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Memantine improves memory and hippocampal proliferation in adult male rats

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Abstract

Neurogenesis occurs during the embryological development of the brain. However, it is universally accepted that in all adult mammalian brains, there are two sites of high-density cell division: the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone (SGZ) of the dentate gyrus of the hippocampal formation.

Doxorubicin (DOX) is an anthracycline agent which results in cognitive deterioration and memory impairment, whereas memantine (MEM) is an NMDA receptor antagonist which is approved for the treatment of Alzheimer's dementia. Many studies have revealed MEM's positive impact on memory and demonstrated that it stimulates neuronal division in the hippocampus.

This study aimed to assess the effect of MEM on spatial memory and neural proliferation in the hippocampus in adult male rats treated with DOX. For this purpose, forty male Sprague-Dawley rats were divided into four groups of ten rats each according to the agent: control, MEM (2.5 mg/kg), DOX (2 mg/kg), and DOX with MEM. The rats were given seven intraperitoneal injections every other day. We tracked the rat's weights to assess the weight-reducing effects of the drugs. In order to test spatial memory, the rats were subjected to the novel location recognition (NLR) task 30 minutes after the last injection. Additionally, Ki67 immunohistochemistry was performed to examine hippocampal proliferation.

The results showed a significant reduction in discrimination index (DI) in the DOX-treated group compared to MEM- (p < 0.001) and MEM with DOX-treated groups (p < 0.001). There was a significant increase in Ki67-positive cells in the MEM-treated group compared to the saline-treated group. Treatment with DOX impaired hippocampal proliferation compared to treatment with MEM or saline. The co-administration of MEM with DOX ameliorated the decline in hippocampal proliferation compared to treatment with DOX alone. There was a significant weight reduction in the DOX group in comparison to the control group, but MEM attenuated DOX-induced weight loss.

Rats treated with DOX displayed a drop in memory, hippocampal proliferation, and weight compared to the MEM-treated group, whereas the co-administration of MEM with DOX protected memory, hippocampal proliferation, and doxorubicin-induced weight loss.

Key words: *memantine, doxorubicin, memory, hippocampus, neurogenesis, novel location recognition.*

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Introduction

According to the World Health Organization (WHO), cancer is the second leading cause of death worldwide after cardiovascular diseases, and it is expected to cause 13.1 million deaths in 2030 [51,56]. Doxorubicin (DOX) is a chemotherapeutic agent that is classified as an anthracycline antitumor antibiotic. It is used in the treatment of many cancers including lung cancer, breast cancer, gastric cancer, paediatric cancer, and multiple myeloma [61].

An estimated 17-75% of cancer patients receiving chemotherapy experience cognitive impairment stemming from agents including DOX [29]. Some patients have shown improved cognition over time after the completion of chemotherapy treatment; however, more than 50% continue to experience impairment [53]. In addition, some animal studies using the novel location recognition (NLR) task have found that chronic exposure to DOX impairs memory function consistently with disrupting hippocampal neurogenesis [8].

The hippocampus, a formation of densely packed neurons in the limbic lobe, plays a fundamental role in learning, memory, and spatial navigation [3,46,60]. It facilitates learning *via* its connections to the neocortex; in particular, it is involved in acquiring new memories and then solidifying them, thus transforming short-term memory into long-term memory. The hippocampus itself is composed of two major grey matter elements: the cornu ammonis and the dentate gyrus [22]. Each component consists of distinct types of cells that, together with the entorhinal cortex, interact with each other through circuits and contribute to the learning and memory process [2,19,30,49,50]. Furthermore, high expression of NMDA receptors on pyramidal cells is observed in the hippocampus, which are required for spatial memory [44,45].

It is universally accepted that in all adult mammalian brains, there are two sites of high-density cell division: the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone (SGZ) of the dentate gyrus of the hippocampal formation [18]. Hippocampal neurogenesis in adults is of particular interest in our research since it has been shown to significantly influence cognition and the formation of new memories, thereby considerably affecting learning. Several papers have shown that adult hippocampal neurogenesis improves memory and cognition [15,20,33]. Adult hippocampal neurogenesis has also been associated with improved pattern separation, which is increasingly recognized as the underlying mechanism of memory and learning [9,12,58]. Furthermore, brains experiencing a reduction in neurogenesis have shown decreased memory performance, whereas those with increased rates of neurogenesis have displayed increases in cognitive function [13,65].

