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Progressive Evaluation of Memory and Hippocampal Histomorphology in Adult Wistar Rats Following Lithium-Pilocarpine-induced Temporal Lobe Epilepsy

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ABSTRACT
Epilepsy is the third most common neurologic disorder, following stroke and Alzheimer's disease. It affects an estimated 1% of the world's population and has no respect for age and gender. This study was designed to examine the histomorphology of neurons in the different sub-fields of the hippocampus, assess the neurobehavioral changes of memory and investigate the time-line dynamics of the above parameters at different phases of epileptogenesis following lithium-pilocarpine-induced TLE in Wistar rats. Sixty 12 weeks old Wistar rats were randomly assigned into 3 groups viz; Control group (15 rats) received 2ml/kg of normal saline intraperitoneally. Sham group (15 rats) was administered diazepam (10 mg/kg) and normal saline 2mL/kg intraperitoneally. Experimental group (30 rats) was administered lithium chloride (127 mg/kg) subcutaneously 24 hours before administration of pilocarpine (30 mg/kg) intraperitoneally. At 90 minutes after seizure onset, the rats received diazepam injection (10 mg/kg) intraperitoneally. The rats were observed for 7-day transient seizure-free period (latent phase), followed by the 7-day spontaneous recurrent seizure period (chronic phase). 5 rats each (from the control and sham groups) and 10 rats from the experimental group were sacrificed at the end of the acute, latent and chronic phases of epileptogenesis. In the acute, latent and chronic phases, there is a significant increase in degenerating neuronal density in the experimental group when compared with control [($p<0.001$), ($p<0.001$) and ($p<0.001$) respectively] and sham [($p<0.001$), ($p<0.001$) and ($p<0.001$) respectively] groups, but no significant difference was noted between the control and sham groups [($p=0.11$), ($p=0.62$) and ($p=0.57$) respectively] across the phases. Memory impairment was only observed when experimental groups were compared with control and sham groups across the three phases of epileptogenesis. Neurodegenerative features observed in different hippocampal sub-fields gives a better explanation about memory impairment associated with this condition and further unravels the scientific basis of epileptogenesis following lithium-pilocarpine-induced TLE

Key words: TLE, lithium-pilocarpine, epileptogenesis, memory, hippocampus

INTRODUCTION
Epilepsy is the third most common neurologic disorder, following stroke and Alzheimer's disease[1].It affects an estimated 1% of the world's population without any age or gender discrimination [2]. Temporal lobe epilepsy (TLE) is the most common form of epilepsy in humans[3]. In TLE, seizures spread to neighboring cortices and there is hippocampal neuronal loss[4]. It is one of the most common form of drug-refractory epilepsy [5]. Affected patient often have similar clinical history, including an initial precipitating injury such as febrile convulsions, status epilepticus (SE), or trauma[6]. Between this injury and the emergence of recurrent complex partial seizures, there is usually a latent period of several years. Epilepsy is usually associated with hippocampal sclerosis [7]. The hippocampus has been identified as a major structure related to cognitive function such as memory [8, 9]. The cellular integrity of the hippocampus have been directly related to the memory status of the individual. In human studies, it's been noted that resection of the temporal lobe alongside the hippocampus results in impairment of memory [10, 11].The degree of sclerosis of hippocampal neurons prior to and post-resection have been found to be directly related to the degree of memory impairment. The lithium-pilocarpine model of TLE in rats is one of the most commonly used experimental model of epilepsy. It reproduces most of the clinical and neuropathological features of human TLE [12, 13].

The state of hippocampal neuron and its relationship with impairment of spatial memory across the phases of TLE is the focus of this study.

MATERIALS AND METHODS
Chemicals and Drugs: Lithium chloride was procured from Sigma-Aldrich (USA). Pilocarpine hydrochloride was purchased from Sigma-Aldrich (USA). Diazepam was procured from F. Hoffmann-La Roche Ltd, Basel (Switzerland). Other reagents used were of analytical grade.

Rat Care and Management: Sixty adult Wistar rats of 12 weeks old were used for this study. The rats were bred at

the Animal Holding of the Department of Anatomy and Cell Biology where they were housed in plastic cages. The animal room was kept under standard laboratory condition of temperature, humidity and light. Rats fed on standard laboratory rat chow (ACE feed, Osogbo, South-West Nigeria, West Africa) and had access to water *ad libitum*. Ethical clearance was obtained from Health Research and Ethics Committee (HREC) of Institute of Public Health, Obafemi Awolowo University, Ile-Ife. The rats received humane care according to the guidelines for the use of animals documented by National Research Council of Thailand, 1999.

Experimental design: Sixty 12 weeks old Wistar rats were used for this study. The rats were randomly assigned into 3 groups {control (15 rats), sham (15 rats) and experimental groups (30 rats)}. The experimental rats were observed for features of seizures (acute phase). At 90 minutes after seizure onset, the rats received diazepam injection at a dose of 10 mg/kg body weight intraperitoneally. Following diazepam administration, the animals were observed for 7-day transient seizure-free period (latent phase), followed by the 7-day spontaneous recurrent seizure period (chronic phase). 5 rats each (from the control and sham groups) and 10 rats from the experimental group were sacrificed at the end of the acute, latent and chronic phases of epileptogenesis.

Experimental acute group served as onset of seizure, experimental latent group is the free interval when there is no seizure and experimental chronic group is characterized with spontaneous recurrent seizures. The experimental grouping explains the progression of epileptogenesis.

Control and sham rats in all groups received normal saline intraperitoneally which served as vehicle for the administration of pilocarpine and lithium chloride. In addition, sham control rats were also administered diazepam at a dose of 10 mg/kg body weight intraperitoneally. Experimental rats were administered lithium chloride at a dose of 3 mEq/kg (127 mg/kg) body weight subcutaneously 24 hours prior to administration of pilocarpine. Pilocarpine was administered at a dose of 30 mg/kg body weight intraperitoneally. After 90 minutes of onset of acute seizure, the experimental rats received diazepam injection at a dose of 10 mg/kg body weight intraperitoneally to minimize mortality. Convulsion gradually reduced and stopped temporarily when the rats slept off.

Neurobehavioural Assessment of Memory: After completion of each epileptic phase, neurobehavioral assessment was carried out with the aid of Radial Arm Maze to assess memory using a one day neurobehavioral protocol. Four out of the eight arms of the maze were baited alternately with standard chow, which was hidden from view in a recess at the end of the arm. Each rat was placed at the center of the maze, and time taken before identification of chowed arm and consumption of the chow was noted [14]. A trial session was done to familiarize the rats with maze and train them to identify the baited arm. Rats were removed from the maze after failure to consume from any of the four baited arms within 2 mins. The sequence of entries into baited arms of the radial maze were recorded during behavioral trials. Behavioral trials time (T1, T2 and T3) were analyzed and the SEM was obtained.

Animal Sacrifice and Histology: Following the completion of the neurobehavioural assessments of the rats, they were sacrificed using intramuscular ketamine anesthesia (90 mg/kg). The skulls were opened carefully to avoid brain tissue damage. The brains were harvested and fixed in 10% Neutral Buffered Formalin (NBF) by immersion. One mm thick coronal brain slice was obtained at the level of the optic chiasma and processed for routine paraffin embedding.

