Original Research Article

EFFECT OF CALCIUM CARBIDE INDUCED RIPENED FRUIT ON THE HIPPOCAMPUS OF ADULT WISTAR RATS

ABSTRACT

Calcium carbide (CaC_2) produces acetylene gas that quickens the ripening process of fruits because it has similar properties to ethylene. CaC_2 as a toxic substance has deleterious effects on several organs, especially on the nervous system. This study was carried out to investigate the neurotoxicity effect of CaC_2 on the hippocampus. Twenty-eight male Wistar rats were grouped into 4 groups of 7 animals. Group1 (control) received distilled water while Group2, Group3 and Group4 were administered with 100mg/kg CaC_2 in water for 7, 14 and 21 days respectively. The rats in each group were subjected to the Barnes Maze test and observations were recorded. On completion of the tests which lasted for 2 days, the rats were sacrificed, and their brains were extracted and processed for neuropathological examination. The results of the Barnes Maize test showed a significant difference with control on the 7th, 14th and 21st days CaC_2 administered rats ($P<0.05$). Neuropathological examination revealed extensive neuronal degeneration and vacuolation of the pyramidal layer, molecular layer and granular layer of the hippocampus in the calcium carbide group which worsen at the third week. The Natural banana group showed rapid duration dependent proliferation of the Neuronal cells with a well-preserved Neuronal architecture. This may suggest that Natural ripened Banana is composed of some mitotic compositions. The calcium carbide and vitamin C group had a better neuronal architecture which was evident at third week. This also showed that Vitamin C has an ameliorative effect in calcium carbide poison. The In conclusion, this study has demonstrated the neurodegenerative effects of CaC_2 on the hippocampus and concurrent Neurobehavioral changes in hippocampal-related learning and memory ability.

Key words: Wistar Rat, Calcium carbide, Hippocampus, Neuropathology, Behavioral studies

INTRODUCTION

Calcium carbide (CaC_2) ripened fruits and vegetables are consumed on daily basis due to lack of awareness and ignorance (Wasim *et al*., 2010). CaC_2 can induce ripening within 24 hours and the fact that it is cheap makes it a popular ripening agent among banana marketers, especially in developing countries (Ajayi and Mbah, 2007). The fast-ripened fruits contain harmful properties because CaC_2 contains traces of arsenic and phosphorus and the production of acetylene gas has a hazardous effect on human health mainly for the nervous system (Hossain *et al*., 2015).

Impurities like arsenic and phosphorus found in industrial grade CaC_2 cause dizziness, frequent thirst, irritation in the mouth and nose, weakness, permanent skin damage, difficulty in swallowing, vomiting, and skin ulcer among workers who are in direct contact with these chemicals while applying the ripening agent. Higher exposures may even cause undesired fluid build-up in lungs (pulmonary oedema) (Fattah and Ali, 2010). The brain is most vulnerable to oxidative damage compared to other organs due to its biochemical and physiological properties. It consumes an inordinate fraction (20%) of total oxygen consumption for its relatively small weight (2%) of body weight.).According to (Kjuus *et al*., 2007; Kurtoglu *et al*., 2007), acetylene gas released by CaC_2, is an asphyxiant and may affect the nervous system by inducing prolonged hypoxia, which causes headache, dizziness, mood disturbances, sleepiness, mental confusion, memory loss, cerebral oedema (swelling in the brain caused by excessive fluids) and seizure. The hippocampus belongs to the limbic system and plays important roles in the consolidation of information from short-term to long-term memory and spatial navigation (Shashi and Jatinder 2016). Studies have shown that the hippocampus, which is the center for learning and memory, is highly vulnerable to neurotoxins (Bhatnagar *et al*., 2002). The use of CaC_2 as a ripening agent by fruit vendors in Nigeria is still ongoing despite the health hazard. Therefore, the aim of this study was to investigate the neurodegenerative effect of CaC_2 on the hippocampus of a Wistar Rat and the resultant neurobehavioral-related changes

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MATERIALS AND METHOD

Experimental Animal: Twenty-eight adult male Wistar albino rats weighing between 210g and 220g were used for this research. The animals were obtained and acclimatized at the animal house, Faculty of Basic Medical Sciences, University of Port Harcourt, Rivers State, Nigeria. All

animals were handled in accordance with the guidelines for animal research as detailed in the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication,1985).

