



RESEARCH ARTICLE

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## Caffeine Exposure at Puberty: Effects on Hippocampal Structure, Neurochemistry and Short Term Memory in Experimental Wistar Rats

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### ABSTRACT

*Hippocampal structural and functional integrity; and its neurogenesis are crucial to memory and associated neurological functions. Caffeine ingestion or exposure is postulated to have significant influence on the hippocampus, especially at puberty which is a critical stage in postnatal neurological development. To this end, this investigation studies the potential influence of caffeine on hippocampal structure, neurochemistry and short term memory in experimental Wistar rats using thirty two (n=32) young male Wistar rats at puberty. They were divided into four groups labeled A-D. Group A served as control and were fed ad libitum. Group B animals received 10 mg/kg body weight of caffeine daily; Group C received 50 mg/kg body weight of caffeine; Group D received 120 mg/kg body weight caffeine daily. Administration was done using oral cannula. Administration lasted 14 days and animals were tested for short term memory using the Barnes maze. Animals were sacrificed and tissues were processed to observe hippocampal structural integrity using the H&E technique; Nissl bodies using the Cresyl Fast Violet technique; possible astrocyte reactions using the glial acidic fibrillary protein technique. Neurotransmitters and enzymes were assayed. Caffeine had mild effects on the CA (Cornu Ammonis) of the hippocampal formation. Caffeine influenced neurochemistry and short term memory slightly negatively. Dentate gyrus was unaffected. Results showed that caffeine had effects on the hippocampus at puberty.*

**KEYWORDS:** Hippocampus, Short term memory, Neurochemistry, Dentate gyrus, CA

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### INTRODUCTION

The hippocampus is a part of the temporal lobe that belongs to the limbic system. It has two allocortex laminae: the gyrus dentatus and the cornu ammonis-folded to create an out growth in the temporal horn of the lateral ventricle [1]. The role of the hippocampus in humans is primarily learning, consolidation of long term memory and spatial navigation [2]. The input region of the hippocampus is the dentate gyrus. The hippocampal system is a group of highly interconnected brain regions in the medial temporal lobe that serves as the medium through which the hippocampus communicates with wide spread regions of cortex [3]. The hippocampus has a unique feature of structural reorganization [4]. The development of the brain is sequential in nature starting from neurogenesis to neural migration, maturation, synaptogenesis, pruning and the formation of myelin [5]. Recent findings have shown that during the peri-pubertal period there is increased rate of neurogenesis in the hippocampal region as well as a continuous differentiation of neuronal structure [6]. Thus, this period is very critical in the development of the hippocampal system and of course in its functions.

It has also been reported that as much as there is an increased neuronal proliferation there is also increased neuronal cell death which suggests that a large amount of cell can be rescued by learning around puberty which further explains that the brain at that period is more receptive to learning [7]. The rescued cells however remains in the dentate gyrus for several months [8] and can be integrated into the existing hippocampal circuit [9]. The ingestion of psychoactive stimulant like caffeine can be associated with alteration to the learning process. It is also important to note that the effects of caffeine are controversial as regard its role on learning and memory and also with respect to the dosages that is safe and unsafe for ingestion.

Caffeine is a psychostimulant drug that is widely consumed by the world population and this is because it is legal. It

is present in most beverages that is commonly ingested for example in form of coffee, tea, cocoa, and drinks. Caffeine is present in the leaves, beans and fruits of over 60 plants [10]. In the past 30 years the rate at which children and adolescents consume caffeine has greatly increased to about 70%. Caffeine is a central nervous system stimulant and it belongs to the group methylxanthine and it is widely used to enhance alertness and improve performance. However, caffeine has a behavioural and biochemical effect in the brain and in the body; it contributes to sleep deprivation and anxiety in humans which in a long run has an inhibitory effect on neurogenesis [11,12]. A recent sample of caffeine consumers between the ages 12-17 showed that the mean amount of caffeine consumed by this population was 69.5 mg per day and this slightly lower than the amount present in one cup of coffee.

Caffeine is a naturally occurring alkaloid that is found in varying quantity in beans, leaves, fruits of more than 60 plants. It is found in the seeds of coffee tree (*Coffea arabica*), leaves of tea bush (*Thea sinensis*), nuts of kola tree (*Cola acuminata*) and seeds of cocoa tree (*Theobroma cacao*). It is usually consumed in beverages like coffee, tea, chocolate and soft drinks which are gotten from the raw material of these sources. Since, it is a metabolite of nitrogen metabolism it could be referred to as an alkaloid [13]. It is usually obtained from its natural sources through a process called extraction and artificially from uric acid as a precursor [14]. Caffeine interacts with adenosine receptors and this is believed to account for most of its pharmacological effects [15]. Therefore the primary pharmacological effect of caffeine is the stimulation of the central nervous system [16].

One of the markers of a possible damage to the hippocampus is the hippocampal atrophy [17]. There is reduced hippocampal volume in patients with minimal cognitive impairment, Alzheimer's disease, schizophrenia, depression and epilepsy, hypertension and Cushing disease [18]. In Alzheimer's disease it has been reported that an early damage to the hippocampus can result in failure of registration of information coming from the hippocampus [19]. In patients that have epilepsy damage to the hippocampus can result in hippocampal sclerosis [20]. Amnesia is also another condition that occur when there is hippocampal damage. Complete amnesia may occur when there is bilateral damage of the hippocampus; memory might be preserved if there is a unilateral damage [21].

Caffeine causes an increase in hippocampal acetylcholine which may block the consolidation by congesting replay of memories [22]. The long term effects of caffeine consumption are seen majorly in slowing hippocampus dependent learning and impaired long term memory which directly inhibits hippocampal neurogenesis [23]. Caffeine-induced glucocorticoid secretion interferes with working memory, short-term memory and formation of long-term memory due to deleterious effects on the normal physiologic processes of the hippocampus [24].