Memantine (MEM) acts as an NMDA receptor antagonist. Previous studies have shown that NMDA receptor antagonists enhance neurogenesis in the brains of adult rats. Accordingly, MEM is being prescribed by clinicians for the treatment of Alzheimer's disease (AD). The excitotoxic process that results from overactive NMDA receptors, which is mediated by the excessive influx of calcium during a sustained release of glutamate, has been associated with strokes, trauma, and chronic degenerative diseases such as AD [11]. Memantine has shown a functional role in the improvement of memory and learning processes after neuronal damage, and it prevents the damage from progressing [5,11].

Memantine acts as a neuroprotective agent rather than a disease-reversing agent [64]. In addition, since it has low affinity to the receptor-associated ion channel, it detaches from its binding site relatively quickly. This results in the alleviation of undesired adverse effects as it is not active for prolonged time periods [35]. Hence, we can deduce that MEM does not alter normal brain signalling and can be predicted to be well tolerated in clinical trials [36]. Due to these properties and its unique mechanism of action, clinical trials have been initiated to study the effects of MEM on other forms of dementia, depression, glaucoma, and severe neuropathic pain [36]. Furthermore, MEM's effect on malignant diseases, like breast and prostate cancer, is being investigated [1,59].

Memantine offers hope for improved quality of life for patients by preventing or at least minimizing the toxic effects of DOX on neural cells and memory.

The current study examined the effects of MEM on both memory and hippocampal proliferation in DOX-treated adult male rats.

Material and methods

Ethics statement

All experiments and animal care were performed in accordance with the University of Jordan's guidelines and with the approval of the local ethics committee.

Animals and drug preparations

Forty male Sprague-Dawley rats (190-225 g) were bought from the University of Jordan's animal office and randomly allocated to four groups: control ($n = 10$), MEM ($n = 10$), DOX ($n = 10$), and DOX with MEM ($n = 10$). The animals were allowed to habituate for two weeks prior to drug administration.

Rats in the MEM group were administered 7 i.p. 2.5 mg/kg doses of memantine (Lundbeck, Denmark) every other day. This dose was modified from a study conducted by Cole *et al.* [10]. Rats in the control group were given an identical volume of 0.9% sterile saline (i.p.). Rats in both the DOX and DOX with MEM groups were administered 7 i.p. 2 mg/kg doses of doxorubicin (EBEWE Pharma, Egypt) every other day. The rats were provided with a 12-hour light/dark cycle (7.00/19.00 h) and free access to food and water.

Behavioural testing

Novel location recognition

In order to test spatial memory, the rats were subjected to the NLR task 30 minutes after the last injection [37]. The NLR task was a spatial variant of a two-trial object recognition task adapted from Dix and Aggleton [14] (see Fig. 1). The apparatus consisted of an arena (a semi-transparent perspex box 49 cm wide × 66 cm long × 40 cm high) and objects (pink, weighted water bottles 15 cm high and 7 cm in diameter). The boxes and the water bottles were cleaned with 20% ethanol prior to each experiment and between trials to remove olfactory cues. A square black card was displayed on the wall of the room during the trials to provide prominent cues for spatial orientation.

This apparatus was modified from a previous protocol [14] and was recorded by video camcorder as in our previous study [48]. The procedure consisted of habituating the animals for one hour in the box on the day prior to testing. The following day, as a familiarization trial, two identical objects (water bottles) were placed in separate locations in the box, and the animals were allowed three minutes to explore. The animals were returned to their home cage for a five-minute inter-trial interval, during which the box was cleaned with 20% ethanol. In the choice trial, the animals were returned to the box for three minutes. One object remained in its original position (the familiar location), while the other object was moved to a new position (the novel location) (see Fig. 1).