Formulation of Toxicant: Samples of CaC_2 were obtained from a welding and fabrication workshop in Rumuosi/Rumekini, Obio/Akpor Local Government Area of Rivers State, Nigeria. 2g of CaC_2 was mixed in 50ml distilled water. The LD50 oral of CaC_2 is >2000mg/kg (Toxicology information on Safety data sheet from Thermofisher scientific, 23rd January 2018).

Figure 1: calcium carbide

Experimental Design: A total number of twenty-eight Wistar rats were grouped into 4 groups of 7 animals each. Group 1 (control) were fed with standard rat chow and water. Group 2 (naturally ripened banana) were fed with standard rat chow, water and 2ml of naturally ripened banana juice. Group 3 was given 2ml of calcium carbide ripened banana juice orally and fed with standard rat chow and water. Group 4 were given 2ml of the calcium carbide ripened banana and 200mg/kg body weight of vit C and fed with standard rat chow on daily basis.

Neurobehavioral Study

Barnes Maze test assesses cognitive deficits in rodent models of CNS disorders. It measures learning abilities without forcing the subjects to perform a task under unnatural conditions, i.e. swimming in water. Testing occurs on a circular platform with numerous escape holes ringed around the center of the platform. Bright overhead lighting creates an aversive stimulus, encouraging the animal to seek out the Target Escape Hole, which is attached to an escape tube, and escape from the light.

Group	Treatment	Barnes maze Test (Week 1)	Barnes maze Test (week 2)	Barnes maze Test (week 3)

Figure 2. Barnes Maze Test.

Histological staining

On completion of the passive avoidance test, the rats were sacrificed and perfused transcardially. The brains were extracted, post fixed overnight in 10% formal saline and then embedded in paraffin wax. Sagittal sections were prepared at 5µm thickness and collected for histological staining with toluidine blue stain.

RESULTS

Findings were tabulated and analyzed with results expressed as mean ± SEM. Statistical analysis was done using one-way Analysis of Variance (ANOVA). The results were compared using Post-hoc (LSD) test. Results were considered significant at $p< 0.05$.

		Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Group 1	Control groups	183.20 ± 71.53 a, b	127.0 ± 25.72 a	85.60 ± 34.05 b	14.80 ± 1.96 b	46.40 ± 3.92 a, c	55.80 ± 2.94 a, b	47.20 ± 11.20 b, c	54.80 ± 16.71 b	51.80 ± 4.82 b
Group 2	Natural banana group	26.80 ± 1.96*c	11.0 ± 2.45* b	41.40 ± 0.25 B	21.20 ± 6.61 b	14.80 ± 2.94* b	14.60 ± 2.2* b, c	17.0 ± 5.39 b, c	13.60 ± 2.40 b, c	14.0± 2.16 b
Group 3	Carbide + banana group	34.0 ± 7.35* C	200.40 ± 60.99 a, c	232.60 ± 19.84* a, c	148.20 ± 61.97* a, c	44.0 ± 11.02 a, c	300.0± 0.0* a, c	216.20 ± 44.39* a	136.40 ± 30.02* a	243.40 ± 56.6* a, c
Group 4	Carbide+ banan+ Vit C	172.20 ± 52.17 a, b	45.80 ± 0.49 b	73.20 ± 0.49 B	31.40 ± 12.49 b	17.40 ± 2.21* b	58.80 ± 9.31 a, b	204.0± 58.79* a	78.80 ± 18.86 a	41.60 ± 7.10 b

Table 1: The visual memory and cognition assessment in the control and test groups using Barnes maze Task.

Values are presented as mean ± sem. N=5.