The aim is to study the effects of caffeine exposure at puberty on the morphology and organisation of cells in the hippocampus and behavioural changes that occur in then Wistar Rat models.

#### MATERIALS AND METHODS

A total of 32 male rats, just attaining puberty, were used for this research; they were 35 days old and the average weight was 70 g.

The experimental animals (rats) were housed in plastic cages and they were kept in a well-ventilated room in the Babcock University Animal House, and allowed to acclimatise. Caffeine was purchased from JT Baker and dissolved in distilled water to obtained correct doses based on research design. The animals were grouped into 4 groups. The groups were labeled A-D and treated as follows:

Group A: Feed and water only

Group B: 10 mg/kg body weight caffeine

Group C: 50 mg/kg body weight caffeine

Group D: 120 mg/kg body weight caffeine

The oral method of administration was adopted using the oral cannula and was done for 14 days.

Behavioural Studies were carried out after the 14<sup>th</sup> day of substance administration using the Barnes maze to observe the short term memory.

***Procedures for Barnes Maze test [25]***

The training period lasted for four days after which the main experiment was performed on the sixth day. The maze was placed in a well-illuminated and ventilated room and it was cleaned properly using 70% ethanol. During the four days of the training there was a rewarding system in place for each animal when they locate the escape box. On the day of the experiment all forms of visual cues and olfactory cues was removed and a good video recording was put in place to capture each of the animals. Each animal was given a maximum time of 3 min to locate the escape box, after which if the box was not located it has been said to have failed the experiment. The parameters for the experiment are; primary latency, secondary latency, total latency, primary pokes, secondary pokes and total pokes.

***Animal sacrifice***

Animals were sacrificed by cervical dislocation; the whole brains were excised through cranial surgical incisions. Tissues were preserved in formal saline fixative while some parts were homogenized in preparation for biochemical assays.

***Histological preparations***

Generally, fixed tissues were paraffin embedded and sectioned. They were thereafter taken through the various procedures of histological demonstrations.

***Haematoxylin and eosin stain [26]***

Slides were immersed in haematoxylin, ammonium hydroxide, Eosin-y and then dehydrated in the ascending order of ethanol 70%, 80%, 95% and 100%. Finally the slides were immersed in xylene and afterwards cover slipped. It is important to note that after every immersion before dehydration the slides were rinsed in water.

***Cresyl fast violet***

Slides were removed from the freezer, covered and left to dry at least 60 min at room temperature. The sections were rinsed in 10 mM PBS. It was then dipped in Nissl stains. Afterwards it was dipped in 90%, 95%, 100% ethanol. Finally it was dipped in xylene and then mounted on a per mounting media. It is important to note that before the dehydration the slides were rinsed in distilled water after every immersion. Also, the per mount is allowed to cure before the slides are viewed under the microscope.

***GFAP (glial acidic fibrillary protein) immunohistochemistry [27]***

Xylene was used to Deparaffinize the sections and it was hydrated in 95% and 100% ethyl alcohols. Endogenous peroxide was quenched with 2 drops of freshly made 3%hydrogen peroxide. The slides were thoroughly washed in distilled water and tris buffered saline. Glial Fibrillary acidic protein primary antibody was applied and incubated at room temperature. After excess buffer was tapped off the amplifier was used to incubate, HRP was applied and then the required quantity of DAB substrate chromogen was applied and then incubated. When appropriate staining has been achieved, the slides were rinsed in deionized water and counterstained lightly with Mayer's Hematoxylin. Afterwards it was rinsed under running tap to blue the sections. Dehydration was done with 95% and 100% ethyl alcohol and cleared with xylene. Finally glycerol gelatin or a compatible aqueous mounting media was applied and cover slipped.

***Enzyme assay***

Tissues were excised and placed in 30 gdm<sup>-3</sup> Sucrose and then homogenized at a low temperature. The supernatant was assayed for the following enzymes and neurotransmitters using spectrophotometric methods. Measurements were recorded in microliters [ $\mu$ L].

***Cytochrome C oxidase assay [28]***

0.95 ml of 1' Assay Buffer was added to a cuvette and the spectrophotometer was placed at zero. A suitable volume of enzyme solution or mitochondrial suspension was added to the cuvette, the reaction volume was brought to 1.05 ml with 1' Enzyme Dilution Buffer. The reaction was started by adding 50  $\mu$ l of Ferrocytochrome C Substrate solution. As a result of rapid reaction rate of the enzyme the  $A_{550}/\text{min}$  was read immediately. The expected background values were between 0.001 and 0.005  $A_{550}/\text{min}$ . Afterwards the activity of the sample was then calculated. It is important to note that all mixtures are gotten by inversion.

**Glucose 6 phosphate dehydrogenase (G6PDH) [29]**

Enzyme suspension diluted to 1.0 mg/ml in 5 mM glycine buffer or lyophilized enzyme was dissolved, pH 8.0. Caution was taken to prevent shaking the solution so as to avoid the formation of precipitate. Immediately it was diluted before it was used in 5mM glycine buffer, containing 0.1% albumin, in order to obtain a rate of 0.02-0.045  $\Delta A/\text{min}$ .  $\text{mgP}/\text{ml} = A_{280} \times 0.87$

**Neurotransmitter assays**

Neurotransmitters activities were assayed for in the prepared homogenates of the brain tissues.

