) showed a non-significant increase (1.608 ± 0.08 ng/mL) from the healthy control group. Furthermore, amifostine treatment showed a highly significant decrease in acetylcholine esterase activity in the induction group (1.475 ± 0.25 ng/mL), with no significant difference from either donepezil or healthy control, as presented in Figure 2.

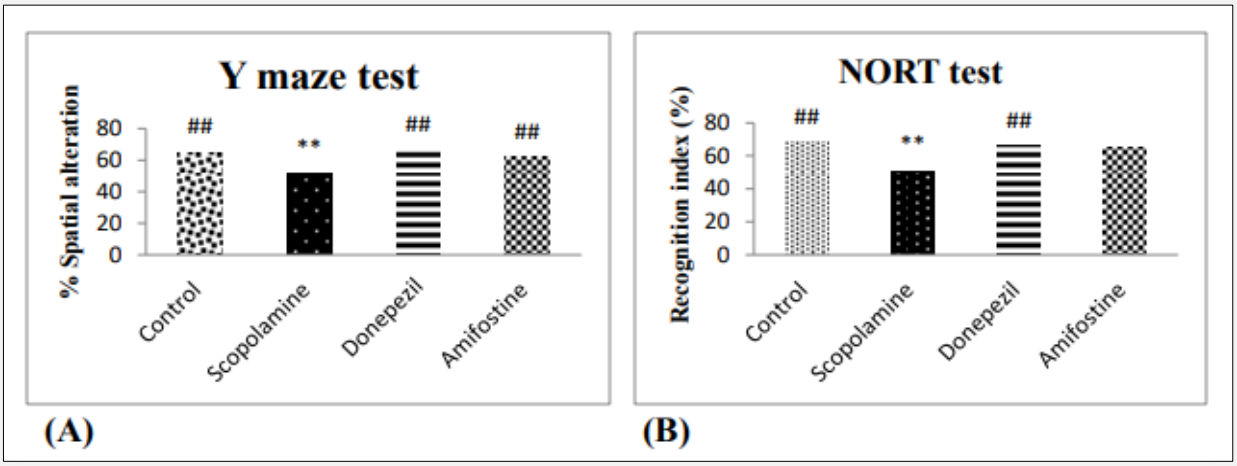

Figure 1. Effects of amifostine on the behavioral patterns of the experimental mice.

A - effect on % spatial alteration according to the Ymaze test, B - effects on recognition index according to the novel object recognition test (NORT), each column represents mean ± SD, n = 10 mice, ## - statistically significant ($p < 0.05$) compared to the induction group, ** - statistically significant ($p < 0.05$) compared to the control group.

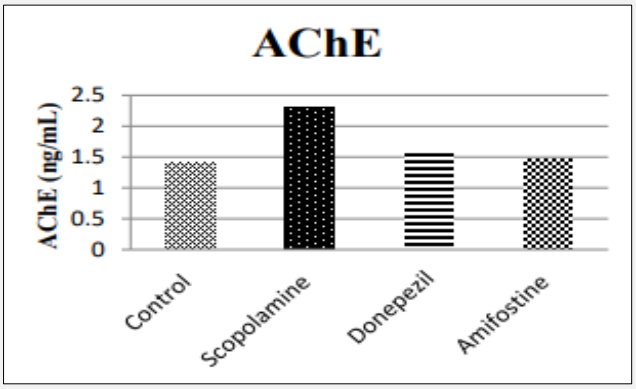

Figure 2. Amifostine reduced choline esterase enzyme (AChE) level.

Each column represents mean ± SD, n = 10 mice, ### - highly statistically significant ($p < 0.05$) compared to the induction group, * - highly statistically significant ($p < 0.05$) compared to the control group.**

Effects of amifostine on tissue inflammatory markers in the experimental mice

As illustrated in Figure 3A, induction of AD with scopolamine increased TNF-α level significantly ($p < 0.05$), with a value of 202.523 ± 75.50 ng/mL compared with the control group, which had a value of 135.509 ± 18.81 ng/mL. Meanwhile, daily treatment with 5mg/kg of donepezil attenuated the increase in brain tissue TNF-α, with no significant difference from the control group (134.291 ± 18.71 ng/mL). Furthermore, amifostine attenuated the increase in TNF-α level (142.050 ± 24.23 ng/mL) in mice brain tissue significantly ($p <$