The rat was considered to be exploring the object when it sniffed, licked, or chewed the object or directed its nose at a distance ≤ 1 cm from the object [48]. Exploration was scored based on the total time spent on each object (familiar and novel locations). Data were converted to discrimination indices (DI) which means the time spent exploring the novel object minus the time spent exploring the familiar object divided by total exploration time [6,14,16].

Histology and immunohistochemistry

The day after behavioural testing was completed, the rats were put down by rapid stunning and cervical dislocation. Their brains were extracted, trimmed, and fixed in 3% glutaraldehyde overnight. The next day the brains were sectioned using a Leica vibrating microtome. The 4 um sections were placed onto positively charged slides for routine staining with haematoxylin and eosin and for Ki67 immunohistochemical analysis. The tissues were dewaxed with xylene and rehydrated through a series of graded ethanols. To retrieve the antigens, the samples were autoclaved in 0.01 M sodium citrate, pH 6.0, at 100°C for 20 minutes and then were heated in a microwave oven (800 W) for 5 minutes [55]. Endogenous peroxidase activity was quenched by incubating the slides in H_2O_2 (3% in methanol) at room temperature for 20 minutes. Non-specific immunoglobulin binding was blocked with 3% bovine serum albumin (manufactured by Merck) in phosphate buffer solution (PBS) at 37°C for 20 minutes.
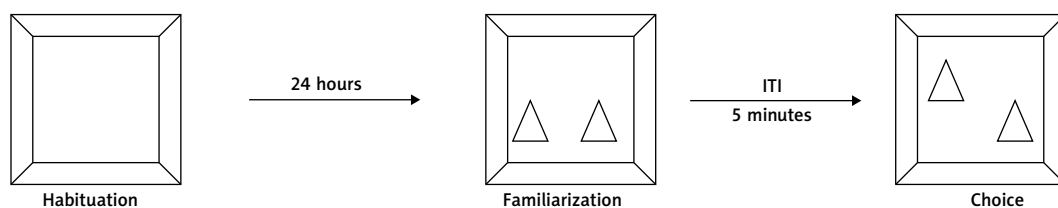

Fig. 1. Schematic representation of the novel location recognition task protocol.

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The polyclonal antibody against Ki67 was bought from Thermofisher, Cat. RB-9043. The primary antibody was diluted in phosphate-buffered saline at a dilution rate of 1 to 50 and incubated at 4°C for 1 h followed by 10 minutes incubation with Goat anti-Rabbit secondary antibody, Thermofisher Cat. A32732 (1 : 250) in PBS for 10 minutes. The slides were counterstained with Mayer's haematoxylin [55].

A systemic random sampling technique [41] was used to choose every twentieth section throughout the length of the dentate gyrus, selecting 10 sections in total. A Zeiss Primo Star microscope (Oberkochen, Germany) equipped with a Canon EOS 550D camera (Tokyo, Japan) was used to confirm the integrity of the selected sections and for counting the proliferating cells.

Counting was done by two independent observers using a double-blind method. Count of Ki67-positive cells was carried out within the SGZ, defined as the zone within three cell diameters of the inner edge of the dentate gyrus (see Fig. 2). Counts from all sections of one dentate gyrus were averaged and multiplied by twenty to provide an estimate of the total number of positive cells in the dentate gyrus [16].

Statistical analysis

Statistical analysis was undertaken and graphs were created using GraphPad Prism 4.0. $P < 0.05$ was regarded as significant. Student's paired t-tests were used to compare the exploration times for rats in each group in the NLR task choice trials. A one-way ANOVA with Bonferroni's post-test was used to compare the number of Ki67-positive proliferating cells and discrimination indices between groups, and a two-way ANOVA with Bonferroni's post-test was used to compare the replicate means of the rat's weights over the injection period between all groups.