* means values are statistically significant when compared to the control

“a” means values are statistically significant when compared to the natural banana group

“b” means values are statistically significant when compared to the calcium carbide + banana group

“c” means values are statistically significant when compared to the calcium carbide + banana + vit. C group

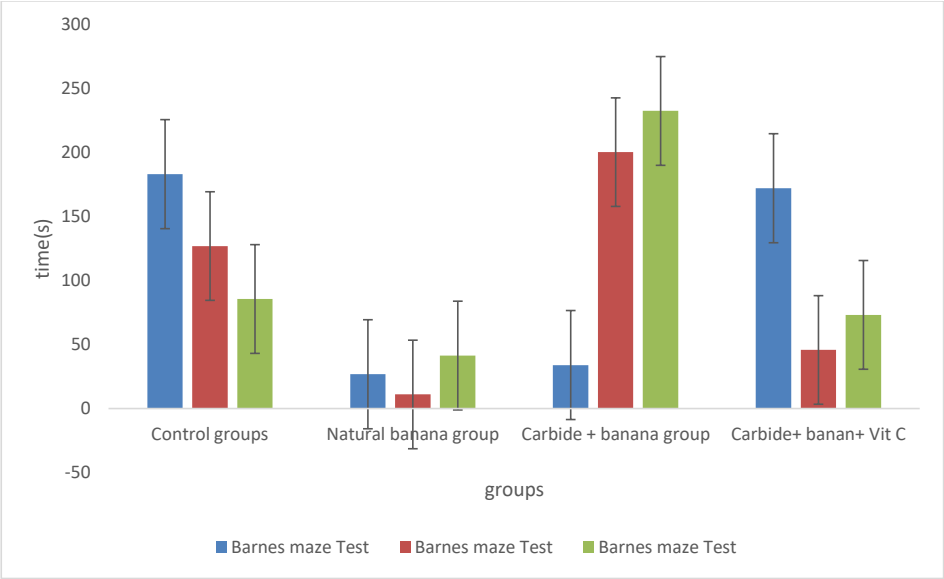

Figure 3: Visual memory and cognition assessment in week 1 using Barnes maze Task

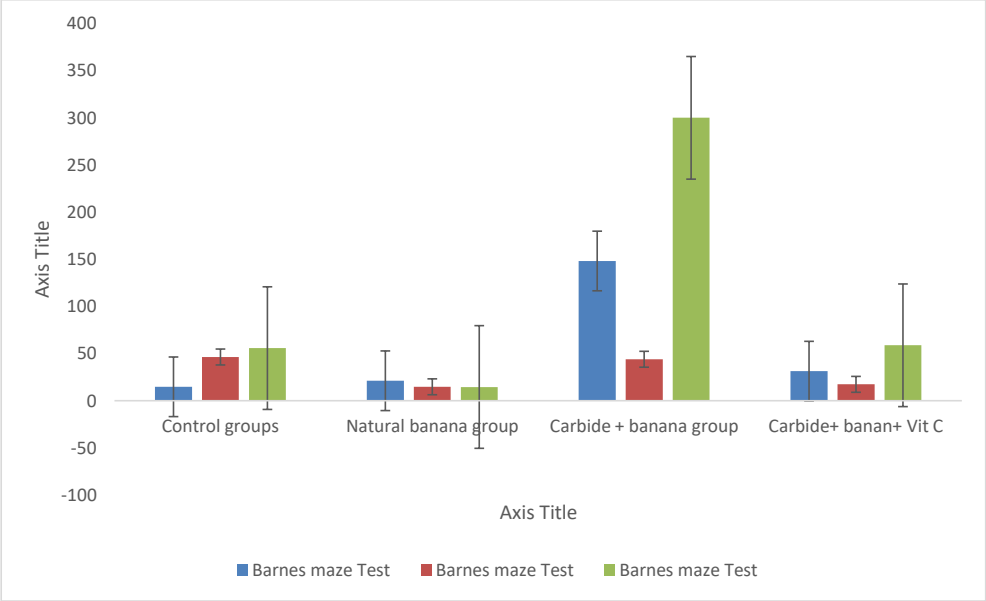

Figure 4: Visual memory and cognition assessment in week 2 using Barnes maze Task

