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Progesterone Reversed the Trimethyltin-Induced Injury on the Histo-Architectural Integrity of the Hippocampus of Adult Male Wistar Rat

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ABSTRACT

The generation of new neurons occur throughout life in specific parts of the central nervous system. In order to further understand the concept of neuroregeneration and the mechanisms involved in these parts, this study focused on creating a disease model of the hippocampus of adult male Wistar rats using trimethyltin, which was further treated with progesterone to aid possible regeneration. Twenty four adult male Wistar rats were divided into three groups; Control (0.2 mL of normal saline), trimethyltin (TMT, 8 mg/kg stat dose only) and trimethyltin and progesterone (TMT-PROG, 8mg/kg stat dose and subsequently 16 mg/kg of progesterone). All administrations were intra-peritoneal. The animals were perfused with 4% paraformaldehyde, brains were excised and taken for haematoxylin and EOSIN, Cresyl Violet stain, Ki-67 and neuron specific enolase (NSE) staining. The results showed defragmented nuclei, disintegrated Nissl bodies, reduced number of Ki-67 positive cells and reduced NSE positive cells count in the hippocampus of the TMT group; these neuronal insults were more in Cornus Ammonis (CA2) and CA3 compared to CA1 and CA4. The rats in TMT-PROG showed cell resuscitation; presence of intact nuclei and of Nissl bodies, and significant increased number of positive NSE and ki-67 proteins positive cells in the hippocampus compared to the rats in TMT. The resuscitation of these cells were better in CA1 and CA4 compared to CA2 and CA3. This study concludes that progesterone has the potential to restore the integrity of hippocampal cells after trimethyltin induced hippocampal injury in adult male Wistar rats.

Key words: *Hippocampus, Progesterone, Neuroregeneration, Trimethyltin, Ki-67*, *Neuron specific enolase*

INTRODUCTION

The adult central nervous system (CNS) has been shown to undergo cell division throughout life in some areas, for example, the olfactory bulb and the hippocampus (Seri et al. 2006). Damage to the CNS leads to neurodegeneration, which forms the basis of most brain disorders, such as Alzheimer's disease, Huntington's disease and Parkinson disease. The mechanism(s) that underline the process of neurode-generation is not well-understood. At present, there seems to be limited treatment options for the management of brain damage that is related to neurodegenerative diseases. The medications that are currently used for patients with certain brain disorders and damage are either expensive or not readily available. Therefore, the treatment of patients with conditions that originate from neurodegeneration are still under intense investigations by researchers in the field of neuroscience (Rubinsztein 2006).

Trimethyltin (TMT) is a colourless to white, sand-like solid with a strong and unpleasant odour, having the

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structural formula $(CH_3)_3Sn$. It is widely used in agro-allied companies, primarily as insects, bacteria and fungi control agent, and also in preserving wood, textiles, leather and paints. It has been known to be a potent neurotoxicant which produces a dose-dependent degeneration of neurons, primarily by inducing neuronal death in the hippocampus and other parts of the limbic system (Whittington et al. 1989). These deaths can lead to conditions like reactive gliosis, epilepsy and neurobehavioural alterations (Geloso et al. 2004).

Progesterone has been implicated in brain functions, where it is synthesized and secreted by the nervous tissues (King and Brucker 2010). Progesterone has been reported to have regenerative potentials on the damaged neurons (Zhang et al. 2010), by increasing the concentration of macrophages and microglia at injured sites (Schneider et al. 2003), and also the circulation of endothelia progenitor cells in the brain (Espinoza and Wright 2011). Therefore, this study sets out to investigate the effects of progesterone on the trimethyltin-induced hippocampal damage in adult male Wistar rats.

MATERIALS AND METHODS

Animal Treatments

Twenty four adult male rats (*Rattus novergicus*), weighing between 220-250g were used for this study. The rats were procured from the animal holdings in the Department of Zoology, University of Ilorin, and were allowed to acclimatize in the animal house of the College of Health Sciences, University of Ilorin for 14 days prior to commencement of the experiments. The animals were housed in cages under normal light/dark cycle and at room temperature/humidity. Food and water were available *ad libitum*. Ethical approval for this study was obtained from the University of Ilorin Ethical Review Committee.