**Gamma amino butyric acid (GABA) assay**

In this assay, the tissues were rinsed first in ice-cold PBS (0.01 mol/L, pH 7.0-7.2) to thoroughly remove all forms of blood and also weighed before homogenization. The tissues were minced to small pieces and homogenized in 5-10 mL of PBS using a glass homogenizer on ice. For breakage of cell membrane to occur the suspension gotten was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles. Thereafter the homogenates were centrifuged for 5 min at 5000  $\times g$ . the supernatant was removed and then assayed for. 50  $\mu\text{L}$  standard or sample was added to each properly; then 50  $\mu\text{L}$  prepared detection reagent A was added immediately; shaking and mixing occurred and then it was incubated. 100  $\mu\text{L}$  prepared detection reagent B was added and then incubated. 90  $\mu\text{L}$  substrate solutions was added. It was then incubated. 50  $\mu\text{L}$  Stop solution was added, followed by Reading at 450 nm immediately. Some of the precautions taken were aspiration which was done after every step alongside washing. Also, incubation was done at temperature of 37°C.

**Acetylcholine assay [30]**

Biolabs Acetylcholine Assay Kits was used; 250 mg of wet tissues or cell pellet samples were homogenized in 4.5 mL of chloroform/methanol at the ratio 2:1. The samples were centrifuged to remove debris. The homogenates were incubated at room temperature for an hour placed upon an orbital shaker. Added to a 96-well microtiter plate was 50  $\mu\text{L}$  of diluted acetylcholine standards as well as 50  $\mu\text{L}$  of prepared acetylcholine reaction reagent added to each standard and samples and it was thoroughly mixed. To prevent the penetration of light, the plate wells were properly covered and were incubated on an orbital rotator at room temperature. A spectrophotometric micro plate reader set in the 540-570 range was used to read the plate. The final results were gotten by comparing the absorbance with the standard curve of acetylcholine.

**Glutamate assay [31]**

The reaction mixes was set up. It is important to note that 100  $\mu\text{L}$  of reaction mix is required for every reaction. 100  $\mu\text{L}$  of the appropriate reaction mix was added to each well and it was mixed using a horizontal shaker either by pipetting or by incubation at 37°C. The absorbance was measured at 450 nm ( $A_{450}$ ). One of the precautions put in place is that during the incubation, light penetration was prevented.

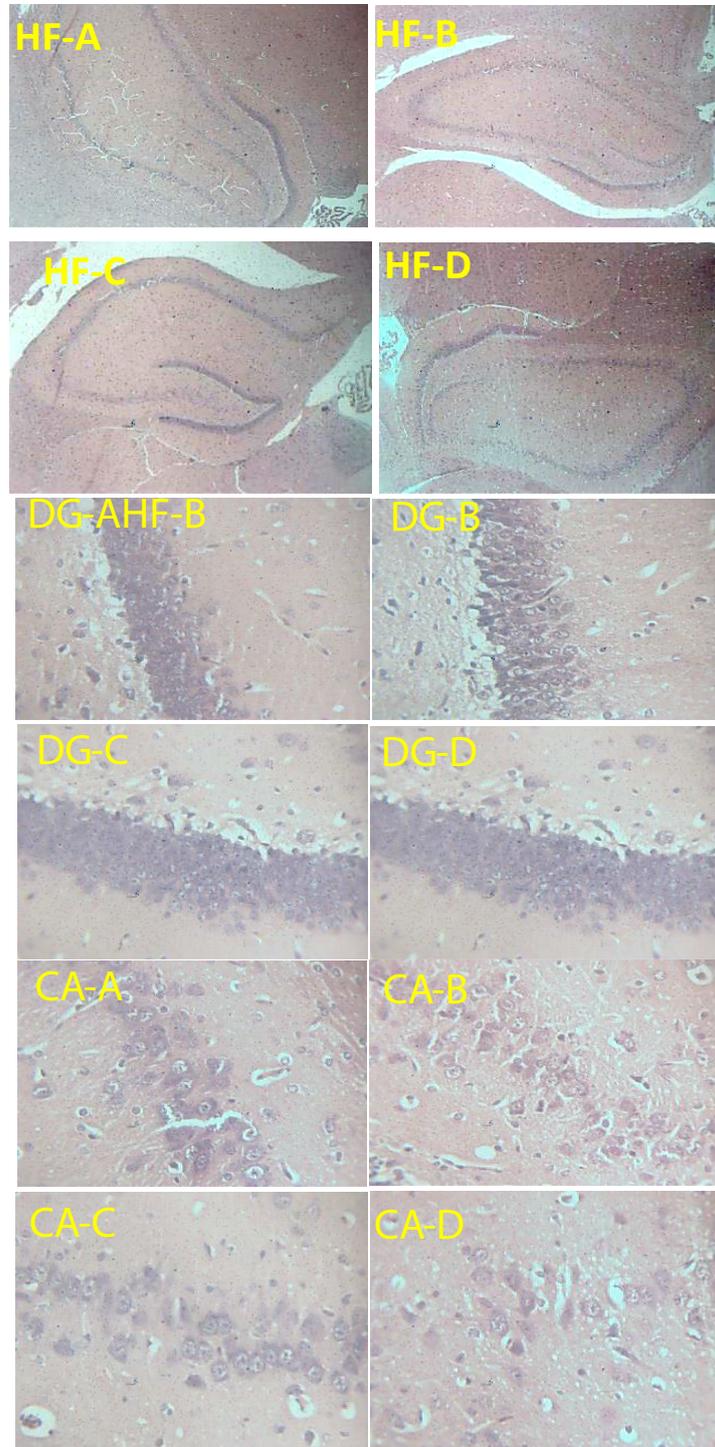
**Statistical analysis**

All the results were represented as a grouped data and analyzed using the Graph pad Prism 5.0 software using one way analysis of variance (ANOVA). The results were expressed as Mean  $\pm$  SEM in charts. Dunnett tests were used to compare all other columns with the standard column Group A there by identifying differences. The confidence interval was placed at 95% such that in all cases a value of  $P < 0.05$  was considered significant.