The brain slices were processed via paraffin wax embedding method [15]. Sections of 5µm thickness were obtained on a rotary microtome (Leica RM 2125 RTS) and were stained with Hematoxylin and Eosin for the demonstration of hippocampal neuroarchitecture, LEICA DM750 microscope interfaced with LEICA ICC50 digital camera. Permanent photomicrographs were obtained with scale bars inserted and archived for analysis.

Histological Scoring: Normal and degenerating neurons were identified and counted in the Hematoxylin and Eosin stained sections in the upper and lower cortical regions using [16, 17] as guide. Count was done in 7 high power fields per slide using X40 objective lens. The area of the higher power fields was measured and noted. Data were analyzed using T-test for comparison between two groups. Significant difference was set at $p<0.05$.

RESULTS

Acute Phase Memory Assessment: In the acute phase, result showed significant increase in time spent to identify baited arms in trials 1, 2 and 3 when experimental group was compared with corresponding control [$p<0.001$, $p=0.04$ and $p<0.001$respectively] and corresponding sham [$p<0.001$, $p<0.001$ and $p<0.001$ respectively] -groups but no significant difference was noted when control (Trials 1, 2 and 3) were compared with corresponding sham ($p=0.13$, $p=0.88$ and $p=0.67$ respectively). However, the result showed a significant decrease in time spent to identify baited arms in the control, sham and experimental groups of Trial 2 when compared with corresponding groups of trial 1 ($p=0.01$, $p=0.02$ and $p<0.001$ respectively). Similarly, comparison of the time spent to identify the baited arm between corresponding groups of Trials 1 and 3 showed a significant decrease in Trial 3 across groups (control; $p<0.001$, sham; $p=0.0001$ and experimental; $p<0.001$). Similar decrease in time was noted when corresponding groups of Trial 3 were compared with Trial 2 (control;

p=0.0061, sham; p=0.0019 and experimental; p<0.001) (Fig. 1).

Latent Phase Memory Assessment: The result showed significant increase in time spent to identify baited arms in Trial 1 when experimental group was compared with control (p<0.001) and sham (p<0.001) groups (Fig 1) but no significant difference was observed when control was compared with sham (p=0.90). In trials 2 and 3 the result showed no significant difference in time spent to identify baited arms in experimental group when compared with corresponding control (p=0.14 and p=0.19 respectively) and corresponding sham (p=0.34 and p=0.77 respectively) groups, also no significant difference was observed when control was compared with sham control (p=0.50 and p=0.47). However, the result showed a significant decrease in time spent to identify baited arms when control, sham and experimental groups of Trial 2 were compared with control (p<0.001), sham (p<0.001) and experimental (p<0.001) groups of trial 1. A significant decrease in time spent was also observed when control, sham and experimental groups of trial 3 were compared with control (p<0.001), sham (p<0.001) and experimental (p<0.001) groups of trial 1. Similar reduction in time spent was noted when experimental and sham control groups of trial 3 were compared with experimental (p<0.001) and sham (p<0.001) groups of trial 2. However, there was no significant difference in time spent when control group of trial 2 was compared with control (p=0.11) group of trial 3 (Fig. 1).

Chronic Phase Memory Assessment: The result showed significant increase in time spent to identify baited arms in trial 1 when experimental group was compared with control (p<0.001) and sham (p=0.01) groups but no significant difference was observed when control was compared with sham control (p=0.65). In trial 2 the result revealed a significant increase in time spent to identify baited arms in experimental group when compared with sham (p<0.001) but not control (p=0.31) group but a significant increase was observed when control was compared with sham (p<0.001) group (Fig. 1). Trial 3 showed no significant difference in time spent to identify baited arms in experimental group when compared with control (p=0.33) and sham (p=0.08) groups and no significant difference was observed when control was compared with sham control (p=0.25). However, the result showed a significant decrease in time spent to identify baited arms in control, sham and experimental groups of trial 2 when compared with control (p<0.001), sham (p<0.001) and experimental (p<0.001) groups of trial 1 respectively. Also, a significant decrease in time spent was revealed when control, sham and experimental groups of trial 3 were compared with control (p<0.001), sham (p<0.001) and experimental (p<0.001) groups of trial 1 respectively. The result showed a significant decrease in time spent to identify baited arms when control, sham and experimental groups of trial 3 were compared with control (p<0.001), sham (p=0.01) and experimental (p<0.001) groups of trial 2 respectively(Fig. 1).

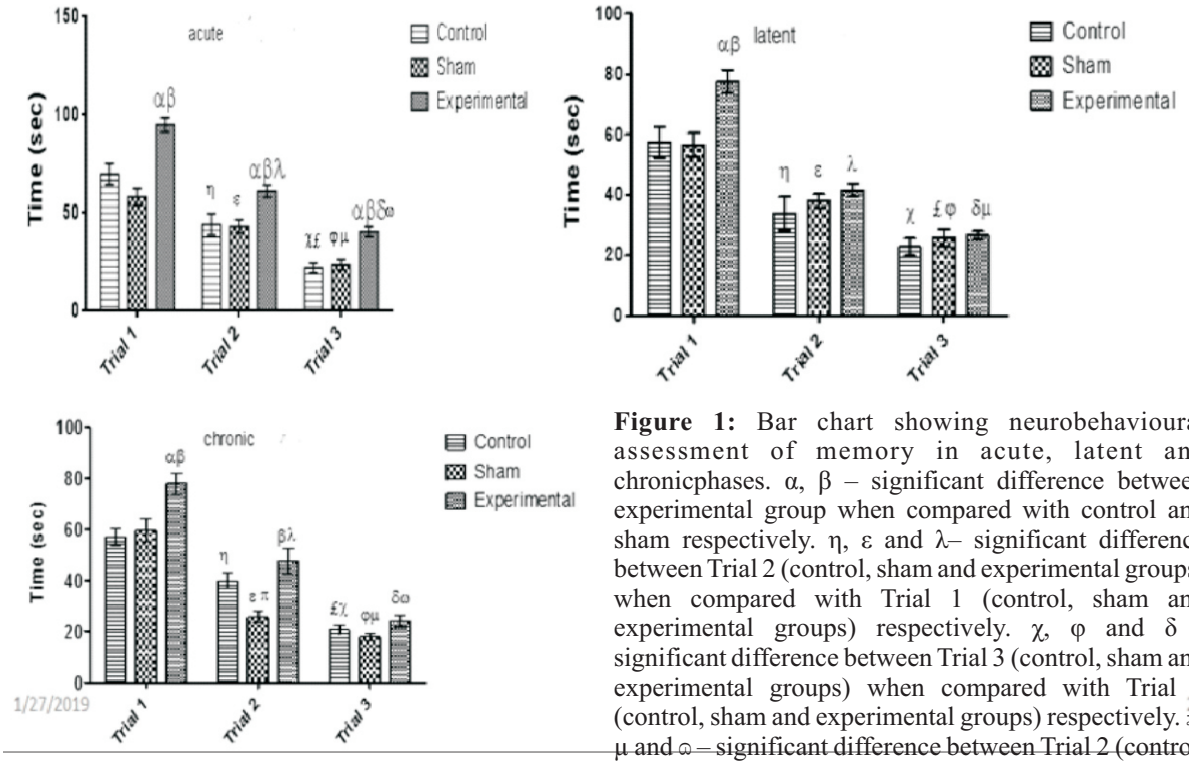

Figure 1: Bar chart showing neurobehavioural assessment of memory in acute, latent and chronicphases. α, β – significant difference between experimental group when compared with control and sham respectively. η, ε and λ– significant difference between Trial 2 (control, sham and experimental groups) when compared with Trial 1 (control, sham and experimental groups) respectively. χ, φ and δ - significant difference between Trial 3 (control, sham and experimental groups) when compared with Trial 1 (control, sham and experimental groups) respectively. £, μ and ω – significant difference between Trial 2 (control, sham and experimental groups) when compared with Trial 3 (control, sham and experimental groups) respectively. Values are expressed as mean±SEM