The rats were randomly divided into three groups (Control, TMT and TMT-PROG, n = 8 per group). The animals in the control group were given 0.2 mL of normal saline (vehicle, i.p.) for 26 days. TMT and TMT-PROG rats were both given trimethyltin (8 mg/kg, i.p.) only on the first day, and were monitored for 21 days (Brock and O'Callaghan 1987). TMT rats was sacrificed on day 21, while TMT-PROG group additionally received progesterone (16 mg/kg, i.p.) for 5 days starting from day 22 as stated below. The administration of progesterone on the first day, was given as follows; first dose at 1-hour post-injury for rapid absorption; the second dose at 6 hour after the 1st progesterone dose, for gradual absorption, and subsequently in other days a single dose of progesterone was given every 24 hours for 5 days (Cutler et al. 2006; Chen et al. 2008; Gilmer et al. 2008; Li et al. 2012).

Tissue Collection

After perfusion has been completed, the whole brain tissues were excised and were post-fixed in 4% paraformaldehyde overnight. The whole hippocampal CA regions were excised and equilibrated in 30% sucrose solution, before histological, histochemical and immunohistochemical analysis. The sections were taken at 2 µm on paraffin wax embedded tissue blocks and mounted on a glass slide.

All antibodies were procured from Dianova (GmbH/ Warbugstr. 45/20354 Hamburg. Also, reagents and buffers used in this study were molecular biology grade (99.9% pure) from Sigma-Aldrich.

Histological Studies

The haematoxylin and eosin (H and E) staining technique was used to demonstrate the general histo-architecture of the cells; to show location of normal or abnormal nucleus of the hippocampal cells.

Cresyl violet: This technique was used to demonstrate Nissl bodies (endoplasmic reticulum and ribosomes) in the cells as well as normal or abnormal protein synthesis in the cytoplasm of the cells of the hippocampus.

Immunohistochemistry

Ki-67 antibody and Neuron Specific Enolase (NSE) antibody (human monoclonal; Elisa and microarray) were used to identify proliferation and regeneration of cells respectively, in the hippocampus of adult male Wistar rats. The method employed was the avidin biotin complex method also referred to the avidin biotin immunoperoxidase method. The antibody dilution factor used was 1:100 for all the antibody markers. The processed tissues were sectioned at two microns on the rotary microtome and placed on a hotplate at 70 °C for at least an hour.

Quantitative and Statistical Analysis

The images were acquired using an Optronics Digital Camera connected to a computer interface (MagnaFire) and an Olympus BX-51 research microscope. The total cell count was determined using a 10 × 10 grid graticle at 400× magnification. For the corona sections, n = 2 fields were captured to obtain n = 4 fields per section. Subsequently, the average count for n = 4 fields was determined as the representative value (Ogundele et al. 2015). Immunopositive cells in both Ki-67 and NSE were counted using software package called imageJ (1.48v). The statistical package for social sciences (SPSS, version 20) was used for statistical analysis and Graphpad Prism (version 5.01) was used to draw the bar charts. All data were analyzed using one-way analysis of variance. All values were reported as Mean ± standard error of mean (SEM) and $p < 0.05$ was considered statistically significant.

RESULTS

Pattern of histological changes of the effects of progesterone on the hippocampus after trimethyltin induced cellular damage. In the trimethyltin treated rat, cellular injury was observed in the pyramidal layer of the hippocampus, which was demonstrated by nuclear fragmentation and vacuolization (Figure 1B). The rats which were exposed to trimethyltin and were treated with progesterone, showed reduction in the progression of cellular injury in the cells of the pyramidal layer of the hippocampus (Figure 1C).

Pattern of changes of the effects of progesterone on the hippocampus after trimethyltin induced cellular damage showed dispersed and disintegrated Nissl bodies in the cytoplasm of the pyramidal cells (Figure 2B). This is evidence of cellular chromatolysis (a precursor of apoptosis) occurring in the pyramidal layer of the hippocampus. The introduction of progesterone after trimethyltin exposure was able to restore the integrity of these cells, by the presence of Nissl bodies that were observed in the cytoplasm of the cells (Figure 2C).

The proliferating cells of the hippocampus of adult male Wistar rats, were marked by Ki-67 antibody. This is evident in Figure 3A, which indicated a significant decrease in hippocampal cell proliferation in rats treated with trimethyltin alone (TMT group). Also, there was a significant increase in number of proliferating cells in the hippocampus of rats that received progesterone after trimethyltin administration (TMT-PROG) at $p<0.05$ compared to the control and TMT groups.

