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Atomoxetine improves hippocampal cell proliferation but not memory in Doxorubicin-treated adult male rats

Ahmed Salman[1,2] | Maha El Beltagy[1,2] | Amjad Shatarat[1] | Loai Alzghoul[1] | Liyana Oweis[1] | Nada Al Antary[1] | Safa Al Fegie[1] | Maram Mohsen[1] | Salma Salman[3]

[1]Faculty of Medicine, The University of Jordan, Amman, Jordan

[2]Faculty of Medicine, Menoufia University, Shebin El-Kom, Egypt

[3]Faculty of Medicine, Ain Shams University, Cairo, Egypt

Correspondence
Ahmed S. Salman, Faculty of Medicine, The University of Jordan, Queen Alia street, 11942, Jordan.
Email: ahmedsalman1971@gmail.com

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Abstract

Atomoxetine (ATX) is a noradrenaline reuptake inhibitor used to treat Attention deficit hyperactive disorder (ADHD), or improve cognition in normal subjects. Cancer patients treated with systemic adjuvant chemotherapy have described experiencing deterioration in cognition. Doxorubicin (DOX, Adriamycin) is one of the anthracycline families used in chemotherapy, which has a deteriorating effect on both cognition and proliferation. The cognitive effects of ATX require inputs from the hippocampus. The aim of this study was to examine spatial memory and proliferation in the subgranular zone (SGZ) of the DG in adult Lister Hooded rats treated either alone or with a combination of Atomoxetine (30 mg kg^{-1} day^{-1}, six i.p. doses, one injection every other day) and Doxorubicin (DOX) (2 mg kg^{-1} day^{-1}, six i.p. doses, one injection every other day). Spatial memory was tested using the Novel location recognition (NLR) test, and proliferation of hippocampal cells was quantified using immunohistochemistry for the proliferative marker Ki67. Results showed that ATX treatment has improved the NLR task and increased cell proliferation in the SGZ of the DG, compared with saline-treated controls. Animals treated with DOX only showed deficits in NLR task, and co-administration of ATX along with DOX did not improve their performance. DOX chemotherapy caused a significant reduction in the number of proliferating cells in the SGZ of the DG compared with saline-treated controls. This reduction was reversed by co-administration of ATX. The above findings suggest that DOX can negatively affect both cell proliferation and memory and ATX co-administration improves proliferation, but not memory in the adult male rat hippocampus.

KEYWORDS

atomoxetine, doxorubicin, neurogenesis, novel location recognition

Ahmed Salman and Maha Elbeltagy contributed equally in the work

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1 | INTRODUCTION

Neurogenesis is defined as the formation of new neurons from neural stem and progenitor cells, which occurs in various brain regions such as the Sub granular zone (SGZ) of dentate gyrus (DG) in the hippocampus and the subventricular zone of lateral ventricles (Begega, Alvarez-Suarez, Alvarez-Suarez, Sampedro-Piquero, & Cuesta, 2017). It has been proved that the hippocampus has a main role in long-term episodic memory, particularly in spatial memory, which is provided by place cells that are located in areas CA1, CA3 and DG (Bird & Burgess, 2008; Park, Dvorak, Dvorak, & Fenton, 2011). Furthermore, long-term potentiation (LTP) occurs in the DG by high frequency stimulation to monosynaptic excitatory pathways, which increase the efficiency of synaptic transmission. Moreover, LTP is the main form of synaptic plasticity reflecting the activity of synaptic information storage processes, and has been identified as the prime candidate to be the cellular correlate of learning and memory (Bliss & Collingridge, 1993; Leal, Bramham, Bramham, & Duarte, 2017). Different factors, such as pharmacological drugs, diet, exercise and ageing, have been known to alter neurogenesis (Lee et al., 2012; Lloyd, Balest, Balest, Corotto, & Smeyne, 2010; Park & Lee, 2011).