Results

The effect of treatment on the novel location recognition task

The NLR task shows interactions with objects either in familiar or novel locations within a test arena. During the familiarization trial, in which the rats explored two identical objects, both the control and the treatment groups showed no preference for either object in terms of the total exploration time (data not shown). During the choice trial, in which one object had been moved to a new location, saline, MEM and MEM with DOX injected groups all explored the novel object significantly more than the old location while rats in the DOX group failed to differentiate between the two locations (data not shown). The discrimination index was calculated as the time spent exploring the novel object minus the time spent exploring the familiar object divided by total exploration time and compared between groups (Fig. 3). There was a significant reduction in discrimination index (DI)
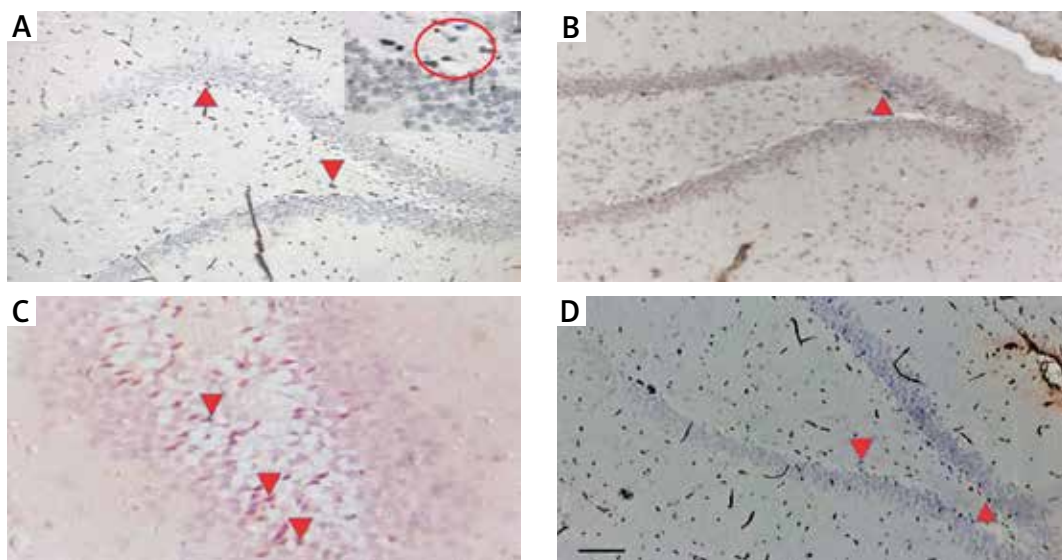

Fig. 2. Representative micrographs of anti-Ki67 immunostaining in the dentate gyrus of **A**) control group, **B**) DOX group, **C**) MEM group, **D**) DOX with MEM group. Ki67-positive cells (arrows) appear dark, indicating proliferation (scale bar = 100 μm).

in the DOX-treated group compared to MEM- ($p < 0.001$) and MEM with DOX-treated groups ($p < 0.001$).

These findings indicate that the rats treated with MEM along with DOX had protected memory compared to those treated with DOX only and that, compared to MEM treatment, treatment with DOX impaired hippocampal recent memory.

The effect of treatments on proliferating cell counts

There was a significant increase in the total number of Ki67-positive cells in the MEM group compared to the control group ($p < 0.05$). DOX treatment impaired hippocampal proliferation compared to both saline and MEM treatment ($p < 0.001$) for both. The DOX with MEM group showed increased hippocampal proliferation compared to the DOX group ($p < 0.01$). Figure 4 shows these findings correlated with the results obtained from the NLR task.

The effect of different treatments on the rats' weight

As shown in Figure 5, there was significant difference between groups due to both treatment ($p < 0.0001$) and injection periods, which are indicated by arrows ($p < 0.0001$). DOX reduced the weights of the rats after each injection. Overall, there was a significant reduction in weight for rats treated with DOX compared to rats treated with saline throughout the injection period ($p < 0.0001$). Memantine attenuated this weight loss ($p < 0.0001$).

Discussion

This study aimed to assess the effect of MEM on spatial memory and neural proliferation in hippocampus in adult male rats treated with DOX.

Previous animal studies have shown that chronic administration of MEM improved spatial cognitive function as evidenced by decreased errors in a Morris water maze [4,42]. This was also suggested to be true in a transgenic mouse model of AD [43]. Pietá Dias et al. demonstrated that chronic MEM administration (20 mg/kg i.p.) over three weeks decreased age-induced spatial memory deficits as investigated through the NLR task. Different test regimens and doses may elucidate this controversy [54].