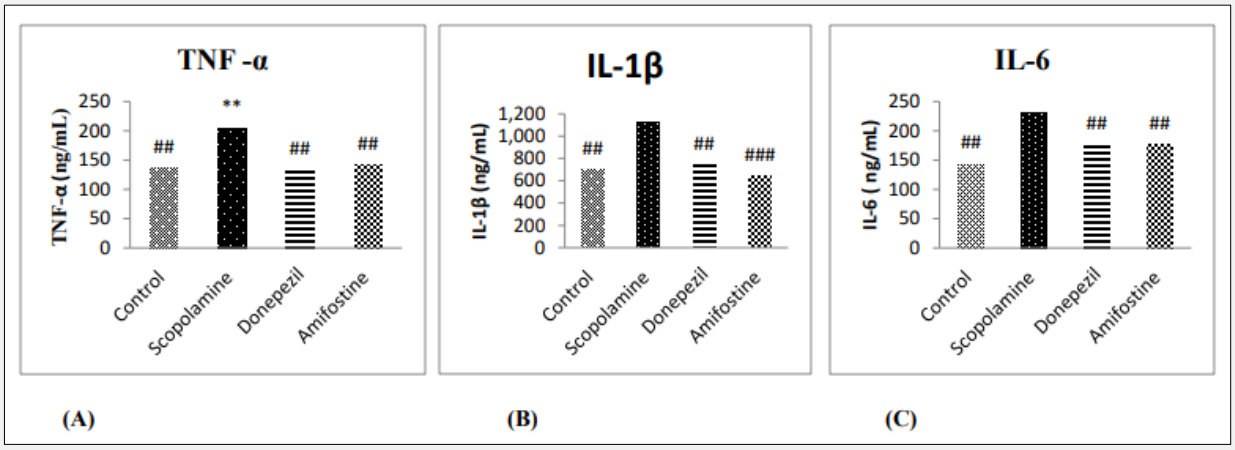

Figure 3. Amifostine reduced the level of the proinflammatory cytokine.

Each column represents mean ± SD, n = 10 mice, A – TNF-α, B - IL-1β, C - IL-6, ## - statistically significant ($p < 0.05$), ### -highly statistically significant ($p < 0.001$) compared with induction group, ** - statistically significant ($p < 0.05$) compared to control group.

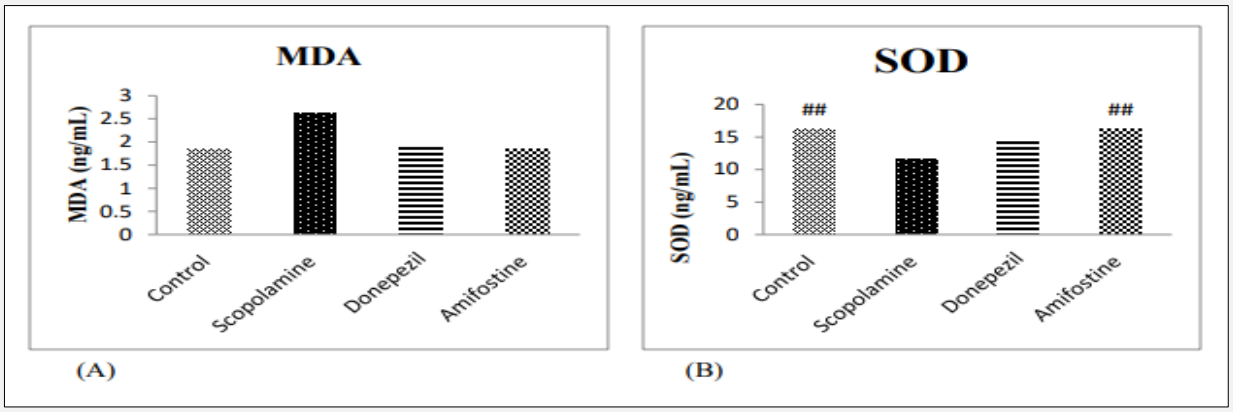

Figure 4. Amifostine pretreatment increases SOD activities and alleviate MDA release.

Each column represents the mean value, A - MDA level, B - SOD level, ## - statistically significant ($p < 0.05$) compared to the induction group.

0.05) compared to the scopolamine group and showed no significant difference from the healthy control group or donepezil group. Brain tissue level of IL-1β in the induction group (1,118.541 ± 171.60 ng/mL) was significantly ($p < 0.05$) lower compared with the healthy control group (693.562 ± 150.19 ng/mL). Compared to the induction group, donepezil significantly ($p < 0.05$) decreased IL-1β level (744.480 ± 34.94 ng/mL). Additionally, amifostine achieved the lowest attenuation in IL-1β level (638.853 ± 399.64 ng/mL), which was highly statistically significant from the induction group ($p < 0.001$), and both donepezil and amifostine showed no significant difference from the control group (Figure 3B). Concerning the effect of amifostine on IL-6 level (Figure 3C), the control group showed the lowest level of IL-6 (142.9 ± 46.27 ng/mL), while scopolamine in-

duction enhanced its production significantly (229.81 ± 32.93 ng/mL). The attenuation of IL-6 in the amifostine treatment group (175.75 ± 15.80 ng/mL) was comparable to that in the donepezil treatment group (176.28 ± 24.82 ng/mL). These values were significantly lower compared to the induction group, but not significantly different from the control group.