Histomorphological analysis of the hippocampus: In the acute phase, CA 1,CA 2, CA 3 and DG hippocampal regions showed significant decrease in the density of normal neuron present in the experimental group when compared with control (p<0.001, p<0.001, p<0.001 and p<0.001 respectively) and sham (p<0.001, p<0.001, p=0.03 and p<0.0001 respectively) groups but no significant difference was observed when control was compared with sham control (p=0.21, p=0.56, p=0.96 and p=0.30 respectively]. However, degenerating neuronal density in the above stated hippocampal regions was significantly increased in the experimental group when compared with control (p<0.001, p<0.001, p<0.001 and p<0.001 respectively) and sham (p<0.001, p<0.001, p<0.001 and p<0.001 respectively) groups but no significant difference was noted when control was compared with sham in the above stated hippocampal regions (p=0.13, p=0.80, p=0.01 and p=0.90 respectively)(Figs. 2,3,4 and 5).

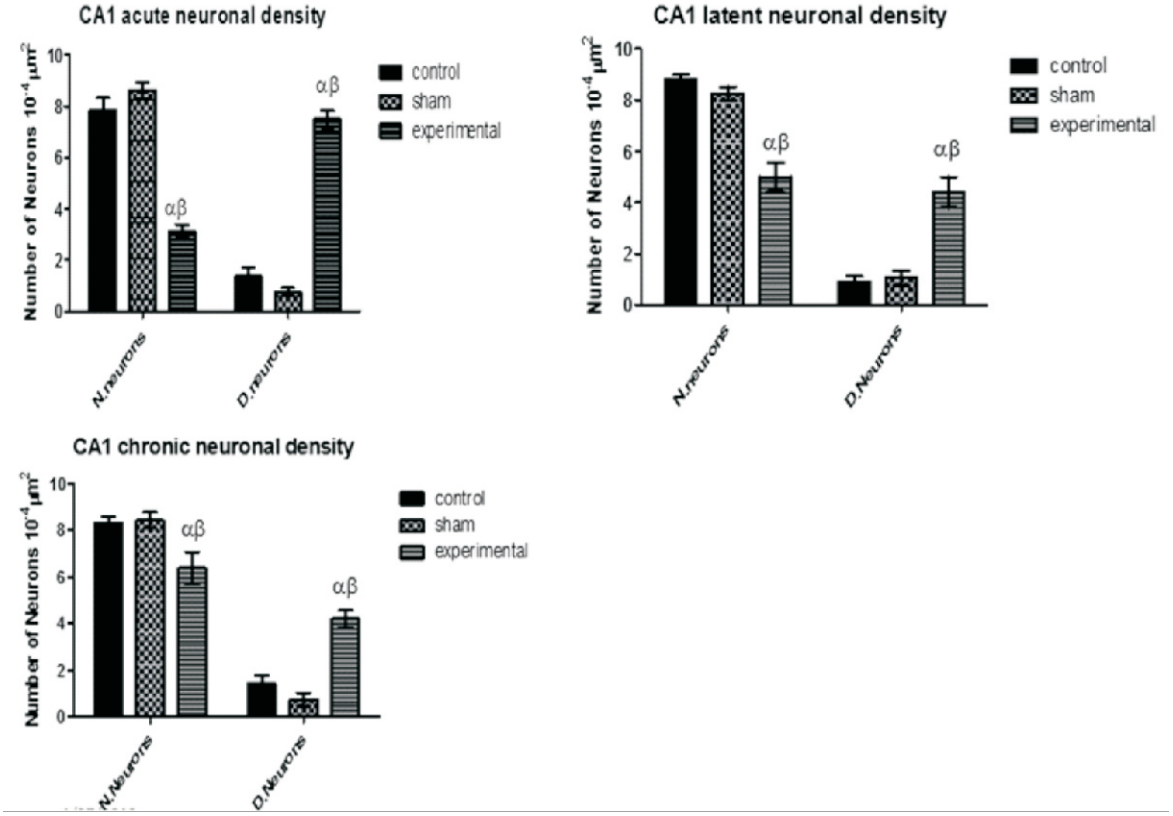

Figure 2: Bar chart showing neuronal density of CA1 hippocampal sub-field of acute, latent and chronic phases.
α, β – significant difference between experimental group when compared with control and sham groups respectively. N. Neurons – Normal Neurons; D. Neurons – Neurons showing degenerating features. Values are expressed as mean±SEM