Doxorubicin (DOX, Adriamycin) is one of the anthracycline family of chemotherapy medications, that is used in the treatment of breast cancer, lung cancer, gastric cancer, ovarian cancer, thyroid cancer, non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma, sarcoma and paediatric cancers (Thorn et al., 2011). It has been proposed that DOX acts by two mechanisms in the cancer cell; disruption of topoisomerase-II-mediated DNA repair due to intercalation into DNA, and generation of free radicals, which damage cellular membranes, DNA and proteins (Gewirtz, 1999; Thorn et al., 2011). Chemotherapies are associated with a variety of neurocognitive deficits that include impaired learning, memory, attention and speed of information processing (Monje & Dietrich, 2012). DOX specifically results in impairment of cognitive function and hippocampal neurogenesis.(Christie et al., 2012; Kitamura et al., 2015).

Atomoxetine (ATX, Strattera) is a non-stimulant selective norepinephrine transporter (NET) inhibitor that is used to treat Attention deficit hyperactivity disorder (ADHD) or to improve cognition in normal subjects by inhibiting presynaptic norepinephrine (NE) reuptake, thus increasing NE levels (Garnock-Jones & Keating, 2009).

Several studies have discussed the importance of NE signalling for consolidation and retrieval of spatial memories (Murchison et al., 2004; Thomas, 2015; Zhang, Ouyang, Ouyang, Ganellin, & Thomas, 2013). The role of NE in retrieval requires signalling through the β_1-adrenergic receptor in the hippocampus (Korz & Frey, 2007; Murchison et al., 2004). The effect of NE on cellular proliferation has been shown to be of positive impact (Kodama & Togari, 2013; Ma et al., 2015; Murphy, Campbell, Campbell, Araki-Sasaki, & Marfurt, 1998; Yang, Zhang, Zhang, Liu, Song, & Tang, 2008). Studies have shown that NE and stimulation of adrenergic receptors increased levels of hippocampal neurogenesis (Jhaveri et al., 2010, 2014).

Both DOX and ATX may be altering hippocampal function, and an understanding of this may provide a mechanism for improving cognition during cancer treatment.

The aim of this study was to examine the effect of DOX chemotherapy on spatial memory and proliferation in the SGZ of DG of the hippocampus in adult male Lister Hooded rats, and the possible protective effect of ATX on the same parameters. To test this, the NLR task was used to investigate the effect of treatment on recent memory of the adult rat hippocampus. In addition, immunohistochemistry for the proliferative marker Ki67 was used to examine the effect of both drugs on proliferation of the DG of the hippocampus.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments and animal care were in accordance with The University of Jordan Home office guidance regulations and with local ethical committee approval.

2.2 | Animals and treatment

All experiments were on male Lister hooded rats of 150–200g at the start of experiments. Animals were allowed to habituate for 2 weeks before treatment and housed in groups of three or four under standard conditions of 12 hr light–12 hr dark cycle (from 8.30 a.m. to 8.30 p.m.) with free access to food and water. Behavioural testing of all animals was performed between 8.30 a.m. and 2 p.m.

Twenty-eight Male Lister hooded rats were randomly allocated to four groups, seven animals each, as follows: Saline, ATX, ATX + DOX and DOX only.

Rats in either ATX or ATX + DOX groups were administered ATX (30 mg kg^{-1} day^{-1}, 6 i.p. doses, one injection every other day, Manufacturer Lilly S.A. Industria) (ElBeltagy et al., 2019). Rats in either DOX or DOX + ATX groups were administered DOX, (2 mg kg^{-1} day^{-1}, 6 i.p. doses, one injection every other day, Manufacturer Ebewe Pharma, Egypt). This dose was modified from Liao et al., 2018. Rats in the control group were given an identical volume of 0.9% sterile saline (6 i.p doses, one injection every other day).

2.3 | Novel location recognition (NLR)

The NLR test used here is a spatial variant of a two trial object recognition task adapted from Dix and Aggleton (Dix & Aggleton, 1999) (Figure 1). The apparatus consisted of an arena (a semi-transparent Perspex box, dimensions; 49-cm wide × 66-cm long × 40-cm high) and pink, weighted water bottles (replicas, 15-cm high, 7-cm diameter). Arenas and water bottles (objects) were cleaned with 20% ethanol prior to each experiment and between trials to remove olfactory