Effects of amifostine on tissue oxidative stress markers in the experimental mice

In the present study, two oxidative stress markers (MDA and SOD) were measured as a quantification method to assess the level of oxidative stress and lipid peroxidation in the brain tissues of the study groups. The group that was exclusively given scopolamine showed a significant ($p < 0.05$) increase in lipid peroxidation, as evidenced by a considerably higher level of MDA (2.628 ± 0.249 nmol/mL) compared to the healthy control group (1.853 ± 0.54 ng/mL). Amifostine treatment achieved a comparable MDA level (1.855 ± 0.37 nmol/mL) compared to donepezil treatment (1.937 ± 0.37 nmol/mL), and both were significantly ($p < 0.05$) lower than the MDA level in the induction group, with no significant difference toward the control group (Figure 4A). Induction of AD with 1 mg/kg scopolamine for one week resulted in a significant ($p < 0.05$) decrease in SOD level (11.6 ± 3.14 ng/mL) compared with the healthy control group (16.25 ± 2.83 ng/mL). Meanwhile, treatment with donepezil showed a non-statistically significant decrease in SOD level (14.45 ± 4.44 ng/mL) compared to either healthy control or scopolamine-only group. Amifostine was employed to reduce the negative effect of scopolamine on SOD level and resulted in a comparable level with the healthy control group (16.27 ± 4.01ng/mL) and was significantly ($p < 0.05$) higher compared with the scopolamine-only group, as presented in Figure 4B.

DISCUSSION

The current study demonstrated that preconditioning with amifostine protected mice`s cognitive function and spatial memory from scopolamine-induced Alzheimer's disease. The main mode of action of amifostine is to scavenge ROS, augment the antioxidant defense, and reduce oxidative stress [7], as demonstrated in previous findings in heart tissue [14] and the spinal cord [19]. Moreover, preconditioning with amifostine before treatment preserved object recognition and improved neuron angiogenesis in mouse models [13]. One study showed that a single systemic dose of amifostine before treatment provided longterm protection (three months) on novel object recognition [20].

The findings of the present study are in agreement with the previous finding, that amifostine can protect mice's cognitive and memory functions. The highly active metabolism and oxygen consumption in brain tissues makes them very susceptible to oxidative stress and DNA damage. Thus, reducing the expression of key genes required in neuron plasticity and survival [21]. The authors proposed that scopolamine-induced oxidative stress is directly implicated in DNA damage, leading to alterations in the transcriptional machinery and impairment of synaptic plasticity and the expression of memory-related genes in the mouse hippocampus [22]. Amifostine protects cells from DNA damage caused by radiation and chemotherapy by outcompeting ROS and preventing their interaction with DNA. Amifostine also contributes hydrogen to repair existing DNA damage [23], allowing DNA condensation [24]. Moreover, it has a regulatory effect on the cell cycle and an antiapoptotic effect [25].

The findings of the present study showed that amifostine successfully restored oxidative markers reduced MDA levels and increased SOD levels significantly in mice brain tissue homogenates, which confirmed the previous findings regarding amifostine's antioxidant effect [9]. Additionally, the data showed that this positive influence of amifostine on spatial memory and recognition memory was further supported by analyzing AChE activity and antioxidant effects in mice brain tissues. It is well documented that scopolamine affects antioxidant defense and causes oxidative stress, which is associated with cholinergic system dysfunction [26]. In the present study, the findings revealed that amifostine treatment modulated the increased level of AChE that was observed under a state of oxidative stress conditions. The anti-amnesic effect of amifostine is also consistent with its anti-inflammatory effect, as it effectively reduced the levels of TNF, IL-6, and IL-1β compared with those in the induction group.

Similar to the findings of this study, previous investigations have shown a significant decrease in the inflammatory cytokines in response to amifostine treatment [9], supporting the hypothesis that amifostine mediates anti-inflammatory effect. The exact molecular mechanism involved remains unclear; however, it could be a result of reducing ROS-induced neuroinflammation [27]. Furthermore, the NLRP3 inflammasome is an essential aspect of the innate immune system that can be triggered by many stimuli, such as ROS, leading to the release of proinflammatory cytokines, specifically IL-1β [28]. The activation of the NLRP3 inflammasome pathway has been implicated in the etiology of various diseases, including Alzheimer's disease [29]. In a study conducted by Li et al. [27], it was demonstrated that amifostine effectively decreased the activation of microglia and the release of proinflammatory cytokines in the central nervous system and spinal cord. The authors proposed that amifostine exerts its anti-inflammatory effects by partially inhibiting the ROS-NLRP3-pyroptosis pathway.

CONCLUSION

The present investigation showed that pretreatment with amifostine provided effective neuroprotection in a scopolamine-induced mouse model of Alzheimer's disease. Amifostine improves cholinergic system function by lowering AChE activity and improving memory and cognitive functions. These beneficial effects could be possibly due to its antioxidant and anti-inflammatory effects.

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Ethical Approval:
The authorizing committee of the College of Medicine, Al-Nahrain University, assessed the research protocol before granting study permission (committee approval number 219-18/1/2022). The research was performed according to the Declaration of Helsinki guidelines.

Data Availability Statement:
The data supporting the findings of this study are available within the article and its supplementary materials. Raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Interest:
The authors have no conflicts of interest to declare.

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