Enhanced hippocampal proliferation with MEM use was evident in the literature. A study has shown that a single intraperitoneal dose of 50 mg/kg stimu-
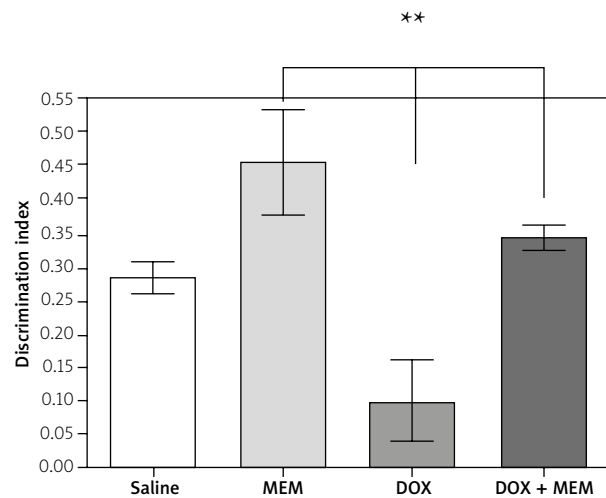
Fig. 3. Discrimination index between groups (DI). The doxorubicin (DOX)-treated group did not discriminate between the familiar and novel location. The DI of the group treated with DOX along with memantine (MEM) was improved compared to DOX treatment ($p < 0.001$). The DI of the MEM-treated group was significantly higher than in the DOX-treated group ($p < 0.001$).
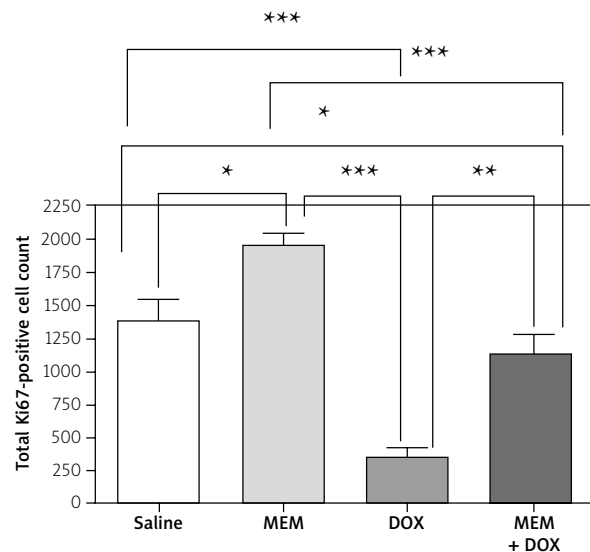
Fig. 4. Effect of treatment on the proliferating cell count. There was a significant difference in the total number of Ki67-positive cells between all groups (one-way ANOVA) ($p < 0.0001$, f = 31.00).

lated hippocampal dentate gyrus proliferation in both young and elderly rats [38]. A similar effect was found by Jin et al. [28].

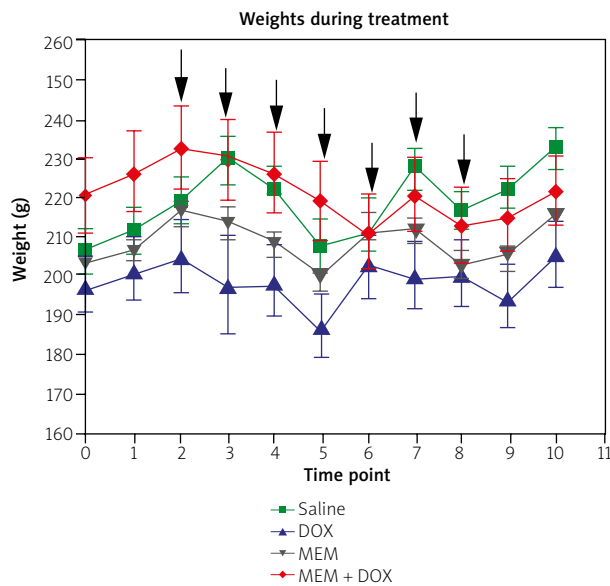Notably, in our study, the group of rats given seven i.p. injections of DOX failed to recognize the