Regenerating cells in the hippocampus of adult male Wistar rats were marked with NSE antibody. Figure 3B revealed that there was significant decrease in regenerating cells in the hippocampus of rats treated with trimethyltin alone (TMT group). Furthermore,

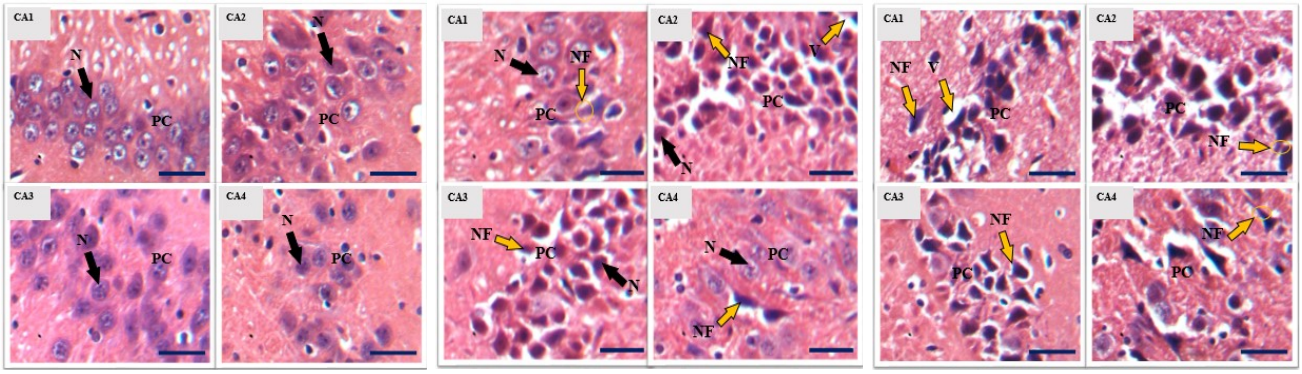

Fig. 1A Fig. 1B Fig. 1C

Figure 1: Representative photomicrographs of histological (haematoxylin and eosin) staining of the Cornus Ammonis (CA) 1, 2, 3 and 4: pyramidal cells (PC) of the hippocampus. Fig 1A; Array of normal neurons (N) with intact nucleus indicated by dark arrows, found within the pyramidal layers of the hippocampus. Fig 1B; Details of nuclei fragmentation (NF) and vacuolization (V) indicated by the yellow arrows, in the pyramidal cells. Fig. 1C; Details of normal neurons (N) indicated by black arrows with few cell undergoing nuclei fragmentation (NF) and vacuolization (V) indicated by yellow arrows in the pyramidal layers of the hippocampus.