**RESULTS**

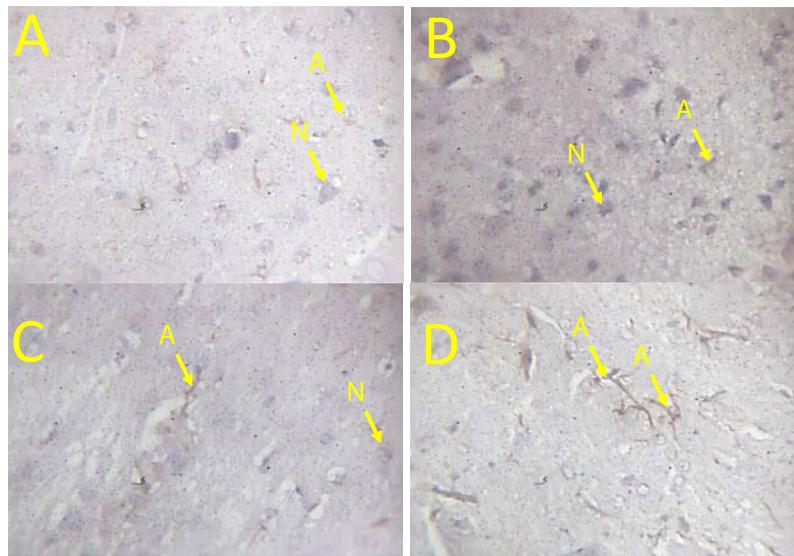
The result obtained from this research includes; morphological analysis, histology, immunohistochemistry, biochemical assays (enzymes and neurotransmitter) and the neurobehavioural studies. The groups of interest in this research are Group A (control) and the groups treated are Group B (low dose caffeine 10 mg/kg Body weight), Group C (medium dose caffeine 50 mg/kg Body weight) and Group D (high dose caffeine 120 mg/kg Body weight).

**Histological results**

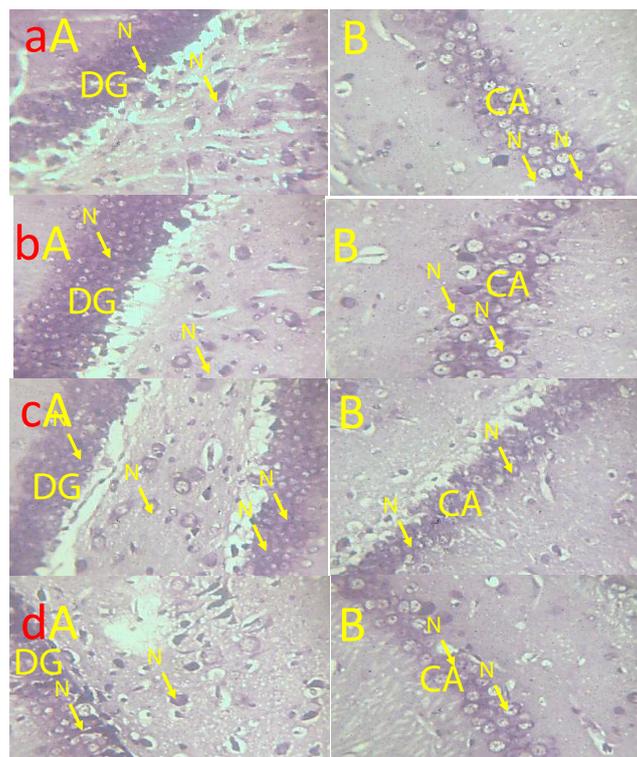


**Figure 1:** Photomicrographs of the hippocampal formation. The outline of the hippocampus formation across the experimental animals groups is normally demonstrated without evidence of extensive disruption. In the dentate gyrus of the experimental animals; neurons are demonstrated normally across the groups in terms of their morphologies and there is no evidence of dentate gyrus disruption due to the effects of the administered substances. On photomicrographs of the hippocampus CA [3] of the Group A-D experimental animals (H&E X1600); the hippocampal CA is normally demonstrated when lower and medium of caffeine doses were administered; CA cells are however sparse when the higher dose was administered

HP: Hippocampus; N: Neuron; DG: Dentate Gyrus; DGC: Dentate Gyrus Cells; CAC: Cornu Ammonis Cell; CA: Cornu Ammonis



**Figure 2:** Photomicrographs of the hippocampus of the Groups A, B, C and D experimental animals showing the expression of the glial fibrillary acidic protein (GFAP) in astrocytes within the hippocampal formation (GFAP X1600). Astrocytic expression of GFAP was observable in treated groups and expression increased with does A: Astrocyte; N: Neuron



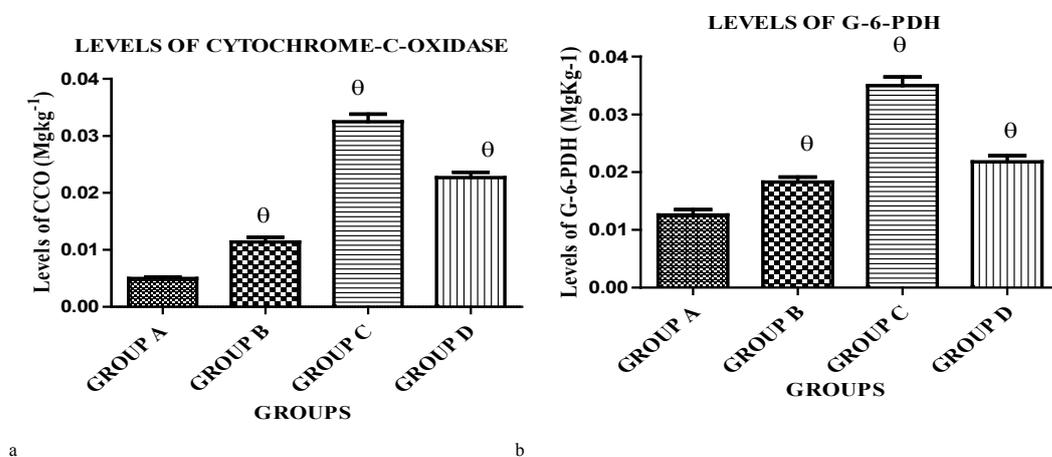
**Figure 3:** Photomicrographs of the hippocampus of the Groups A-D experimental animals. In Group A (Control), hippocampus is demonstrated with structurally normal dentate gyrus and Cornu Ammonis. In Group B, hippocampal dentate gyrus and Cornu Ammonis are relatively normal. In Group C hippocampal dentate gyrus cells stain relatively less intensely for Nissl bodies. Cornu Ammonis is relatively thinner and its cells stain relatively intensely for Nissl bodies. In Group D, hippocampal dentate gyrus cells are sparse and they stained relatively less intensely for Nissl bodies. Cornu Ammonis is relatively thinner and its cells are sparse (CFV, X1600)