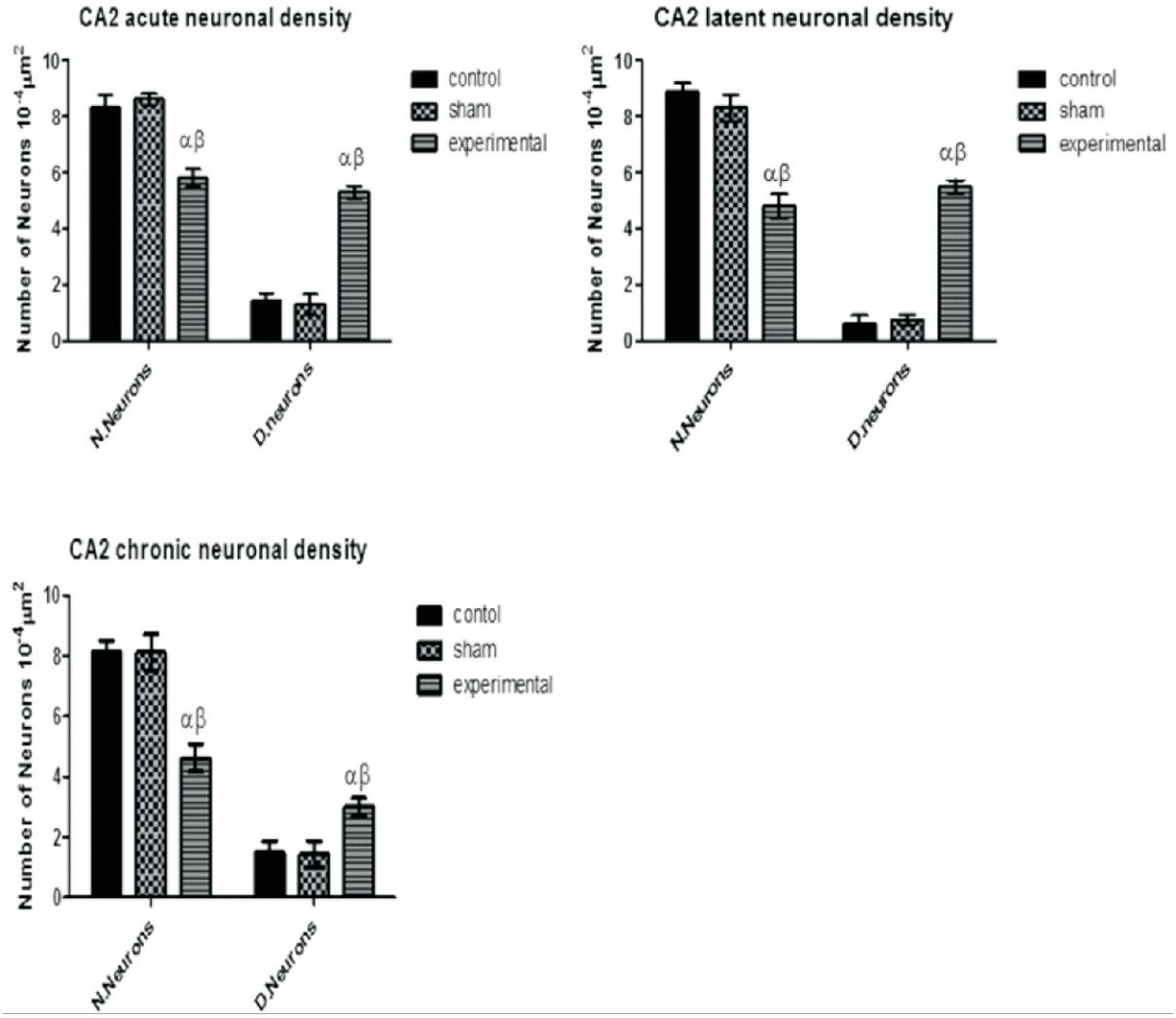

Figure 3: Bar charts showing neuronal density of CA2 hippocampal sub-field of acute, latent and chronic phases. **α, β –** significant difference when experimental group is compared with control and sham control groups respectively. N. Neurons – Normal Neurons; D. Neurons – Neurons showing degenerating features. Values are expressed as mean±SEM

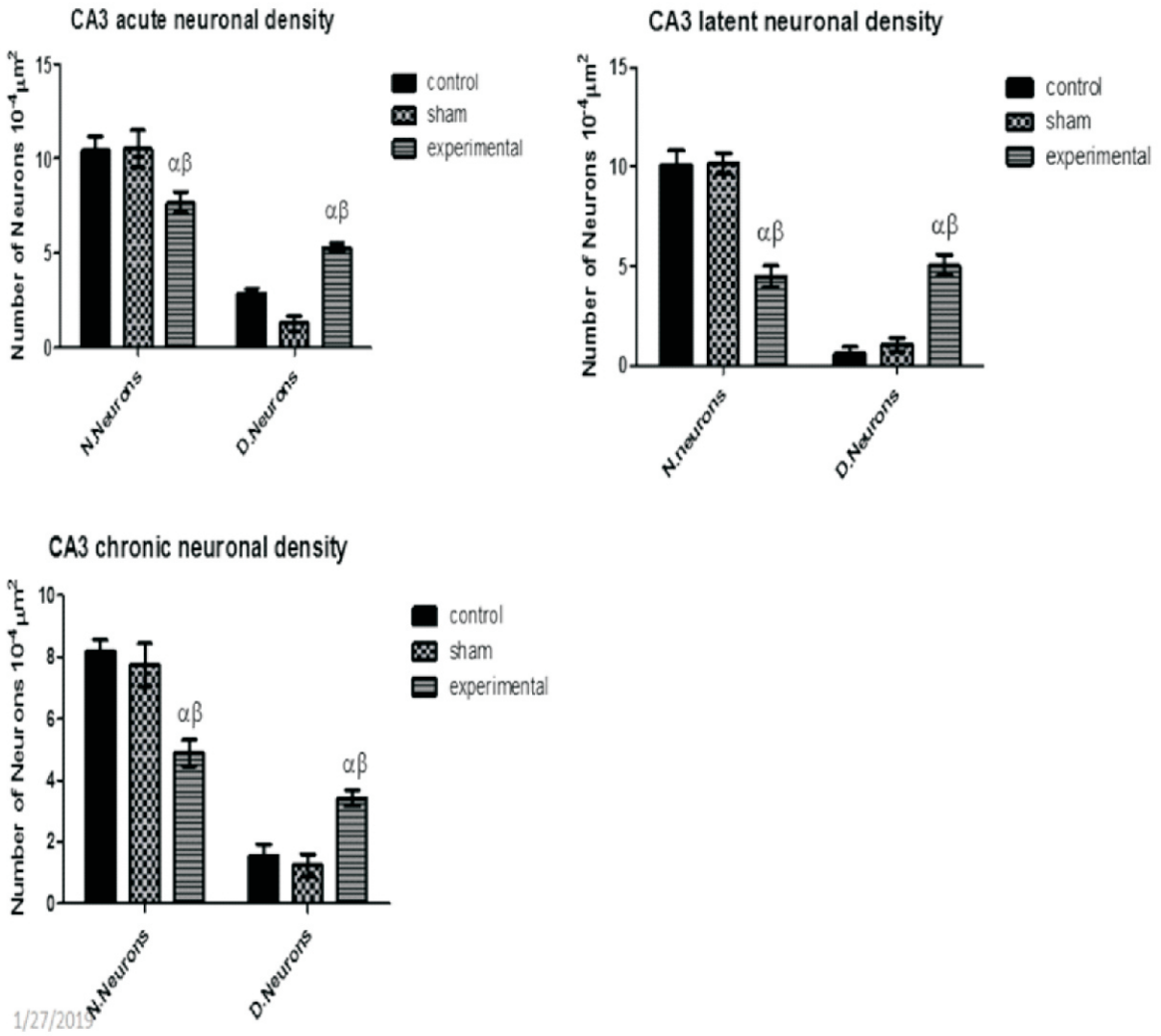

Figure 4: Bar charts showing neuronal density of CA3 hippocampal sub-field of acute, latent and chronic phases. α, β – significant difference when experimental group is compared with control and sham control groups respectively. N. Neurons – Normal Neurons; D. Neurons – Neurons showing degenerating features. Values are expressed as mean±SEM