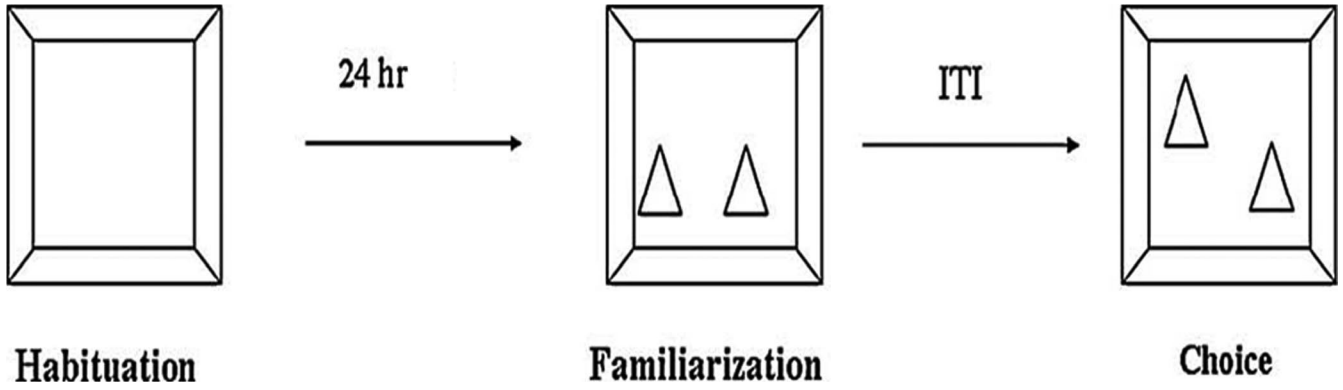

FIGURE 1 The Novel location recognition test protocol was carried out over 2 days. On the first day, animals were allowed to habituate to the test arena for 1 hr. On the following day, two identical objects were placed in different locations of the box with the animal to explore for 3 min (Familiarization trial). Animals were then removed outside the box for 5 min inter-trial interval (ITI) after which the animal was put in the same box after changing the objects location for 3 min (choice trial)

cues. A black square card was on the wall of the room during trials to provide prominent cues for spatial orientation.

This was modified from a previous protocol (Dix & Aggleton, 1999) and was recorded by video camcorder as done previously in our laboratory (Mustafa, Walker, Walker, Bennett, & Wigmore, 2008). The test apparatus consisted of plastic boxes (39 × 23.5 × 30 cm). The procedure consisted of habituating the animals for 1 hr in the box on the day prior to testing. The following day, two identical objects (water bottles) were placed in separate locations in the box and the animals were allowed 3 min to explore (Familiarization trial). Animals were returned to their home cage for 5 min (inter-trial interval) during which the box was cleaned with 20% ethanol. For the choice trial, the animals were returned to the box for 3 min where one object remained in its original position (familiar location) while the other object was moved to a new position (novel location) see (Figure 1).

Exploration of the object was scored when the animal sniffed, licked, chewed or directed its nose at a distance ≤1 cm from the object (Mustafa et al., 2008). (Bruel-Jungerman, Laroche, Laroche, & Rampon, 2005; Dix & Aggleton, 1999).

2.4 | Histology and immunohistochemistry

The day after behavioural testing was completed; rats were put down by rapid stunning and cervical dislocation. The brains were extracted, trimmed and fixed in 3% gluteraldehyde overnight. The following day, the brains were sectioned using Leica vibrating microtome sections (4 mm) and placed onto positively charged slides for routine staining with haematoxylin and eosin (HE) and for IHC. The tissues were dewaxed with xylene and rehydrated through a series of graded ethanols. For antigen retrieval, the samples were autoclaved in 0.01 M sodium citrate, pH6.0, at 121°C for 20 min and then were heated in a microwave oven (800 W) for 5 min (Pisamai, Rungsipipat, Rungsipipat, Kunnasut, & Suriyaphol, 2017). Endogenous peroxidase activity, slides were incubated in H_2O_2 3% and methanol at room temperature for 20 min. Non-specific immunoglobulin binding was blocked with 3%(w/v) bovine serum albumin (Merck, Rockland,Massachusetts, USA) at 37°C for 20 min.

The monoclonal antibody against Ki67 was from Dako (Glostrup, Denmark). The primary antibodies were diluted in Phosphate buffered saline (PBS) at a dilution of 1 in 50. The antibodies were incubated at 4°C for 16 hr. Primary antibody binding was detected by use of a polymer-based non avidine biotin system. The slides were counterstained with Mayer's haematoxylin (Pisamai et al., 2017).