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Fig. 5. Animal body weight during the injection period between groups. There was a significant reduction in body weight of rats treated with doxorubicin (DOX) compared to rats treated with saline throughout the injection period ($p < 0.0001$). Memantine (MEM) attenuated this weight loss ($p < 0.0001$). Injections are indicated by arrows. The analysis was performed using a two-way ANOVA with Bonferroni's post-test to compare replicate means on a GraphPad Prism 4.

novel location. This finding agrees with previous research showing that DOX disrupted many hippocampal-based memory functions including spatial memory and contextual conditioned fear memory [8,31,63]. In contrast, Fremouw *et al.* found that DOX-treated mice showed normal contextual fear memory and normal performance in the NLR task when compared to the control group [17]. This may be due to the different dosing regimens and differences in neuronal processes and biological factors between rats and mice.

In concordance with our study, DOX-induced cognitive decline may be attributed to decreased hippocampal neurogenesis [8], although one study showed that DOX administration alone did not significantly alter hippocampal neurogenesis unless co-administered with cyclophosphamide [31].

Although the co-administration of MEM with DOX ameliorated DOX-induced cognitive decline and inhibition of hippocampal proliferation, no causal relationship can be deduced based on this observation. Memantine was shown to have other neuroprotective effects like enhancement of synaptic plasticity [67]

and inhibition of apoptosis [27]. In addition, recent *in vitro* studies have demonstrated that MEM ameliorated DOX-induced apoptosis in different types of neuronal cell cultures [25,26]. So further research is encouraged to investigate a causal relationship.

The molecular basis of MEM-induced hippocampal proliferation has not been made clear, although one plausible mechanism is through enhanced BDNF local expression and signalling. This hypothesis is based on a previous finding that MEM caused a dose-related increase in the expression of the BDNF gene, a member of the neurotrophin family, and its receptor TrkB in many cortical regions including the hippocampus [40]. Increasing evidence suggests that increased BDNF levels promote hippocampal neurogenesis in response to different stimuli [24,39,57]. Recent studies have demonstrated that enhanced expression of BDNF contributed to MEM-related enhancement of synaptic plasticity [67] and to the anti-apoptotic effect of MEM [27], but further research is needed to elaborate the effect of BDNF on MEM-induced hippocampal proliferation.

Different NMDA receptor antagonists, including MEM, have shown great promise in reversing chemotherapy-induced cognitive deficits in animal models. A study revealed that methotrexate-induced spatial cognitive impairment, which was attributed in part to increased levels of the excitotoxic glutamate analogue homocysteic acid, was ameliorated by the co-administration of MEM [10]. Another NMDA receptor antagonist, Dextromethorphan, has also been shown to decrease negative cognitive outcomes in methotrexate-treated rats [62]. One study showed that MEM mitigated cisplatin-induced impaired performance of rats in the Morris water maze, and it attenuated the cisplatin-related reduction of the expression of PSD95 and ERK1/2 proteins, which are essential for the formation and maintenance of synaptic plasticity [7]. These preclinical data support the hypothesis that NMDA receptor antagonists like MEM may be used for the treatment of chemotherapy-induced cognitive deficits.

Reduction in body weight is a toxic effect associated with the administration of DOX [21,23,34,66]. We noticed a significant weight reduction in the DOX-injected rats in comparison to the control group during the two-week injection period. However, MEM co-treatment effectively improved DOX-induced weight loss. Accordingly, MEM could be a potential agent for the attenuation and prevention

of weight loss induced by DOX in clinical practice. This can be partly supported by a randomized controlled trial done on Alzheimer's patients showing that MEM was associated with a significant increase in body weight when compared to placebo [52]. In addition, two studies have shown that MEM attenuated weight loss in animal models of Huntington's disease and ulcerative colitis [32,47].

Conclusions

Rats treated with DOX showed a deterioration in memory and an inhibition of cellular proliferation in the hippocampus, in addition to a noticeable decrease in weight. In contrast, the co-administration of MEM and DOX revealed a significant enhancement of memory, a promotion of hippocampal proliferation, plus a remarkable improvement of DOX-induced weight loss.

Acknowledgements

Disclosure

The authors report no conflict of interest.

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