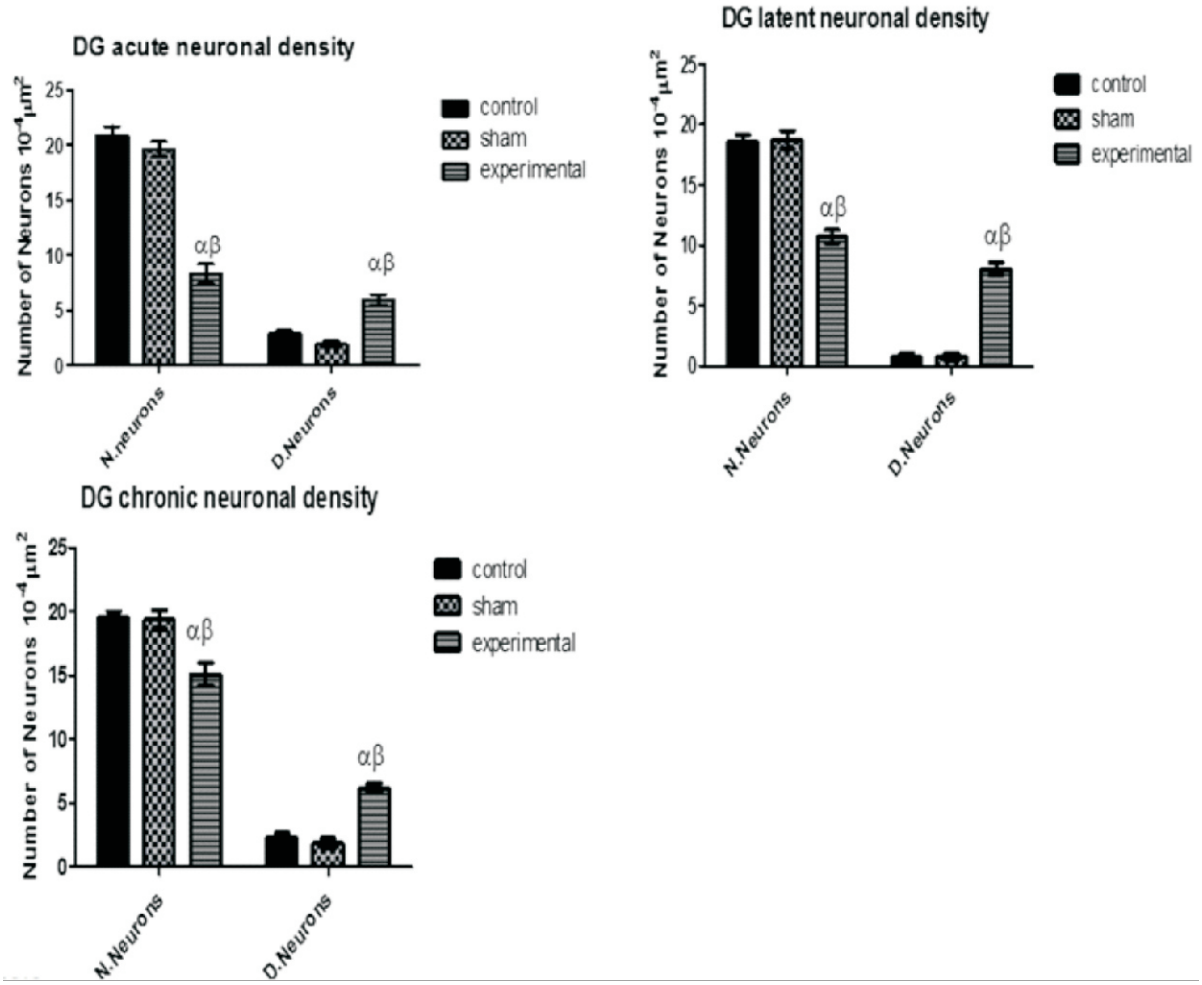

Figure 5: Bar chart showing neuronal density of DG hippocampal sub-field of acute, latent and chronic phases. α, β – significant difference when experimental group is compared with control and sham control groups respectively. N. Neurons – Normal Neurons; D. Neurons – Neurons showing degenerating features. Values are expressed as mean±SEM

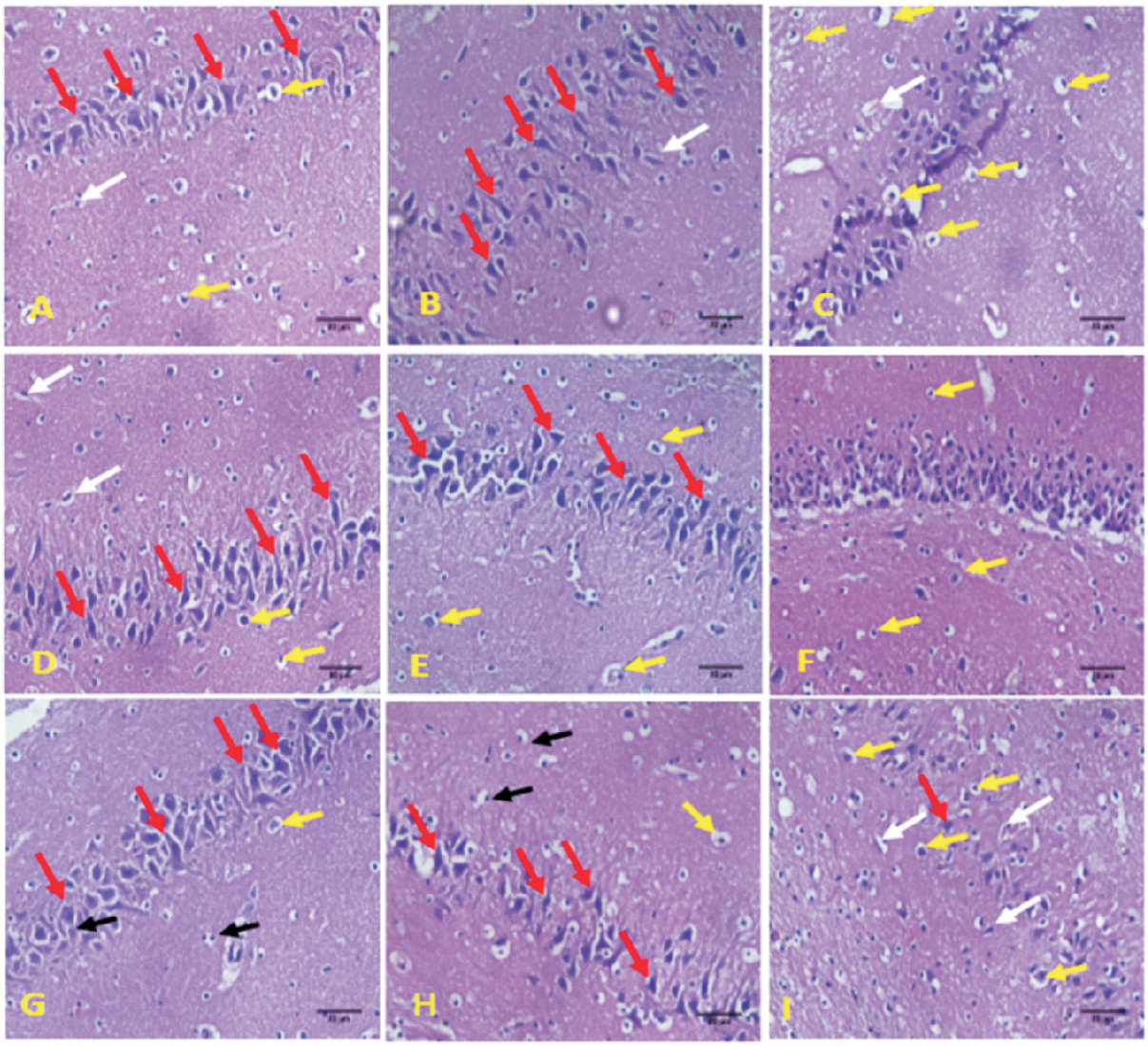

Plate 1: Photomicrographs of sections of hippocampal CA1 sub-field subjected to H&E stain. Upper panel represents Acute phase (Control, sham and experimental {A, B and C}respectively). Middle panel represent latent phase (Control, sham and experimental {D, E and F} respectively). Lower panel represents chronic phase (Control, sham and experimental {G, H and I}respectively). White arrow- degenerating (pyknotic) neuron, vacuolated neuron- yellow arrow, Red arrow– intact neuron (pyramidal cell), black arrow – oligodendrocytes. Scale bar- 50µm