A systemic random sampling technique (Mayhew & Burton, 1988) was used to choose every 21st section throughout the length of the DG (overall 10 sections) using a Zeiss Primo Star microscope (Zeiss) equipped with a Canon EOS 550D camera (Canon).

Ki67 positive cells were counted within the SGZ, defined as within three cell diameters of the inner edge of the DG. Counts from all sections of one DG were averaged to provide a number per section (ElBeltagy et al., 2010).

2.5 | Statistical analysis

Statistical analysis and graphs were created using GraphPad Prism 4.0 and significance was regarded as $p < .05$. The Student *t*-test was used to compare exploration times of animals in the familiarization or choice trials in each group separately. ANOVA with Bonferroni post hoc tests were used to compare the number of proliferating cells between the four groups.

3 | RESULTS

3.1 | Effect of treatment on the Novel location recognition (NLR) task

The NLR test measures interactions with objects either in familiar or novel locations within a test arena. During the familiarization trial, when animals explore two identical objects, both saline and drug treated groups showed no preference for either object or the total

exploration time (Figure 2). Following a 5-min inter-trial interval, one object is moved to a new location (choice trial) and object preference is recorded. Saline injected controls explored the novel object significantly more than the old one ($p = .0001$) Figure 3. Animals treated with ATX explored the novel location significantly more than the old one ($p = .0076$), Figure 3. On the other hand, Animals treated with DOX failed to differentiate between the two locations ($p = .13$), as shown in Figure 3. Co-administration of ATX and DOX did not improve recent memory of the animals ($p = .2$), as shown in Figure 3.

Figure 2 included the results of NLR task in all groups (Familiarisation trial) plotted on one graph. Figure 3 included the results of NLR task in all groups (Choice trial) plotted on one graph.

These findings indicated that animals treated with ATX demonstrated improved memory compared with those saline treated, and that DOX impaired hippocampal recent memory. Our results also suggested that co-administration of ATX with DOX did not improve the memory of adult male rats.

3.2 | Effect of treatments on proliferating cell counts

There was a significant difference in the total number of Ki67 positive cells between all groups ($p < .0001$), as shown in Figure 4 (one way ANOVA). The Bonferroni post hoc test revealed that there was a significant increase in the mean number of proliferating cells in the ATX-treated group compared with saline-, DOX- and ATX + DOX-treated groups ($p < .001$), as shown in Figure 4. Moreover, co-administration of ATX and DOX significantly increased the mean number of Ki67 positive cells compared with the DOX-treated group ($p < .05$). The saline-treated group showed a significant increase in the mean number of Ki67 positive cells compared with the DOX-treated one ($p < .001$), as shown in Figure 4. Figure 5 shows a representative image of Ki67 positive proliferating cells within the DG counter-stained with haematoxylin stain.

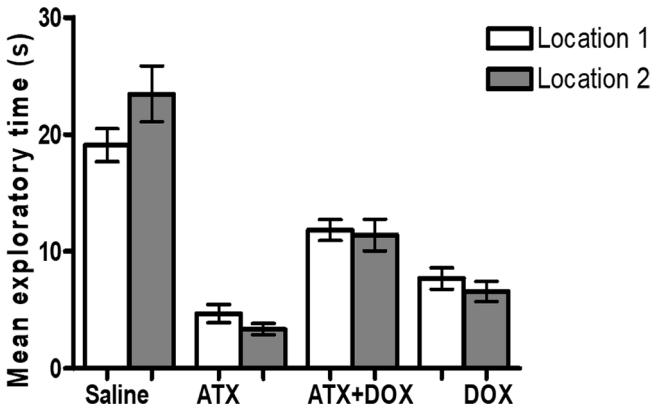

FIGURE 2 Results of the Novel location Rrecognition test during the familiarization trial. There was no difference between either locations in any of the four groups ($p > .05$). The analysis was done by GraphPad Prism 4.0 using student *t*-test to compare between location 1 and location 2 in each group separately