HPL: Hippocampus; N: Neuron; DG: Dentate Gyrus; DGC: Dentate Gyrus Cells; CAC: Cornu Ammonis Cell; CA: Cornu Ammonis

**Enzyme tests****Cytochrome – C – oxidase (CCO) and glucose-6-phosphate dehydrogenase (G-6-PDH)**

The cytochrome-c-oxidase level of Group A (control group) was  $0.0050 \pm 0.0002582$ . The level of cytochrome-c-oxidase in Group B (10 mg/kg low dose) was significantly ( $p < 0.05$ ) higher when compared to the control at  $0.0114 \pm 0.0008055$ . Cytochrome-c-oxidase level was significantly higher ( $p < 0.05$ ) in Group C (50 mg/kg medium dose) at  $0.0325 \pm 0.001319$  when compared to the control. The level of cytochrome-c-oxidase for Group D (high dose 120 mg/kg) was significantly ( $p < 0.05$ ) higher than the control at  $0.0227 \pm 0.0009315$ .

The G-6-PDH level of Group A (control group) was  $0.0126 \pm 0.0009092$ . The level of G-6-PDH in Group B (10 mg/kg low dose) was significantly ( $p < 0.05$ ) higher when compared to the control at  $0.0183 \pm 0.0008439$ . G-6-PDH level was significantly higher ( $p < 0.05$ ) in Group C (50 mg/kg medium dose) at  $0.0350 \pm 0.001483$  when compared to the control. The level of G-6-PDH for Group D was significantly ( $p < 0.05$ ) higher than the control at  $0.0218 \pm 0.001041$ .



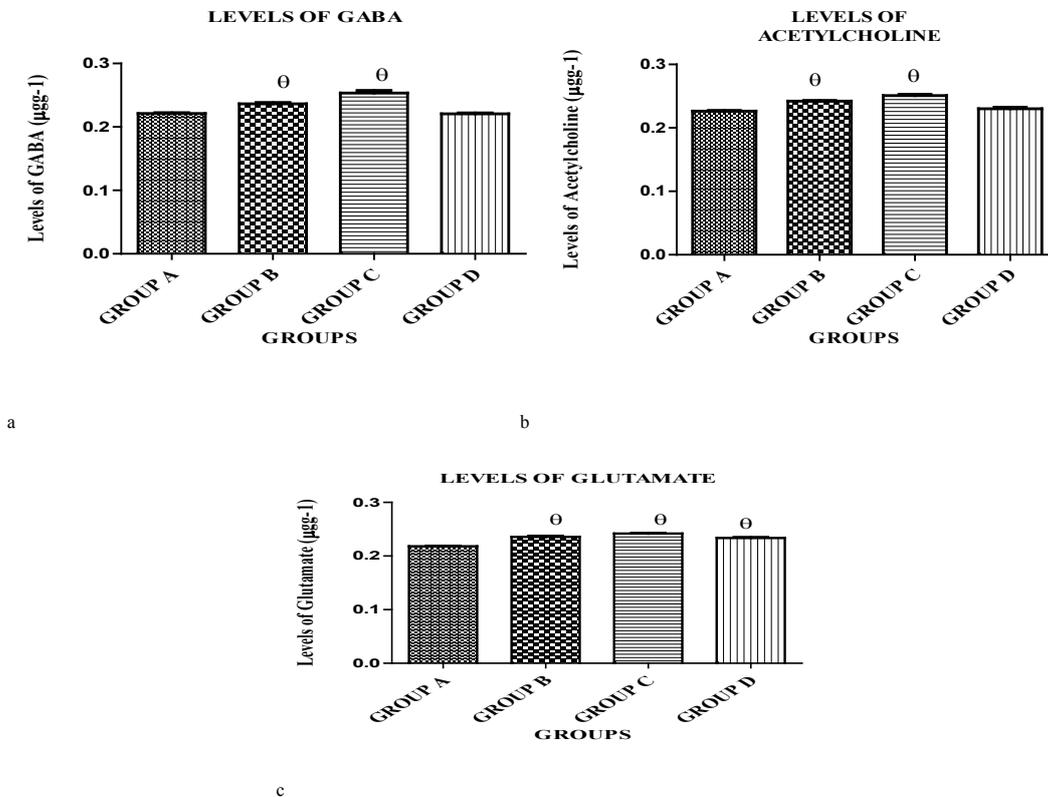
**Figure 4:** A bar chart showing the levels of cytochrome-C-oxidase and G-6-PDH in experimental animals after 14 days of treatment  
 θ: Indicates statistical significance when compared with Group A at  $P < 0.05$

**Neurotransmitters assays****Gamma amino butyric acid (GABA), acetylcholine and glutamate**

GABA level of Group A (control group) was  $0.2210 \pm 0.001220$  as shown the level of GABA in Group B (10 mg/kg low dose) was significantly ( $p < 0.05$ ) higher when compared to the control at  $0.2364 \pm 0.001746$ . GABA level was significantly higher ( $p < 0.05$ ) in Group C (50 mg/kg medium dose) at  $0.2533 \pm 0.003887$  when compared to the control. The level of GABA for Group D was slightly lower than the control at  $0.2205 \pm 0.001400$ .