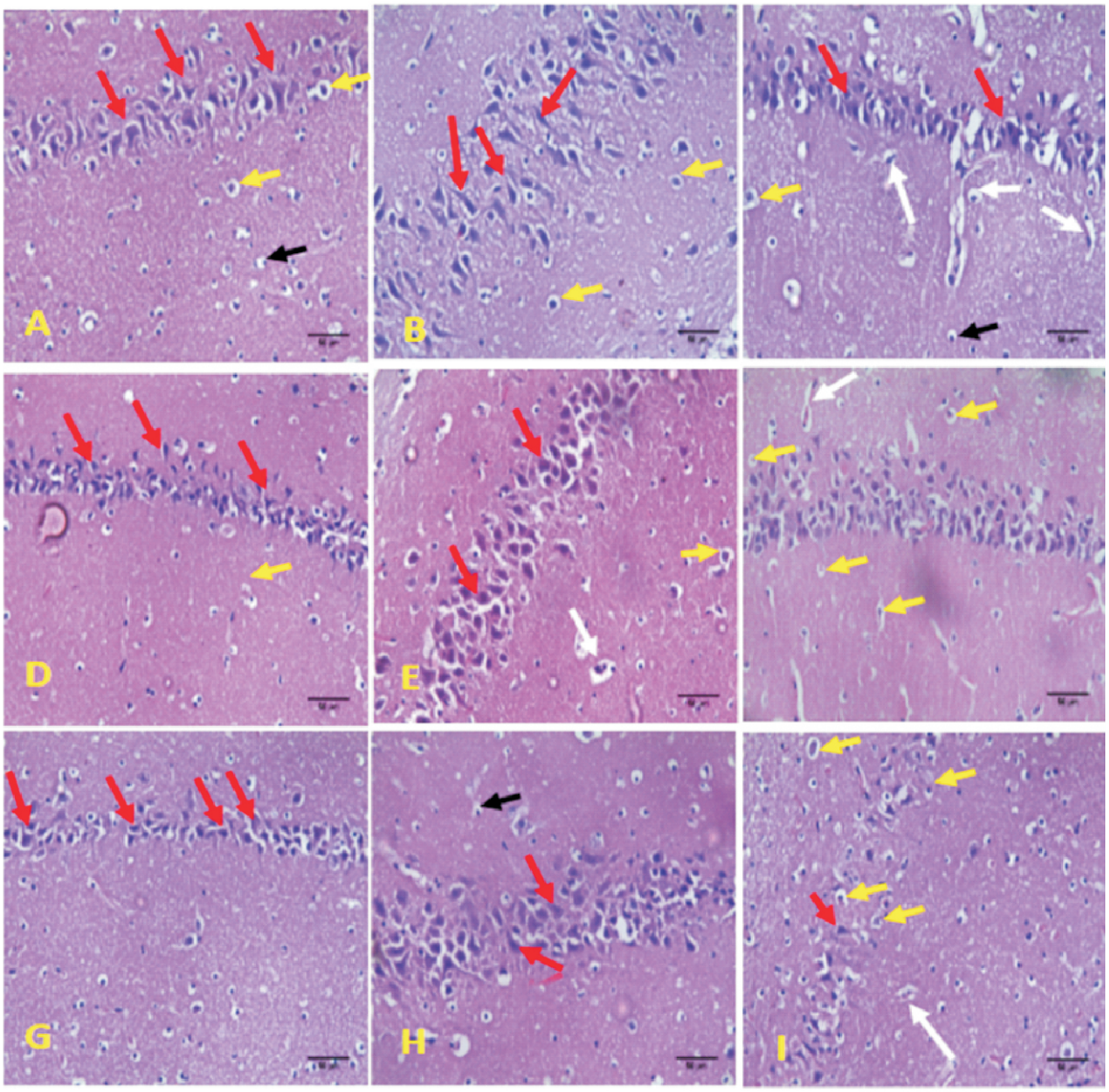

Plate 2: Photomicrographs of sections of hippocampal CA2 sub-field subjected to H&E stain. Upper panel represents Acute phase (Control, sham and experimental {A, B and C}respectively). Middle panel represent latent phase (Control, sham and experimental {D, E and F}respectively). Lower panel represents chronic phase (Control, sham and experimental {G, H and I} respectively). White arrow- degenerating (pyknotic) neuron, vacuolated neuron- yellow arrow, Red arrow– intact neuron (pyramidal cell), black arrow – oligodendrocytes. Scale bar-50μm.