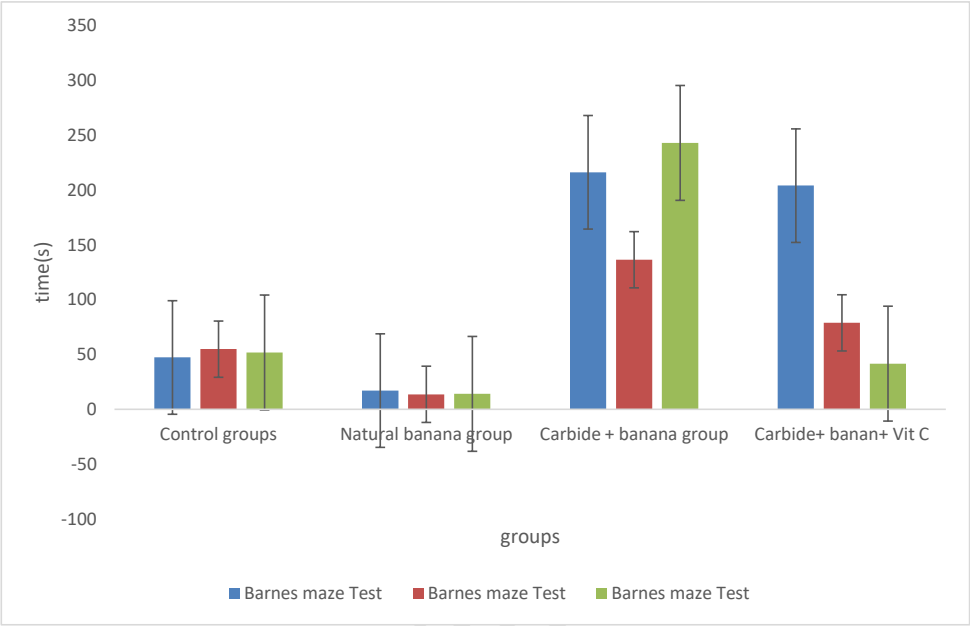

Figure 5: Visual memory and cognition assessment in week 3 using Barnes maze Task

Barnes Maze Task

The effect of application of different group treatment and visual memory and cognition assessment on rat in various trials is also demonstrated in figure 3 Mean level of visual memory and cognition assessment in the control and test groups were 183.20 ± 71.53, 127.0 ± 25.72 and 85.60 ± 34.05 for trial 1, 2, and 3 respectively, which increased significantly. Application of Barnes maze task treatment also significantly increased visual memory and cognition assessment of rat between 26.80 ± 1.96*, 11.0 ± 2.45* to 21.20 ± 6.61 for Natural banana group, 34.0 ± 7.35*, 200.40 ± 60.99 to 232.60 ± 19.84* for Carbide + banana group and 172.20 ± 52.17, 45.80 ± 0.49 and 73.20 ± 0.49 for Carbide+ banana+ Vit C.

The result for week two for treatment group and visual memory and cognition assessment on rat is demonstrated in figure 4 Mean level of visual memory and cognition assessment in the control and test groups were 14.80 ± 1.96, 133.4±68.2s, 46.40 ± 3.92 and 55.80 ± 2.94 for trial 1, 2, and

3 respectively, which increased significantly. Application of visual memory and cognition assessment treatment significantly increased between 21.20 ± 6.61, 14.80 ± 2.94* to 14.60 ± 2.21* for Natural banana group, 148.20 ± 61.97*, 44.0 ± 11.02 to 300.0± 0.0* for Carbide + banana group. But showed 31.40 ± 12.49, 17.40 ± 2.21* to 58.80 ± 9.31 for Carbide+ banana+ Vit C which was statistically insignificant.

Conversely, result for week three on application of different treatment group and visual memory and cognition assessment treatment on rat is demonstrated in figure 5. Showed the visual memory and cognition assessment in the control group were 47.20 ± 11.20, 54.80 ± 16.71 and 51.80 ± 4.82 for trial 1, 2, and 3 respectively, which increased significantly. Application of various Barnes maze task treatment also significantly increased visual memory and cognition assessment of rat between 17.0 ± 5.39, 13.60 ± 2.40 to 14.0± 2.16 for Natural banana group, 216.20 ± 44.39*, 136.40 ± 30.02* to 243.40 ± 56.6* for Carbide + banana group and 204.0± 58.79*, 78.80 ± 18.86 to 41.60 ± 7.10 for Carbide+ banana+ Vit C.

We observed significant changes in the hippocampal morphology across different groups with massive duration dependent changes in the cytoarchitecture of the hippocampus.