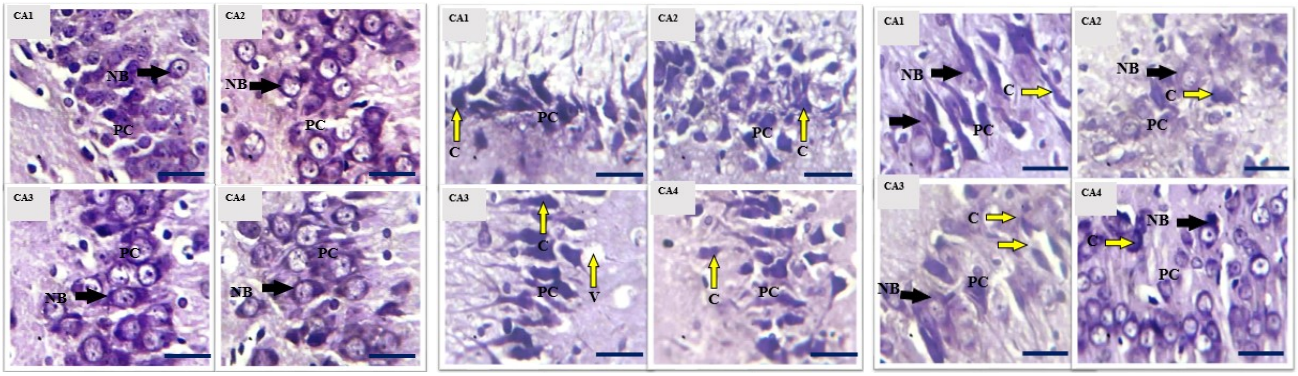

Fig. 1A Fig. 1B Fig. 1C

Figure 2: Representative photomicrographs of Cresyl violet staining of Cornus Ammonis (CA) 1, 2, 3 and 4 pyramidal cells (PC) of the hippocampus. Fig. 2A; Indicated by the black arrows are normal neurons with presence of Nissl bodies (NB). Fig 2B; The yellow arrows point to the location of neurons that have lost their Nissl bodies (disintegration) and also to neurons with dispersed Nissl bodies in their cytoplasm i.e. chromatolysis (C) in the cells of pyramidal layer of hippocampus. Also, there is evidence of vacuolization (V) of neurons. Fig 2C; Presence of Nissl bodies (NB) indicated by the black arrows and yellow arrows point to the location of neurons with dispersed Nissl bodies and neurons that have lost their Nissl bodies indicating chromatolysis (C) and vacuolization (V).

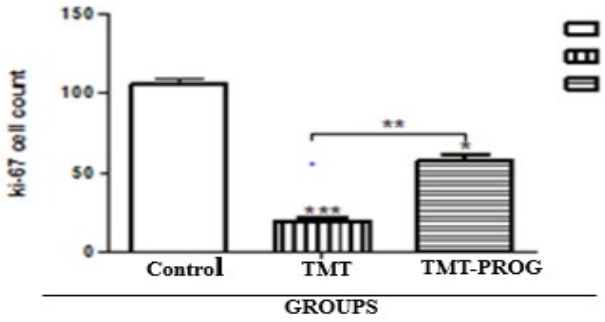

Fig. 3A

there was a significant increase in the number of regenerating cells in the hippocampus of rats that received progesterone after trimethyltin administration (TMT-PROG) group at $p < 0.05$ compared to control and TMT groups.

DISCUSSION

Trimethyltin is a neurotoxin that affects the limbic system and associated structures (Whittington et al. 1989). The intraperitoneal introduction of trimethyltin

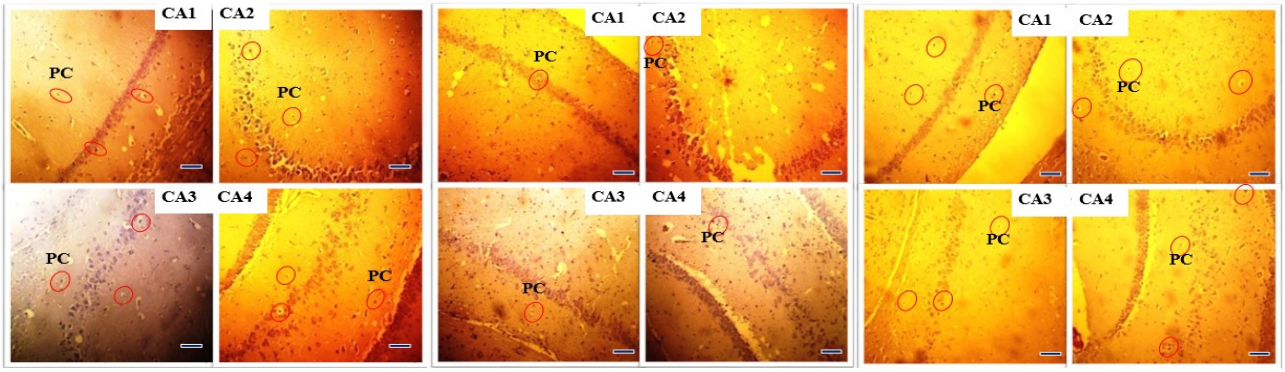

Fig. 3A1 Fig. 3A2 Fig. 3A3

Figure 3A (1-3): Representative photomicrographs with Ki67 antibody on Cornus Ammonis (CA) 1, 2, 3 and 4 of the hippocampus of adult male Wistar rats. Encircled in the red rings are cells undergoing proliferation in the hippocampus