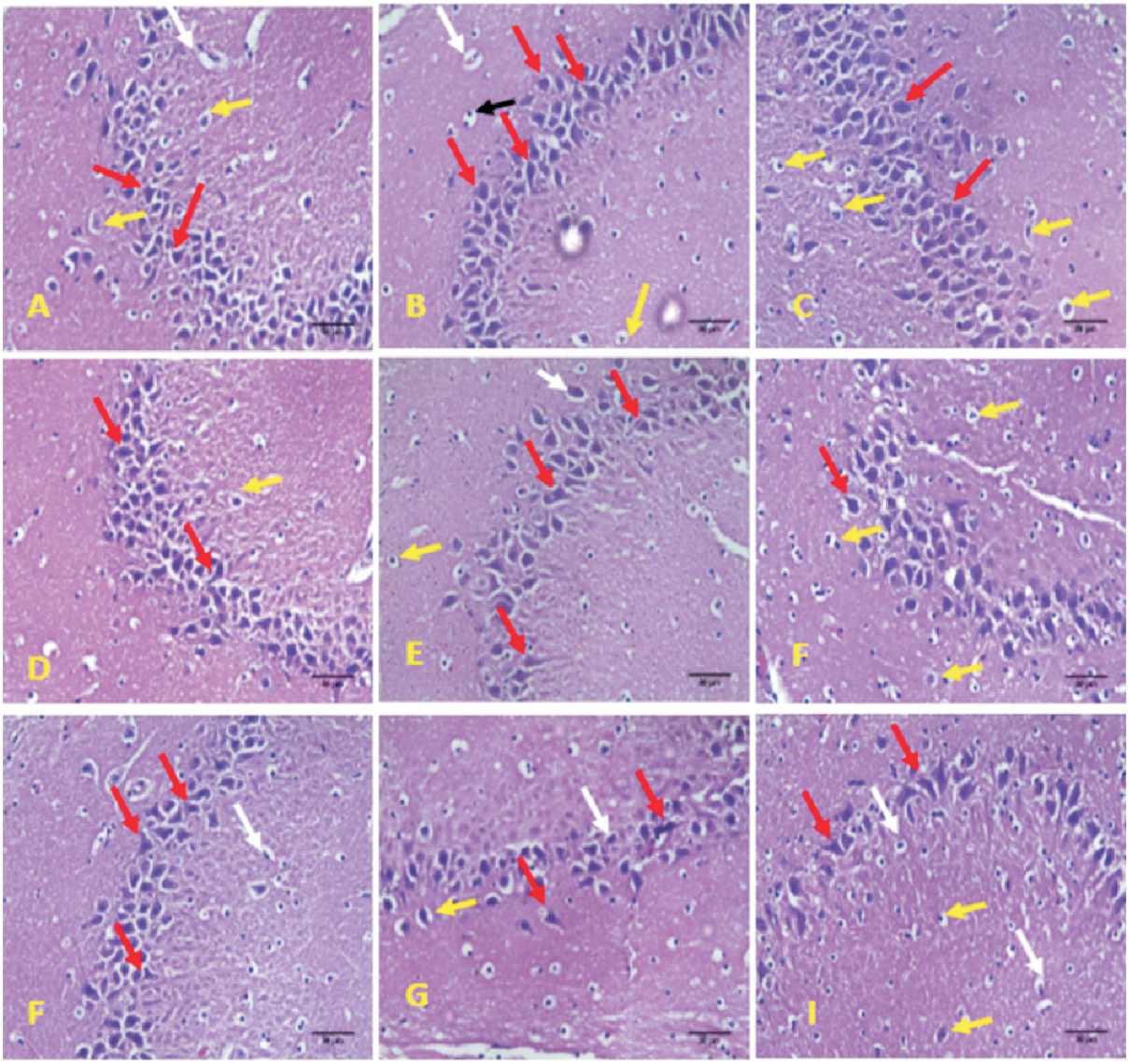

Plate 3: Photomicrographs of sections of hippocampal CA3 sub-field subjected to H&E stain. Upper panel represents Acute phase (Control, sham and experimental {A, B and C}respectively). Middle panel represent latent phase (Control, sham and experimental {D, E and F}respectively). Lower panel represents chronic phase (Control, sham and experimental {G, H and I} respectively). White arrow- degenerating (pyknotic) neuron, vacuolated neuron- yellow arrow, Red arrow– intact neuron (pyramidal cell), black arrow – oligodendrocytes. Scale bar- 50µm.

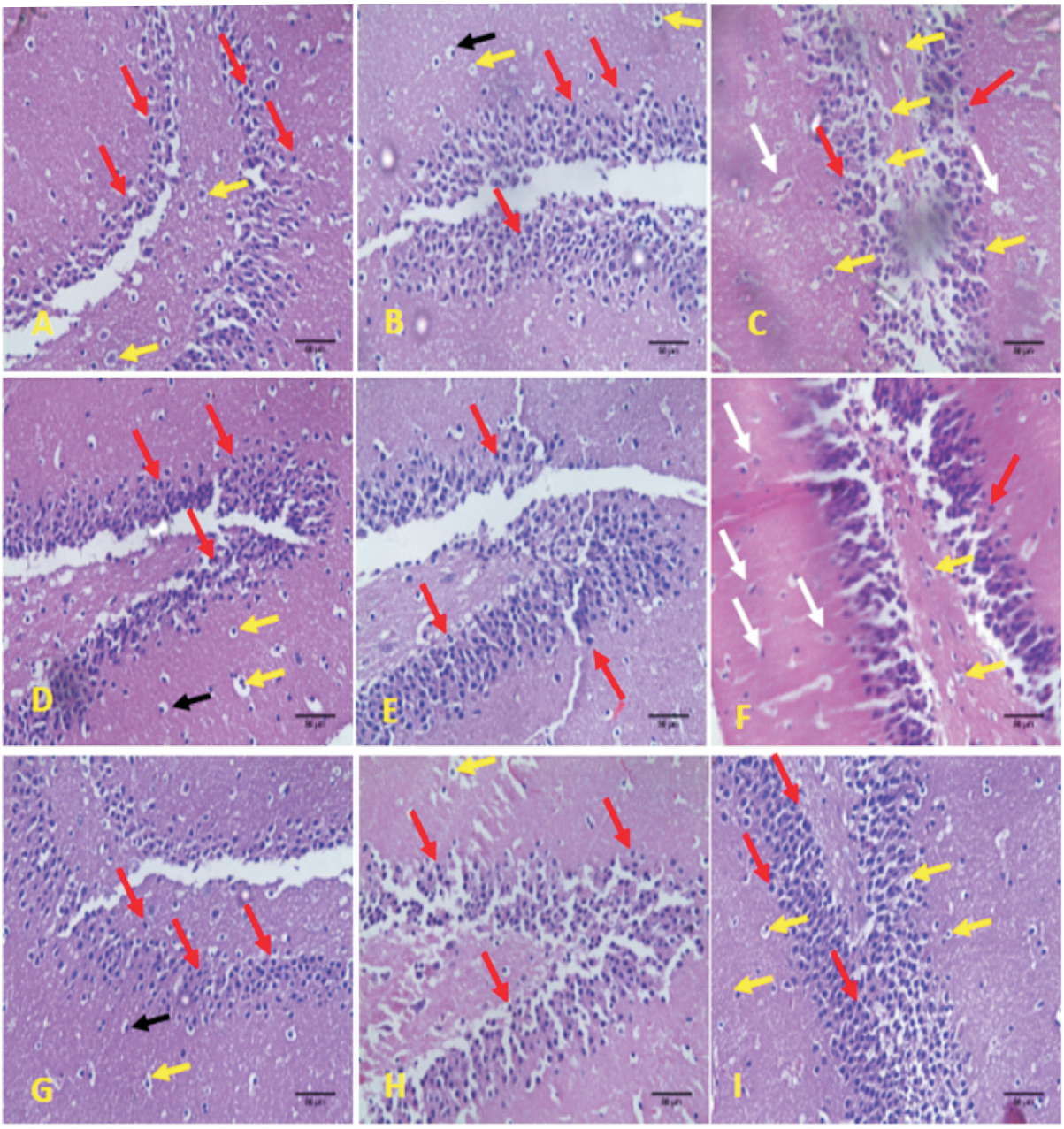

Plate 4: Photomicrographs of sections of hippocampal DG sub-field subjected toH&E stain. Upper panel represents Acute phase (Control, sham and experimental {A, B and C}respectively). Middle panel represent latent phase (Control, sham and experimental {D, E and F}respectively). Lower panel represents chronic phase (Control, sham and experimental {G, H and I} respectively). White arrow- degenerating (pyknotic) neuron, vacuolated neuron-yellow arrow, Red arrow– intact neuron (pyramidal cell), black arrow – oligodendrocytes. Scale bar- 50μm.

DISCUSSION

In the acute group, the experimental group showed significant increase in mean time taken to identify chow in baited arms of the radial arm maze in trials 1, 2 and 3 when compared with the control and sham groups (Figure 1).