Week 1: The control group showed the normal cytoarchitecture of the regions of the hippocampus. The of pyramidal cells in CA1, 2, 3 and 4 regions were visible and well persevered, while dentate gyrus (DG) showed normal granular cells (Group 1, Figure 6 A&B). The group 2 which is the Natural ripened banana group showed a well preserved cytoarchitecture of the hippocampal neurons which was duration dependent (Figure 6 C&D.) The calcium carbide group showed destruction of the pyramidal cell layer of the CA1 region (necrosis), with fewer cells observed in the molecular layer when compared with the control group. There were vacuolations and disintegration of granule cells of the DG with no obvious change in its overall cytoarchitecture (Fig 6, E& F). The group 4 (Calcium carbide and Vitamin C, showed a restored cytoarchitecture of the neurons with densely packed cells. (Fig 6, G& H).

Week 2: The control group showed the normal cytoarchitecture of the regions of the hippocampus. The CA1, 2, 3 and 4 regions are composed of pyramidal cells, while the dentate gyrus (DG) presented normal granular cells (Fig 7, I& J). The group 2 (Natural Banana),

showed increase in Neuronal density (Fig 7, K& L). The group 3 (calcium carbide group), the hippocampus showed fewer cells in the molecular layer, decrease in neuronal density, more vacuolations of the pyramidal cells and disintegration of granule cells of the dentate gyrus were when compared with the control observed (Fig 7, M & N). The group 4(Calcium carbide and Vitamin C showed more restored cytoarchitecture of the neurons with densely packed cells (Fig 7, O& P)

Week 3: The control group showed the normal cytoarchitecture of the regions of the hippocampus. The CA1, 2, 3 and 4 regions showed well preserved pyramidal cells, while the granular cells of dentate gyrus (DG) were intact (Fig 8, Q& R). The Group 2 (Natural ripened Banana) showed more densely packed cells (Fig 8, S&T). In Group 3, there was extensive vacuolation of hippocampal cells and disintegration of granule cells dentate gyrus, loosely packed cells, thus indicating neuronal cell death caused by CaC_2 (Fig 8, U& V). The group 4 (Calcium carbide and Vitamin C, showed a restored cytoarchitecture of the neurons with densely packed cells (Fig 8, W& X)

WEEK 1

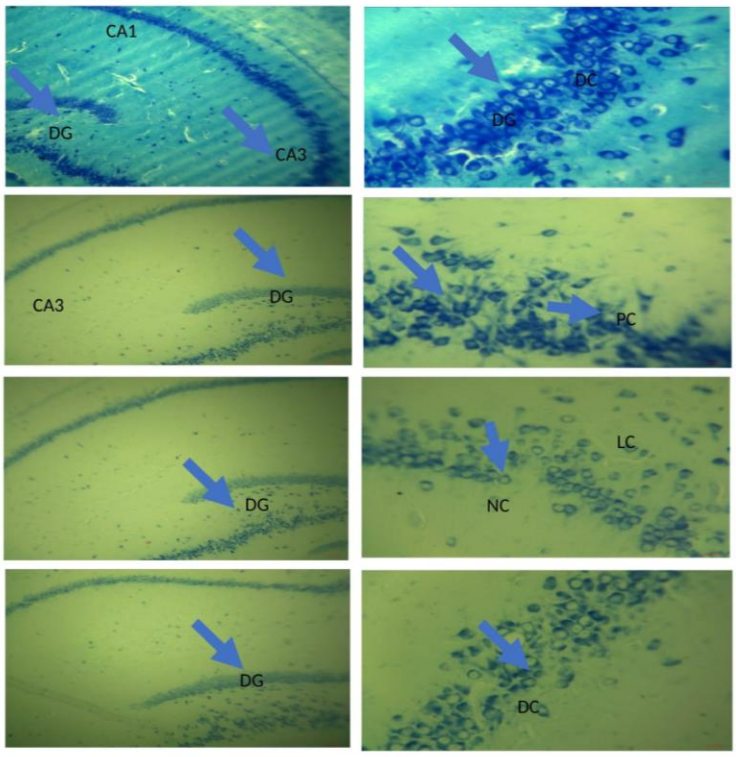

Figure 6: **Photomicrograph of Hippocampus at week 1; Group 1(control)** A& B @ x100 & 400: The hippocampus for the control group showed normal cytoarchitecture of the various regions. The pyramidal cells of the CA regions and the granular cells of the dente gyrus (DG) are preserved.