The acetylcholine level of Group A (control group) was  $0.2264 \pm 0.0006360$ . The level of acetylcholine in Group B (10 mg/kg low dose) was significantly ( $p < 0.05$ ) higher when compared to the control at  $0.2421 \pm 0.0008492$ . Acetylcholine level was significantly higher ( $p < 0.05$ ) in Group C (50 mg/kg medium dose) at  $0.2508 \pm 0.001794$  when compared to the control. The level of acetylcholine for Group D was slightly higher than the control at  $0.2301 \pm 0.002193$ .

Glutamate level of Group A (control group) was  $0.2182 \pm 0.0006633$ . The level of Glutamate in Group B (10 mg/kg low dose) was significantly ( $p < 0.05$ ) higher when compared to the control at  $0.2355 \pm 0.002151$ . Glutamate level was significantly higher ( $p < 0.05$ ) in Group C (50 mg/kg medium dose) at  $0.2419 \pm 0.0005667$  when compared to the control. Also, the level of Glutamate for Group D was significant ( $P < 0.05$ ) than the control at  $0.2337 \pm 0.001230$ .



**Figure 5:** A bar chart showing the levels of GABA, acetylcholine and glutamate in the experimental animals after 14 days of treatment

Θ: Indicates statistical significance when compared with Group A at  $p < 0.05$

### Neurobehavioural results

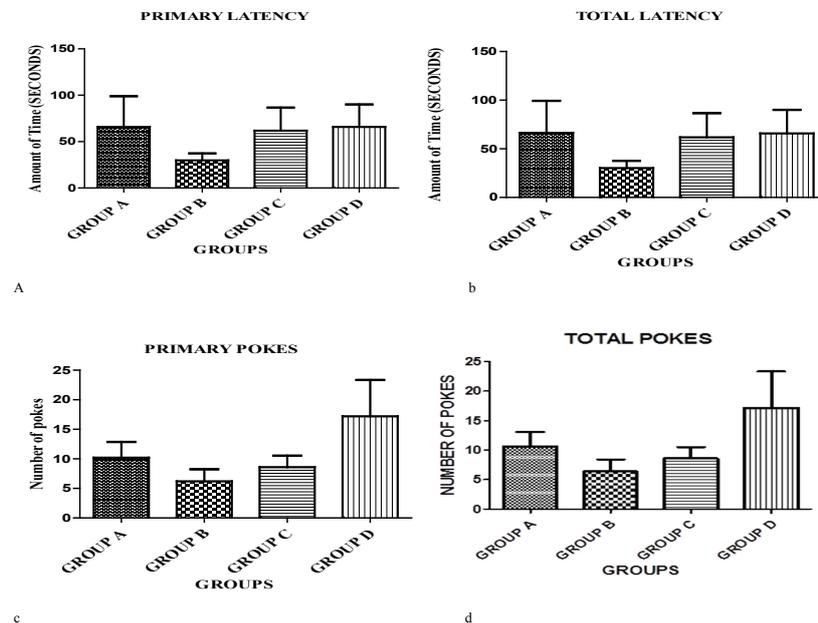
#### Short term memory

Primary latency of Group A (control group) was  $65.75 \pm 33.23$ . The primary latency of Group B (10 mg/kg low dose) was lower when compared to the control at  $29.80 \pm 7.453$ . The primary latency was slightly lower in Group C (50 mg/kg medium dose) at  $61.75 \pm 24.96$  when compared to the control. The primary latency of Group D was slightly higher than the control at  $65.75 \pm 24.28$ .

Total latency of Group A (control group) was  $66.25 \pm 33.02$ . The Total latency of Group B (10 mg/kg low dose) was lower when compared to the control at  $30.20 \pm 7.479$ . The total latency was slightly lower in Group C (50 mg/kg medium dose) at  $61.75 \pm 24.96$  when compared to the control. The total latency of Group D was slightly higher than the control at  $65.75 \pm 24.28$ .

The primary pokes of Group A (control group) was  $10.20 \pm 2.672$ . The primary pokes of Group B (10mg/kg low dose) were lower when compared to the control at  $6.200 \pm 2.035$ . The primary pokes were slightly lower in Group C (50 mg/kg medium dose) at  $8.600 \pm 1.939$  when compared to the control. The primary pokes of Group D were higher than the control at  $17.20 \pm 6.151$ .

The total number of pokes of Group A (control group) was  $10.20 \pm 2.672$ . The total number of pokes of Group B (10 mg/kg low dose) was lower when compared to the control at  $6.200 \pm 2.035$ . The total number of pokes was slightly lower in Group C (50 mg/kg medium dose) at  $8.600 \pm 1.939$  when compared to the control. The total number of pokes of Group D was higher than the control at  $17.20 \pm 6.151$ .



**Figure 6:** A bar chart showing results of the short term memory tests of the experimental animals after 14 days of treatment. At  $P < 0.05$  there was no statistical significance when Groups B, C and D were compared to Group A. aberrations in behaviour were however generally observable.

## DISCUSSION

One of the most important parts of the human body is the brain and this is because it is the seat of cognition and thinking. The hippocampus on the other hand plays a very important role in learning and different forms of memory, most especially its role in the consolidation of long term memory. It has been argued over time that caffeine inhibits hippocampal neurogenesis in the postnatal peri-pubertal brain. This research investigated the caffeine effects at different dosages on hippocampal neurogenesis. The result obtained from this experiment includes; morphological analysis, histology, immunohistochemistry, biochemical assays (enzymes and neurotransmitter) and the neurobehavioural studies. The groups of interest in this study includes; Group A which serves as the control, Group B which was administered low dose caffeine for 14 days, Group C which was administered medium dose caffeine for 14 days and Group D which was administered high dose caffeine for 14 days.