However, comparison between the control and the sham rats revealed no significant difference in the time taken to identify the baited arm of the radial arm maze. The implication of these findings is that diazepam which is the main difference between the control and sham groups did not impair memory in this model of temporal lobe epilepsy. The experimental group result revealed that lithium-pilocarpine induced temporal lobe epilepsy results in impairment of spatial memory in Wistar rats. In addition, a comparison of the time taken to identify the baited arm in the control, sham and experimental rats from trial 1 to 3 revealed a progressive reduction in the time when corresponding groups are compared. This

showed that spatial memory was intact in the experimental rats just as it was expectedly intact in the control and sham rats. Therefore, in the acute phase of the lithium-pilocarpine induced temporal lobe epilepsy, memory was intact in relation to time but impaired when experimental rats were compared with the corresponding control and sham rats.

In the latent phase, assessment of memory revealed no significant difference in the time taken to identify the baited arm across the trials with the exception of the first trial where the time taken by the experimental group rats was significantly increased when compared with that of the control and sham. However, the time taken by the 3 groups was progressively reduced from trial 1 to trial 3 when corresponding groups were compared. These pictures showed that memory was not impaired in the latent phase of lithium-pilocarpine induced TLE. The memory status in this phase of TLE in Wistar rat was different from the picture obtained from that of the acute phase, confirming the fact that the response of the brain to the lithium-pilocarpine induced epilepsy is phase dependent.

In the chronic phase, significant increase in the time taken to identify the baited arm was noted in the experimental group of trial 1 when compared with control and sham groups. In trial 2, the increase in time was only noted in the experimental group when compared with the sham group while there was no significant difference in the time taken to identify the baited arm when the 3 groups were compared. The overall picture of memory assessment in lithium-pilocarpine induced temporal lobe epilepsy in Wistar rats showed that impairment of memory is unequivocal in the acute phase while the latent and chronic phases did not show an impairment of spatial memory in the epileptic rats. It is important to note that lithium-pilocarpine-induced TLE causes widespread damage in the brain affecting the hippocampus, amygdala and thalamic nuclei [18, 19, 20, 21]memory impairment does not appear to be progressive with time, it actually appeared to be restricted to the acute phase. Such non-progressive nature of memory impairment in this model of TLE indicate that the underlying mechanism may not be structural damage of the cellular components of the hippocampus which will be expected to be irreversible and long standing. One major characteristic of the acute phase of TLE in this model is preponderance of seizures which are secondary to an imbalance between the excitatory and inhibitory signals that the neurons are subjected to. Since the latent phase is usually devoid of seizures and the frequency of seizures in the chronic phase is reduced compared with the acute phase, it is therefore possible that memory impairment secondary to TLE in lithium-pilocarpine model is directly related to the frequency of seizure. This is in consonance with the claim in a human study that memory loss is closely related to young age of onset of epilepsy and frequent seizures[22]. This indicates that a significant reduction in the frequency of seizures in this model may significantly ameliorate memory impairment. The claim that memory impairment is related to degeneration of neurons and oligodendrocyte [23]might therefore need a second look. Though there was a constant neuronal degeneration in this study across the phases of TLE, such structural hippocampal neuronal degeneration does not appear related to memory impairment. It is possible that the same mechanism might underlie the 2 events, there are no strong evidence from the findings of this study that they are interconnected.

This study evaluated the neuronal density of different hippocampal subfields (CA1, CA2, CA3 and DG). The normal neuronal density of control and sham groups were not significantly different when compared with each other in each of the subfields. This implies that diazepam which is the distinguishing factor between the sham and control groups caused no neuronal degeneration. In the acute, latent and chronic phases, there was a significant reduction in the density of normal neuronal in the experimental group when compared with control and sham groups. Expectedly, there was a significant increase in degenerating neuronal density in the experimental group when compared with control and sham groups. This constant picture of hippocampal neurons in the 3 phases of TLE reiterated the fact that the degree of neuronal degeneration does not appeared tied to the frequency and or intensity of seizures. It also showed that either spontaneous neuronal recovery was not possible in this model of TLE or the mechanism underlining neuronal degeneration remains constant. It is striking that all the different regions of the hippocampus revealed uniform picture of neuronal degeneration across the acute, latent and chronic phases of TLE in this model. Consistent significant density of degenerating neuron in the different region of the hippocampus particularly in the latent phase of this model of TLE showed that the extent of neuronal degeneration cannot be solely explained by frequency of seizure. Aside from the heightened excitation of the neurons, possible involvement of other underlining mechanism which may be secondary to the excitation induced degeneration of the acute phase or a completely different mechanism is involved in the neuronal degeneration that is known to characterize TLE. This study also showed that neuronal degeneration in this model of TLE has global consequences within the hippocampus thereby resulting in widespread manifestations such anomaly. A possible mechanism of hippocampal damage following lithium-pilocarpine-induced SE is glutamate-induced neurotoxicity causing an imbalance between excitatory and inhibitory neurotransmitters. The concept of hippocampal circuitry is based on the fact that majority of hippocampal inputs and intrinsic hippocampal connections operate with excitatory amino acid transmission. The differences in synaptic input may be a cause of interlaminar differences leading to neuronal damage. This result points to the fact that SE can cause various structural changes in brain because of its widespread ability resulting into neuronal degeneration as seen in plates1, 2, 3 and 4 causing an

increase in oligodendrocyte which resulted to early neuronal damage such as appearance of pyknotic neuron with a shrunken, darkly stained or condensed nuclei in dentate granule cells and hilus, followed by a progressive CA3–CA1 damage. This finding is in line with the work of[23]who reported increase severity in neuronal damage at different hippocampal subfields following lithium-pilocarpine-induced SE.

In the latent phase the profile of neuronal degeneration followed a similar pattern. The result revealed a significant reduction in the normal neuronal count of the dorsolateral cerebral cortex in the experimental group when compared with control and sham groups. However, there was a significant increase in degenerating neuronal count in the experimental group when compared with control and sham groups. During the latent phase, cells in the different hippocampal subfields and neocortex confirm that neuronal degeneration continue for at least 6 days after SE. This suggests a time-dependent expression of these neuronal degeneration after lithium–pilocarpine SE as well as the contribution of combined expression of cytokines to the genesis of neuronal damage, as previously suggested with the potential exception of CA1–CA3 pyramidal cell layers.

The chronic phase revealed significant increase in neuronal density of neurons showing degenerating features in the experimental group when compared with control and sham groups while a significant reduction in neuronal count of normal neurons in the experimental group when compared with control and sham groups. The possible reason for this might be that this group is predominantly associated with the onset of occurrence of SRSs. Therefore, development of hippocampal sclerosis is the consequence of the initial excitotoxic event, during the SE and the ongoing episodic seizure activity during the chronic phase. The result obtained from the evaluation of different hippocampal subfields agrees with the study of [24]who reported similar findings. It is well known that hippocampal sclerosis is the predominant feature of TLE and that neuronal degeneration is a hallmark of TLE [25]. In view of these, the progression of neuronal damage observed across the three groups (acute, latent and chronic) showed significant neurodegeneration in this brain region. It is noteworthy to state that histomorphological changes observed in the different hippocampal subfields in this study are reflective of the behavioral (memory) assessment following lithium-pilocarpine-induced TLE. In view of this, the study suggests that the neurodegenerating features observed were debilitating enough to produce a striking consequential deficit in memory, or probably some compensatory mechanisms may have been triggered to cause such deficits in memory as earlier discussed in the neurobehavioural assessment of memory.

Disclosure

None of the authors had any conflict of interest to disclose

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