Group 2(Natural ripened Banana) C & D @ x100 & 400: cell Proliferation, densely packed cells (**DC**), well preserved, cytoarchitecture

Group 3 (CaC_2 ripened Banana) E & F @ x100 & 400: Showed destruction of the pyramidal cell layer of the CA1 region., Necrotic Cell (NC), Loosely arranged cells **(LC)**

Group 4(CaC_2 ripened Banana + Vitamin C) W & X @ x100 & 400: Restored cytoarchitecture of the neurons with densely packed cells

WEEK 2

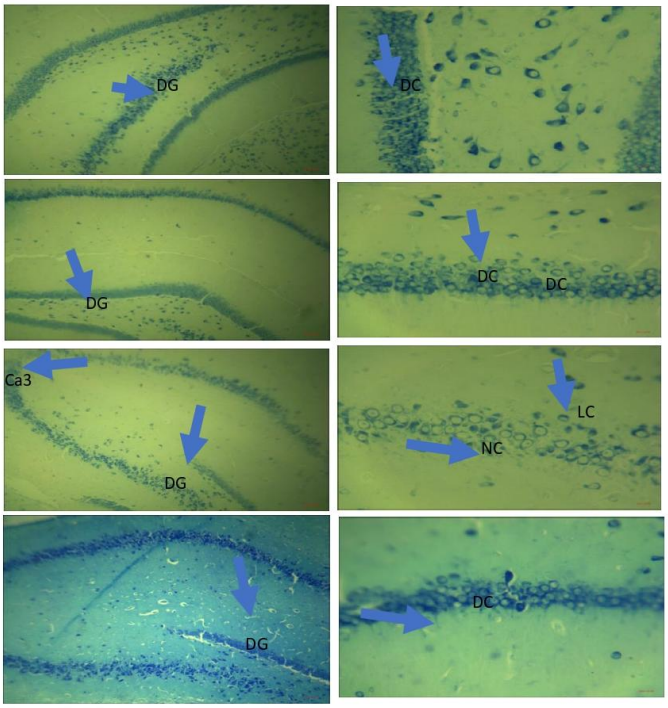

Figure 7: **Photomicrograph of Hippocampus at week 2**. **Group 1(control)** I & J @ x100 & 400: The hippocampus for the control group showed normal cytoarchitecture of the various regions. The pyramidal cells of the CA regions and the granular cells of the dente gyrus (DG) are preserved.

Group 2(Natural ripened Banana) K & L @ x100 & 400: Densely packed cells, well preserved cytoarchitecture

Group 3 (CaC_2 ripened Banana) M & N @ x100 & 400: Showed destruction of the pyramidal cell layer of the CA1 region. Necrotic Cells **(NC)**

Group 4(CaC_2 ripened Banana + Vitamin C) W & X @ x100 & 400: Restored cytoarchitecture of the neurons with evidence of cellular proliferation