### *Histological observation*

The histological demonstration of the hippocampus of the experimental animals using the Haematoxyline and Eosin technique [H&E] provided knowledge on the general histoarchitecture and cell morphology and distribution; helping to appreciate the structural integrity of the brain shown in Figures 1-3. Therefore, aberrations in the animals' brain histological preparations in tissues could be observed in the various groups of experimental animals to determine the nature of treatments effects and consequent changes. The H&E method was generally employed to observe the brains' structural integrity in the experimental groups.

The Cresyl Fast Violet (CFV) staining technique was employed to demonstrate the cytological conditions of the demonstrated cells by demonstrating the Nissl bodies or materials in the cells. Nissl bodies are rough endoplasmic reticulum being demonstrated because of the rRNA materials associated with them. CFV technique provides reliable information the cellular morphology and size, but more importantly, provides information on the functional status of the cell. This is because the level of activities at the level of the rough endoplasmic reticulum is an important indication of protein synthesis of neurons. This gives quality information about neurons in conditions of health and diseases.

### *General structure of tissues*

The photomicrographs of the hippocampus of the control Group A animals serve adequately as standard reference. In

the Group A, hippocampal general histoarchitecture was normally demonstrated. The cells- especially neurons, are well demonstrated and clearly observable. Neurons have normal individual characteristic morphology and the spatial distribution is normal. There are no observable deviations of the basic histological and cyto-morphological features from normal. Photomicrographs of the hippocampus of the Group A experimental animals was clearly demonstrated in terms of its entire outline. The whole hippocampal features were normally demonstrated; so also the dentate gyrus. The cells of the dentate gyrus as well as the Cornu Ammonis are also normally demonstrated.

Photomicrographs of the hippocampus of the Group B experimental animals showed that the whole hippocampus was normally demonstrated. Relative to the control (Group A), the dentate gyrus was normal and cells of the dentate gyrus and Cornu Ammonis were also normally demonstrated and spatially distributed. These observations showed that caffeine at the dose employed did not have significant and observable deleterious effects that could have altered the hippocampal formations and its constituent's cells in the various distinct parts.

The medium caffeine dose was administered to the Group C experimental animals. Photomicrographs of the hippocampus of the Group C experimental animals (showed that the whole hippocampal formation was demonstrated). Relative to the control (Group A), the dentate gyrus is normal and cells of the dentate gyrus and Cornu Ammonis are also normally demonstrated and spatially distributed. This also showed that the effects of caffeine on the hippocampus in this group were not significantly deleterious and neither could it be inferred to have improved hippocampal structural integrity.

In the Group D, photomicrographs of the hippocampus of the Group D experimental animals shows that the hippocampus is demonstrated without extensive disruption. However, relative to the control (Group A), cells of the dentate gyrus and Cornu Ammonis are sparse and less prominent. The lip of the dentate gyrus is also relatively thinner. These observations collectively showed that caffeine could affect the structural integrity of certain parts of the hippocampal formation.

#### ***Demonstration of Nissl bodies***

The Cresyl fast Violet staining technique helps to understand the cytological conditions of the demonstrated cells by demonstrating the Nissl bodies or materials in the cell. Photomicrographs of the hippocampus of the Group A (Control) experimental animals showing the dentate gyrus [A] and the Cornu Ammonis [B] (CFV). Hippocampus is demonstrated with structurally normal dentate gyrus and Cornu Ammonis. Photomicrographs of the hippocampus of the Group B experimental animals showed that the dentate gyrus [A] and the Cornu Ammonis [B] (CFV) were relatively normal relative to the control. Photomicrographs of the hippocampus of the Group C experimental animals showed that the hippocampal dentate gyrus cells stain relatively less intensely for Nissl bodies. Also, Cornu Ammonis is relatively thinner and its cells stain relatively intensely for Nissl bodies. These effects are attributable to caffeine effects at the dose employed. Photomicrographs of the hippocampus of the Group D experimental animals show the dentate gyrus [A] and the Cornu Ammonis [B] (CFV). Hippocampal dentate gyrus cells are sparse and they stained relatively less intensely for Nissl bodies. Cornu Ammonis is relatively thinner and its cells are sparse. Caffeine high dose, as seen in the research, therefore affected hippocampal cells and their expression of Nissl bodies and an indication of cytoplasmic functional integrity relative to the synthesis of protein.

#### ***Immunohistochemical demonstration of astrocytes***

Photomicrographs of the hippocampus of the Groups A, B, C and D experimental animals showed the expression of the glial fibrillary acidic protein (GFAP) in astrocytes within the hippocampal formation (GFAP). Astrocytic expression of GFAP was observable in treated groups and expression increased with dose. Astrocytic reaction was most prominent in the Group D hippocampal formation when the highest dose of caffeine was employed. This observation suggested that caffeine did not induce extensive astrocytic reactions in the groups that were administered the lower caffeine doses, but caffeine induced more prominent astrocytic reactions in Group D.

Caffeine effects on the hippocampal formation did not cause extensive disruption or deleterious effects at any of the doses employed. However, at the high dose, caffeine altered general hippocampal outline and morphologies. Astrocyte reactions in this group also indicate a high stimulation of the brain to have induced

### ***Cytochrome – C – oxidase***

In Figure 4a the level of cytochrome-c-oxidase was statistically significant in the groups administered caffeine when compared to the Group A. there was a significant increase in the level of Cytochrome-C-Oxidase across the groups. This result suggests that the stimulatory effects of caffeine caused a relatively high increase in the amount of metabolism in the body. However, the groups B, C and D administered caffeine showed a varying result.