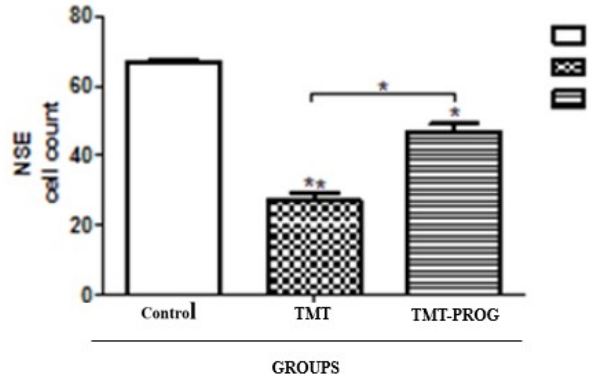

Fig. 3B

in the Wistar rats disrupted the cyto-architecture of the hippocampus of these animals. Also, progesterone was also administered to some of these rats and most of the cyto-architectural integrity of the hippocampus of these animals were restored. Furthermore, progesterone significantly increased the number of proliferating and regenerating cells in these animals. These findings can be linked to one of the properties of progesterone which has been established, that is, progesterone increases the amount of endothelia progenitor cells in the circulatory system (Espinoza and Wright 2011).

To ascertain these findings; histological and immunohistochemical procedures were carried out on the

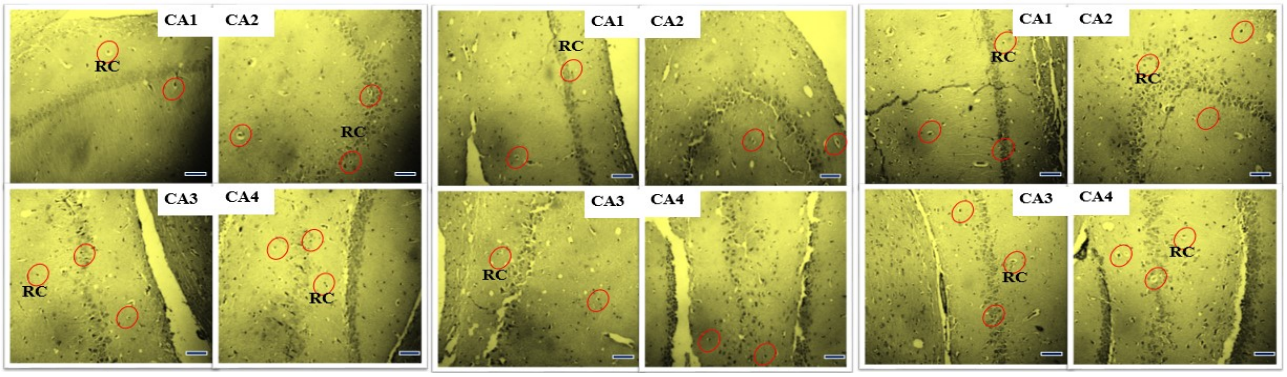

Fig. 3B1 Fig. 3B2 Fig. 3B3

Figure 3B (1-3): Representative photomicrographs of NSE antibody on Cornus Ammonis CA) 1, 2, 3 and 4 of the hippocampus of adult male Wistar rats. Encircled in the red rings are cells undergoing regeneration in the hippocampus.

hippocampus of trimethyltin-administered rats, and possible ameliorative effects of progesterone on these alterations.

The pyramidal cells of the hippocampus of rats showed that normal saline has no deleterious effect on the neurons found in the Cornus Ammonis (CA1-CA4) of the hippocampus. They appeared to have intact nuclei, which is evidence of normal neuronal cyto-architecture. The rats that received trimethyltin alone showed extrusion of the nucleus from the cytoplasm of the cells, which is a characteristic evidence of nuclear fragmentation. The rats that received trimethyltin followed by progesterone, showed no evidence of neuronal nuclear fragmentation and vacuolization in the pyramidal layers of CA1-CA4, as these neurons showed presence of normal nuclei and intact cytoplasm. The degenerative changes observed in this study as a result of exposure to trimethyltin underlie the ability of TMT to initiate cell death (McMillan 1987; Robertson et al. 1987; Thompson et al. 1996; Geloso et al. 1997; Fiedorowicz et al. 2001). These confirmed extensive damage to the pyramidal neurons of the hippocampus of rats after exposure to trimethyltin. The ameliorative effects confirmed some other functions of progesterone that is not reproductive, which exist in the central nervous system. These functions include; mitochondrial function, neurogenesis and neuroregeneration (Brinton et al. 2008). The recovery noticed in the pyramidal cells of the rat hippocampus in this study may be as a result of the influence of progesterone given after trimethyltin in the treatment where progesterone was able to rescue neurons from the insult caused by trimethyltin, that had compromised the integrity of the cells of the hippocampus of rats (He et al. 2004; Carroll et al. 2007; Stein 2008; Li et al. 2012).