WEEK 3

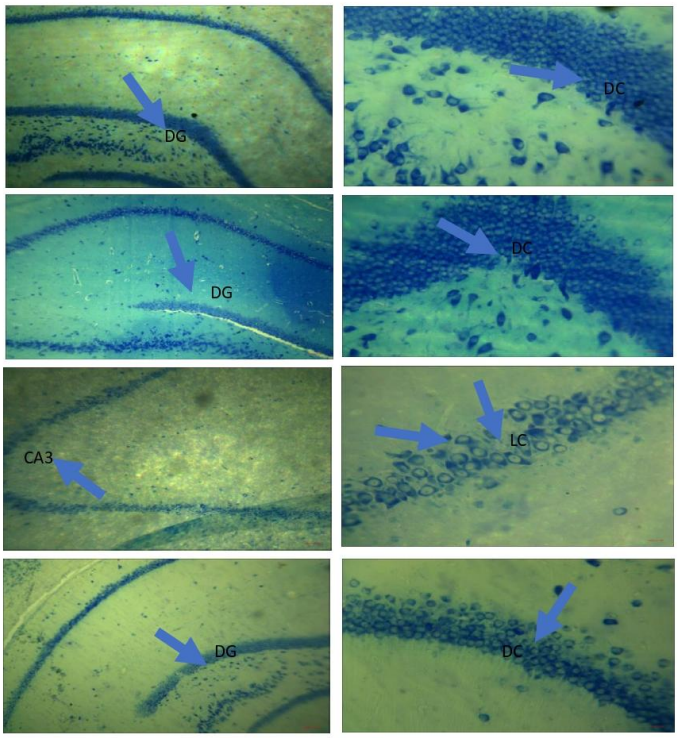

Figure 8: **Photomicrograph of Hippocampus at week 3**. **Group 1(control)** Q & R @ x100 & 400: The hippocampus for the control group showed normal cytoarchitecture of the various regions. The pyramidal cells of the CA regions and the granular cells of the dente gyrus (DG) are preserved.

Group 2(Natural ripened Banana) S & T @ x100 & 400: Densely packed cells, well preserved cytoarchitecture

Group 3 (CaC_2 ripened Banana) U & V @ x100 & 400: Showed destruction of the pyramidal cell layer of the CA1 region. Necrotic Cells (NC) and Loosely Packed Cells (LC),

Group 4(CaC_2 ripened Banana + Vitamin C) W & X @ x100 & 400: Restored cytoarchitecture of the neurons with densely packed cells, Proliferated Cells.

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DISCUSSION

The toxicity of Calcium Carbide cannot be over emphasized, as several studies have revealed its toxicity on body organs including the liver, testis, kidney, spleen and blood parameters (Gbakon, *et al.*, 2018; Patoare, *et al.*, 2007). The use of calcium carbide as a ripening agent in developing, low- and middle-income countries is on the increase not minding its associated health hazards (Dhembare *et al.*, 2011). This unregulated and abuse use of ripening agent may account for the rise in the cases of Neurodegenerative diseases like dementia which was previously seen as a western disease. In Nigeria, it is seen as the cheapest and easiest way of ripening fruits for sales, in other to make large profits (Mariappan, 2002). The key structure of memory formation passes through the hippocampus (Graves and Pickening, 1999). It is also involved in retention and retrieval of memories created (Fortin *et al.*, 2004). This study shows that CaC_2 causes depletion of the essential component of fruit and vegetables ripened with it.

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Neurobehavioural Analysis

Barnes maze study demonstrated that the rats showed good visual memory and cognition in the group treated with naturally ripened banana compared to the other groups. This implies that naturally ripened banana increases the learning and memory capacities to form cognitive maps. This can be likened to the presence of potassium in bananas that function in generating electrical charges that helps the cells to function properly. Also, banana contains tryptophan an essential amino acid important to produce serotonin which has a beneficial impact on learning and memory skills (Cording, 2015; Harbottle, 2016; Jenkins *et al.*, 2016; Lindseth *et al.*, 2015; Sansone and Sansone 2013). In the carbide treated banana group, it was observed that the rats were either reluctant or took longer time to perform a task as shown in the result. This is because calcium carbide has been proven to have very toxic effects such as mental confusion, mood disturbances, sleepiness (Per *et al.*, 2007). It can also cause alterations in haematological and biochemical parameters (Igbinaduwa and Aikpitanyi-Iduitan, 2016).

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The control group showed a general cytoarchitecture of the hippocampus with cellular integrity appearing normal, showing densely packed rounded neurons containing large vesicular nuclei, hence healthy. The natural banana group showed a normal pyramidal layer formed of densely packed round neurons. The calcium carbide banana group, cells were seen to be loosely packed, distortions were observed, degenerative changes in axons of nuclei and neuronal vacuolation. Histological findings in this Calcium carbide group showed higher destruction of cells in the

CA1, DG and CA3 regions of the pyramidal cell layer, molecular layer and granular cell layer in the CaC_2-induced groups. We observed more conspicuous changes in CA1 and CA3 regions than CA2 and CA4. The death of hippocampal cells caused by necrosis, and the loss of pain sensation which have been reported in our previous passive avoidance study, may be due to phosphine present in CaC_2 which hindered the creation, retention and retrieval of memory observed in the subsequent trials. Phosphine a compound found in Calcium carbide causes agitation followed by convulsions, hyperactivity and lethargy in humans (Hüseyin *et al.,* 2007) and what has been referred to as necrosis or anesthesia in animals (Li and Rossman 1989).