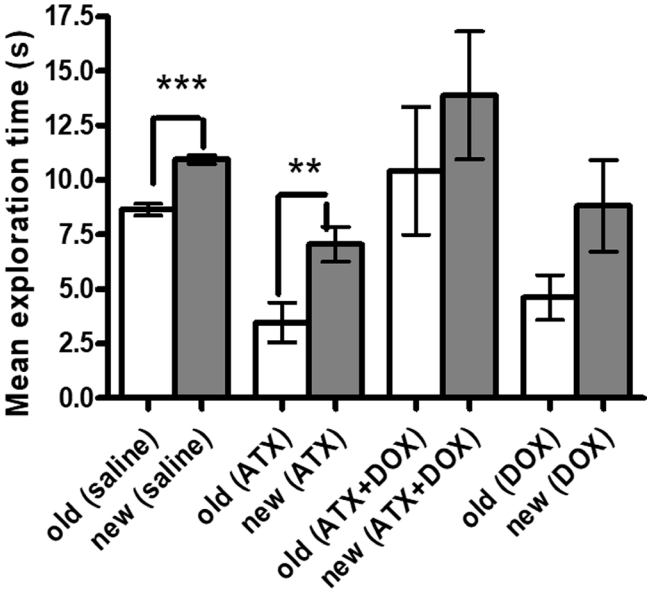

FIGURE 3 Results of the Novel location Rrecognition test during the choice trial. Saline injected controls explored the novel location significantly more than the old one ($p = .0001$). Animals treated with ATX significantly explored the novel location more than the old one ($p = .0076$). Animals treated with DOX failed to differentiate between the two locations ($p = .13$). Co administration of ATX and DOX did not improve recent memory of the animals ($p = .2$). The analysis was done by GraphPad Prism 4.0 using student *t*-test to compare between the old and new locations in each group separately

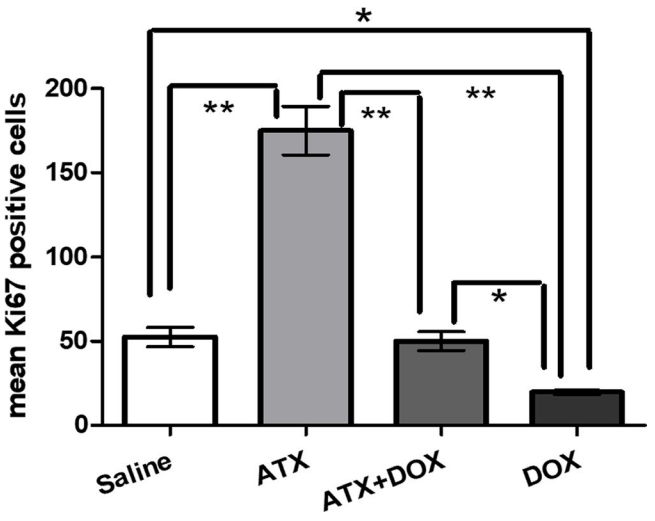

FIGURE 4 Effect of treatment on the proliferating cell count. There was a significant difference in the total number of Ki67 positive cells between all groups ($p < .0001$), (one way ANOVA). Bonferroni post hoc test revealed that there was a significant increase in the mean number of proliferating cells in ATX treated group compared to Saline, DOX and ATX + DOX treated groups ($p < .001$). Co-administration of ATX and DOX significantly increased the mean number of Ki67 positive cells compared to DOX treated group ($p < .05$). Saline treated group showed significant increase in the mean number of Ki67 positive cells compared to DOX treated ($p < .001$)