In addition, this study showed that rats that received normal saline have intact hippocampal cyto-architecture and densely stained Nissl bodies in most of the neurons in the pyramidal layers of CA1-CA4, whereas those that received only trimethyltin, revealed disintegrated and dispersed Nissl bodies (chromatolysis) in the cytoplasm of most neurons in the pyramidal layers of the hippocampus. The rats that received trimethyltin followed by progesterone, revealed that some of the neurons found in the pyramidal layers still possessed Nissl bodies in their cytoplasm. The degenerative changes observed in the hippocampus of the rat in this study on exposure to TMT, and which led to dispersed and disintegration of Nissl bodies, might cause abnormal gene expression due to disrupted protein synthesis in the neurons. Chang et al. (1983), McMillan (1987) and Tandrup (2003) confirmed chromatolytic changes in the hippocampus of rats as a result of TMT exposure. The ability of progesterone to repair neurons damaged by TMT, as shown in this study, is in line with the findings of He et al. (2004b), Stein (2008), Carroll et al. (2007), González et al. (2009) and Li et al. (2012).

Quantitative analysis test was done to count ki-67 positive cells across the treatment groups and compare to the control group. This study revealed significant reduction in Ki-67 positive cells in the hippocampus of rats in groups TMT and TMT-PROG compared to the Ki-67 positive cells in the hippocampus of rats in the control groups. This indicates that trimethyltin caused the inhibition of Ki-67 protein which is necessary for cell proliferation. Although, the number of ki-67 positive cells in the hippocampus of rats in TMT-PROG group was significantly increased when compared to the TMT group, this may suggest that progesterone was able to modulate Ki-67 antigen in trimethyltin-induced hippocampal injury leading to the activation of Ki-67 and initiation of cell proliferation in trimethyltin-induced hippocampal injury in the adult male Wistar rats, as suggested by Wang et al. (2005), who reported that neurosteroid allopregnanolone promotes cell proliferation of rodent, regulates cell-cycle gene and protein expressions. Barha et al. (2011) also reported that treatment with progesterone normalizes the levels of cell proliferation in the dentate gyrus of the hippocampus after traumatic brain injury.

Quantitative analysis test was done to count NSE positive cells across the treatment groups and compare to the control group. Control and TMT groups served as positive and negative controls respectively. NSE antibody was used to determine the numbers of cells that are regenerating as a result of influence of progesterone on the trimethyltin-damaged neurons. The rats in groups TMT and TMT-PROG showed significantly reduced numbers of NSE positive cells in the hippocampus compared to the control group. The rats in group TMT-PROG showed no significant change in the number of NSE positive cells in their hippocampus compared to the control group, but showed significant increase compared to the NSE positive cells in the TMT group. Furthermore, the TMT-PROG group showed significantly increase in NSE positive cells compared with the TMT group, and stimulated regeneration of these neurons. This study supports the findings of Wang et al. (2005) and Zhang et al. (2010), who reported that 'progesterone promotes the survival of newborn neurons in the dentate gyrus of adult male mice, and that treatment with progesterone normalizes the levels of cell proliferation in the dentate gyrus of the hippocampus after traumatic brain injury, respectively. Furthermore, it is reported that progesterone increased circulating endothelial progenitor cells and induced neural regeneration after traumatic brain injury in aged rats (Li et al. 2012).

Conclusion

This work demonstrated the activities of progesterone and trimethyltin in the hippocampus of adult male

Wistar rats. The progesterone acts as an ameliorative agent against the insult or damaging effects of trimethyltin. It was observed that progesterone restored the cyto-architectural integrity in the hippocampus of the adult male Wistar rats by reducing the progression of chromatin condensation in the nucleus of the neurons. It also played a role in adult neurogenesis in the hippocampus of the rats by activating Ki-67 antigen and the glycolytic pathway needed for cell proliferation and cellular regeneration respectively. This indicated that progesterone stimulated neural stem cells growth factors (neurotrophins and cytokines) and also regulated the glucose metabolism of the neurons which are responsible for cell proliferation and regeneration respectively. Thus, this study concludes that intra-peritoneal administration of progesterone showed ameliorative potentials on the hippocampus against the process of apoptosis initiated by trimethyltin.

Conflict of Interest
None declared.

Acknowledgements
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