Histological results of this study clearly support earlier reports that phosphine contained in CaC2 extensively destroys hippocampal neurons (Nwoha *et al*., 2007) and that phosphine induces excitotoxicity in the brain as revealed by Al-Azzawi *et al*., (1990); Potter *et al*., (1993). Based on the works by Alkayed *et al*. (1997); Zonta *et al*. (2003), the above result could be said to be due to the disruption of astrocyte specific Na^+, K – ATPase or its provocation of electrical changes in the hippocampus and cerebral cortex which is like those noticed during generalized seizure

CaC_2 at long duration increases acetylcholine neurotransmission by suppressing acetylcholine esterase. Because acetylcholine is an excitatory neurotransmitter and the role of the esterase is to attenuate acetylcholine signaling, exposure to phosphine contained in CaC_2 would be expected to inhibit the attenuation. The net result would be overactive acetylcholine signaling, which would most likely be expressed as hyperactivity and in extreme cases, excitotoxicity (Valee, *et al.,* 1960) in the limbic regions producing extensive neuronal degeneration in the hippocampus, leading to learning and memory loss (Nwoha *et al.,* 2007). It has been reported that CaC_2 induces neurotoxicity by generating reactive oxygen species and these free radicals cause oxidative stress that result in brain neuronal damage (Anderson, 1994, Prasanna *et al*., (2007). Our previous study on the effect of calcium carbide on the hippocampus also reported neurodegeneration of CaC_2 on the hippocampus and concurrent neurobehavioral changes in hippocampal-related learning and memory ability (Ibeachu *et al*., 2019)

Neuronal distortion can cause information which are carried by the CA1 region from the CA3 region to the subiculum and out of the hippocampus to the entorhinal cortex to be affected and there won't be proper flow of information in the brain. This will lead to anterograde amnesia which implies that the rats might not be able to form new memories. This agrees with the work

done by Di Gennero *et al.*, 2006, Catherine 2006 which showed that damage to the hippocampus will result in both anterograde and retrograde amnesia. The CA1 region is also involved in spatial movement. This implies that rats here might not be able to remember and form memories of a new environment they find themselves. Previous studies in arsenite-treated cultured primary rat hippocampal neurons showed symptoms of apoptosis such as decreased viable cell growth, cytoplasm vacuoles, and nuclear condensation with intact membrane (Yang, *et al*., 2003). Similar changes were observed in the present study. Arsenate compound found in CaC_2 has also been reported to cause cell death in the brain and other body organs by Barret, *et al*., (1989). In the present study the group that received natural Banana showed proliferation of the neuronal cells, which means that the CaC_2, toxicity causes arrest in cellular growth. The reason for the mitotic figure seen could be because of tryptophan present in Banana. This is however in accordance with Brunner (1973) who reported that limitation of tryptophan in the growth medium of mouse LM cells produces a growth arrest, presumably in G1, which is reversible, and which is attended by partially synchronous growth upon restoration of tryptophan. The mechanism by which tryptophan exerts its effects upon cellular proliferation and DNA synthesis in the absence of diminished protein synthesis is not clear (Paul et., al 1974)

Administration of vitamin C was seen to reduce the impairment caused by the calcium carbide. Vitamin C is an antioxidant which implies that it helps mop off free radicals produced by toxins hence the hippocampus appearing to be undergoing healing or regeneration. From this study, the ameliorative effect of the vitamin C was seen to be time dependent. In other words, rats in week three showed better and a more improved structure of the hippocampus than rats in week 1 which were given same doses of vitamin C.

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CONCLUSION

In conclusion, this study has shown the neurodegenerative effects of CaC_2 on the hippocampus and demonstrates that such degenerative effects could lead to neurobehavioral changes such as learning, and memory abilities as observed during the passive avoidance test. The mitotic figure observed in the Banana group proved that CaC_2 causes depletion of the essential nutrients of fruit ripened with it. However, this study also revealed the efficacy of Vitamin C in ameliorating the CaC_2 toxicity.

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