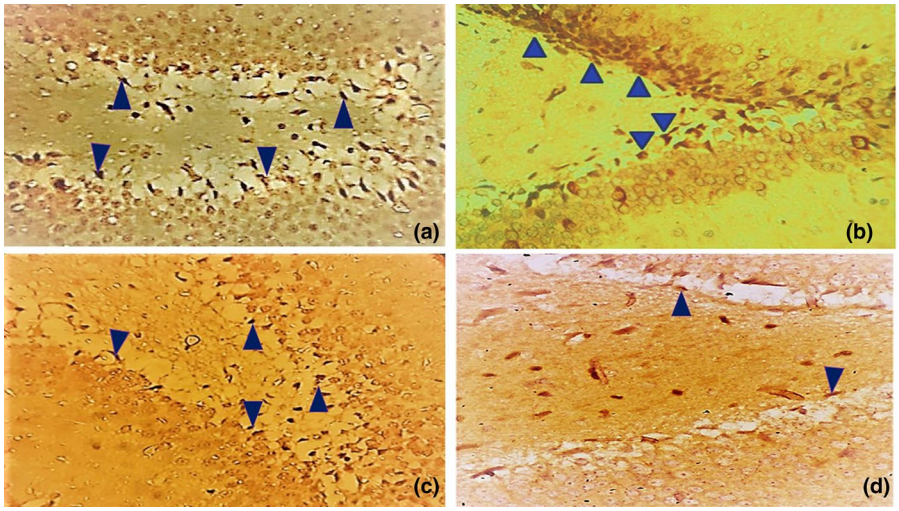

FIGURE 5 Showing representative immunohistochemistry photos taken from the dentate gyrus of (a) saline, (b) ATX, (c) ATX + DOX, (d) DOX treated groups. Ki67 positive proliferating cells (arrows) appear dark indicating proliferation. X400

4 | DISCUSSION

This study aimed to address the effect of ATX, DOX and co-administration of ATX and DOX on memory and proliferation in the adult rat hippocampus.

ATX is a noradrenaline reuptake inhibitor used to treat ADHD (Attention deficit hyperactive disease) and to increase cognition in normal individuals (Fitzgerald, 2011; Pringle, McCabe, McCabe, Cowen, & Harmer, 2013; Sara, 2009).

In this study and also in a previous one, we showed that ATX improves memory of the adult rat brain (ElBeltagy et al., 2019). Spatial memory was tested using the NLR test. Rats that received five intraperitoneal injections of ATX spent significantly more time exploring the novel object than the old one. In agreement with current findings, Tzavara et al., 2006, ElBeltagy et al., 2019 have reported that ATX improved memory deficit in object recognition tests and the radial arm maze test. They investigated the precognitive properties of ATX in two distinct behavioural assays, the 8-arm radial arm maze and the object recognition test, which are used to test memory in animals. Their results showed that ATX increased the time spent by rats interacting with the novel object in the object recognition test and decreased errors in spatial pattern recognition in the radial arm maze, suggesting improved memory performance in both tasks (Tzavara et al., 2006).

The effect of ATX on memory has been investigated in several animal studies and it has been shown to have a positive impact on cognition and memory (Tamburella, Micale, Micale, Mazzola, Salomone, & Drago, 2012; Tzavara et al., 2006; Warner & Drugan, 2012). On the other hand, Adriamycin (DOX) has been shown to impair performance of animals in novel place recognition, memory retention and contextual fear conditioning tasks (Christie et al., 2012) (Liedke et al., 2009; Macleod et al., 2007). Although chemotherapy increases the survival rate of patients with various cancers, such treatment can induce acute or long-term cognitive dysfunction; a phenomenon known as post-chemotherapy cognitive impairment (PCCI) or "chemobrain". Our results were confirmatory to these findings in that animals injected with DOX failed to distinguish between the new and old locations in the NLR task. Moreover, co-administration of DOX and ATX did not improve animal performance in the NLR task.

It is believed that the SGZ of the hippocampal DG and the subventricular zone of the lateral ventricles are two regions of the mammalian brain that are the sites of neurogenesis throughout life (Bekiari et al., 2015). However, there is some controversy whether human neurogenesis persists in the hippocampus with age or not (Sorrells et al., 2018) Some workers report a reduction of adult hippocampal neurogenesis (Dennis, Suh, Suh, Rodriguez, Kril, & Sutherland, 2016; Knoth et al., 2010), while others are supportive of the persistence of neurogenesis with age (Boldrini et al., 2018; Spalding et al., 2013). Newly generated neurons in the DG are closely involved in learning and memory (Abrous, Koehl, Koehl, & Moal, 2005). On the other hand, it has been well known that neurogenesis in the brain can be altered by various factors, such as pharmacological drugs, diet, exercise and ageing (Beltz, Tlusty, Tlusty, Benton, & Sandeman, 2007; Lee et al., 2010; Lloyd et al., 2010; Meng et al., 2006; Park & Lee, 2011; Schiavon et al., 2010; van Praag, Christie, Christie, Sejnowski, & Gage, 1999).