### ***Glucose-6-phosphate dehydrogenase (G6PDH).***

In Figure 4b there was a statistical significance in the level of G6PDH in the Groups B (10 mg/kg), C (50 mg/kg) and D (120 mg/kg) administered caffeine when compared to the Group A; there was a significant increase in the levels of G-6-PDH across the groups administered caffeine. This also indicates the indirect stimulatory effects of caffeine resulting in increased metabolism and increased use of ATP. However, there was a varying result among the Groups administered caffeine.

### ***Gamma amino butyric acid (GABA)***

In Figure 5a the level of GABA across the groups B (low dose caffeine), C (medium dose caffeine) and D (high dose caffeine) treated with caffeine was higher than the Group A (control). This simply means that caffeine increases the level of GABA neurotransmitter in the brain. However, there was statistical significance in groups B and C treated with low dose (10 mg/kg) and medium dose respectively. Interestingly the group treated with high dose (120 mg/kg) of caffeine group D showed no significance when compared to group A; to explain the varying level of GABA in the treated groups with respect to the amount of caffeine administered, a research by Shi stated that at the chronic dosage of caffeine at  $\geq 100$  mg/kg is not known to block extracellular receptors other than the adenosine receptors.

### ***Acetylcholine***

In Figure 5b there was increase in the level of acetylcholine across groups B (low dose caffeine), C (medium dose caffeine) and D (high dose caffeine) treated with caffeine. From the graph it can be deduced that caffeine increases the level of acetylcholine in the homogenates. While the group B (low dose 10 mg/kg) and group C (50 mg/kg) showed a statistically significance increase the group D treated with high dose caffeine (120 mg/kg) didn't show any significance when compared to group A. This supports the statement that caffeine causes an increase in hippocampal acetylcholine which may block consolidation by causing congestion in the replay of memories [22]. In the subgranular Zone of the dentate gyrus GABAergic mechanisms regulate differentiation and also the timing of synaptic integration.

### ***Glutamate***

In Figure 5c, the bar chart showed an increase in the level of glutamate across all the groups treated with caffeine Group B (10 mg/kg), Group C (50 mg/kg) and Group D (120 mg/kg). This signifies that caffeine increased the level of glutamate in the brain homogenate. There was a statistically significant increase in the level of glutamate in all the caffeine treated groups. Glutamate is an excitatory neurotransmitter of the central nervous system, since caffeine is a psychostimulant, it fires up the activities of the excitatory neurotransmitter and glutamate is not excluded. In support of this result Rossi stated that caffeine blocks the A2A receptors by reducing the activation of cAMP-PKA pathways which causes an increase in the level of glutamate, and also activation of metabotropic mGlu5 receptors and the release of endocannabinoid.

### ***Neurobehavioural results: Barnes Maze test for short term memory***

In the experiment conducted the result showed that there were aberrations in parameters, but without statistical significance in all the parameters used which are; primary latency (the amount of time it takes for the animal to first locate the escape box), total latency (total time taken for the rat to locate the escape box), primary pokes (the number of times the animals made errors before first locating the escape box) and secondary pokes (the total number of times the animals made errors before finally making it to the escape box). However, the bar charts in Figures 6a-6d showed that there was a reduction in the values of the Group B (Low dose 10 mg/kg) in the above parameters when compared to group A. This implies that caffeine improved the short term memory and learning of the Group treated with low dose. Also, in the figures mentioned above the group treated with medium dose caffeine (50 mg/kg) showed a slight reduction when compared to Group A in all the parameters stated, this also signifies that caffeine at a moderate dose of 50 mg/kg is known to slightly improve short term memory and learning. In Figures 6a and 6b, the high dose

caffeine (120 mg/kg) showed that there was almost no difference when compared to Group A (control group) using the primary and total latency parameters. This might suggest that caffeine at a high dose inhibits short term memory. However, there was a very high increase in the primary and total number of errors made by the group treated with high dose caffeine (120 mg/kg) when compared to Group A and other groups B and C.

Generally, caffeine can be said to improve short-term memory and learning when ingested at moderate doses from 10 mg/kg to 50 mg/kg, this was supported by Glade that at moderate doses, caffeine enhances short-term memory.

### CONCLUSION

The compilation of the results gotten from these research showed there is little or no effect of caffeine on the physical and morphological parameters. However, results from the histology revealed no defect in structure of the hippocampus and the dentate gyrus at the various doses of caffeine but has an effect on the spatial distribution of cells in the high dose caffeine. There were also visible effects on the Nissl bodies when compared to the normal. Immunohistochemistry revealed an assault on the astrocytes in the treated groups. From the neurotransmitter assay, conclusions derived shows that caffeine has a stimulatory effect on GABA, Glutamate and Acetylcholine of which they are excitatory in nature at the peri-pubertal age. The increase in cytochrome-c-oxidase and glucose-6-phosphate dehydrogenase showed the direct effect of caffeine on metabolism which directly increases the production of ATP. The neurobehavioural study showed the effect of specific graded doses of caffeine that improved short-term memory (10-50 mg/kg) and learning as well as the dose (120 mg/kg) that might have a reverse effect. Caffeine has also been seen to increase anxiety by a relative increase in the close arm duration and entries in all the groups.

It is important to note that from all the results presented, caffeine might have a positive and negative dose-dependent and dose- duration effect on the structure of the hippocampus at peri-puberty.

### RECOMMENDATION

Further research should be done on the effects of long-term consumption of caffeine on hippocampal structure with much emphasis on the structure of the dentate gyrus. Also, researches should be conducted on the behaviour of neurotransmitters in peri-pubertal hippocampal system as this might be an indication for neurogenesis.

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