Persisting proliferation in the adult hippocampus, highly reflects the continuous, interaction between brain structure and function. Correlation of the above presented findings with the change in hippocampal proliferation could further contribute to our current understanding of the mechanism by which ATX and DOX affect memory. Besides, it was suggested that cognitive brain functions, such as learning and memory, involve increased proliferation in the hippocampus (Kempermann, Jessberger, Jessberger, Steiner, & Kronenberg, 2004).

Our results have shown that ATX-treated rats had significantly increased level of hippocampal proliferation compared with saline, DOX and the group co-treated with ATX and DOX, as measured by the positive number of Ki67 cells in the DG of the hippocampus.

It has been well known that hippocampal neurogenesis is suppressed by adjuvant chemotherapy in many animal models (Christie

et al., 2012; ElBeltagy et al., 2010; Lyons, ElBeltagy, Bennett, et al., 2011; Lyons, ElBeltagy, Umka, et al., 2011; Macleod et al., 2007; Mustafa et al., 2008; Mustafa et al., 2008; Seigers et al., 2016). Furthermore, DOX alone or in conjunction with other chemotherapeutics negatively affects hippocampal proliferation, survival and differentiation of new neurons (Christie et al., 2012; Kitamura et al., 2017; Liedke et al., 2009; Orchard, Gaudier-Diaz, Gaudier-Diaz, Weinhold, & Courtney DeVries, 2017; Park et al., 2018; Rendeiro et al., 2016).

Previously, we have shown that ATX injection of rats significantly increased the level of hippocampal neurogenesis compared with saline injection (ElBeltagy et al., 2019),"article In Press". The current finding is confirmatory to our previous results. Besides, co-administration of ATX and DOX significantly improved the level of hippocampal proliferation compared with those treated with DOX alone as measured by Ki67 positive cell counts. It has been proven that ATX significantly up regulated levels of brain-derived neurotrophic factor (BDNF) (Fumagalli et al., 2010) (Banerjee, Aston, Aston, Khundakar, & Zetterstrom, 2009). Moreover, studies have shown that increased NE and DA neurotransmission can increase neuronal BDNF expression within the hippocampus (Ivy, Rodriguez, Rodriguez, Garcia, Chen, & Russo-Neustadt, 2003; Liu et al., 2015; Ramos-Quiroga et al., 2014). On the other hand, BDNF levels were markedly reduced by DOX treatment (Heinen et al., 2016; Jaboin, Kim, Kim, Kaplan, & Thiele, 2002) and this may also contribute to the chemofog symptoms of cancer patients under chemotherapy treatment. One of the possible mechanisms by which ATX increased hippocampal proliferation in our experiment could be the elevation of BDNF levels in the hippocampus, and this should be further assessed. To our knowledge, our study is the first that used both ATX and DOX together, and the picture of the neuroprotective effect of ATX against DOX will be painted more clearly by further investigating the levels of hippocampal BDNF.

5 | CONCLUSION

Our results conclude that DOX chemotherapy can negatively affect both cell proliferation and memory, while ATX co-administration improves proliferation, but not memory in the adult male rat hippocampus, which could be of help in future treatments of cancer patients.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTION

Ahmed Salman: Data curation; Formal analysis; Investigation; Project administration; Resources; Software; Supervision; Visualization; Writing-original draft. **Maha El beltagy:** Conceptualization; Data curation; Methodology; Resources; Software; Supervision; Writing-original draft; Writing-review & editing. **Amjad Shatarat:** Formal analysis; Funding acquisition; Supervision; Visualization. **Loai Alzghoul:** Funding acquisition; Project administration; Visualization. **Liyana Oweis:** Data curation; Methodology; Software; Visualization; Writing-original draft. **Nada AlAntary:** Data curation; Methodology; Software; Visualization; Writing-original draft. **Safa AlFegie:** Data curation; Methodology; Software; Visualization; Writing-original draft. **Maram Mohsen:** Data curation; Methodology; Software; Visualization; Writing-original draft. **Salma Salman:** Writing-review & editing.

ETHICS APPROVAL

All experiments and animal care were in accordance to The University of Jordan Home office guidance regulations and with local ethical committee approval.

ORCID

Ahmed Salman https://orcid.org/0000-0002-4382-3125
Maha El Beltagy https://orcid.org/0000-0002-4777-2388
Amjad Shatarat https://orcid.org/0000-0002-2101-0174
Loai Alzghoul https://orcid.org/0000-0001-9499-4840

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