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Evaluating the Effect of Chloroform Inhalation as a Method of Euthanasia on the Cerebellum and Hippocampus of Adult Wistar Rats

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Authors' contributions

This research work was carried out in collaboration among all authors. Author USA designed and coordinated the research and also drafted the first manuscript. Authors OSN and EDN corrected the first manuscript and drafted the final manuscript. Authors ECE, AO, OBN, OS and OD carried out the bench work. Authors OOF and OKC oversaw the laboratory analysis while authors DO and OC oversaw the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Chloroform inhalation is a common method of rodent euthanasia in Nigeria for research purposes. This work is designed to evaluate the consequence of this method of sacrifice on the cerebellum and hippocampus of Wistar rats. Twenty male Wistar rats weighing between 160 and 180 were divided into 4 groups of 5 rats each. Group A served as control and was sacrificed using cervical dislocation (a widely acceptable non-inhalation method of sacrifice). Groups B, C and D were exposed to chloroform for 5 minutes once a day (group B), once a day for 2 days and once a day

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for 3 days. The brains were removed; four from each group was processed for antioxidant assay while one from each group was fixed in Bouin's fluid for histological studies. Our results show that chloroform inhalation adversely affected the results of the antioxidant parameters studied in a dose-dependent fashion. That means that the adverse effect worsened as the number of days increased. This was also the case with the histology results as there was evidence of cell necrosis in the cerebellar and hippocampal tissues. This also showed dose dependence. We therefore conclude from our results that when studying the brain tissues or carrying out brain related researches, chloroform inhalation is not the method of choice for rat euthanasia.

Keywords: Euthanasia; chloroform; inhalation; cerebellum; hippocampus.

1. INTRODUCTION

Chloroform is a colorless liquid that is not very soluble in water and is very volatile. It has a pleasant, nonirritating odor with an odor threshold is 85 ppm. The chemical formula for chloroform is $CHCl_3$, a molecular weight of 119.38 g/mol, a vapor pressure of 159 mm Hg at 20°C, and a log octanol/water partition coefficient (log Kow) of 1.97 [1,2]. Chloroform is a commonly used chemical in biological laboratories and for industrial processes. It is nonflammable. Most of the population is exposed to very low levels of chloroform every day in the air, food and water we take in. Low levels of chloroform are found in the air and in coastal waters, inland rivers, lakes and groundwater. Exposure to higher levels of chloroform is very unlikely for anyone outside industries using or manufacturing chloroform. Levels can be higher in industrial areas as well as in the air above swimming pools containing chlorine [3].

Chloroform is used as a solvent for lacquers, floor polishes, resins, adhesives, alkaloids, fats, oils and rubber. It is also used in the building, paper and board industries, in pesticide and film production. Until the mid-1900s, chloroform was used as an anesthetic to reduce pain during medical procedures. Today, it is not used in this way due to its harmful effects. Breathing air with chloroform for a short time causes headache, fatigue and dizziness. Longer exposure to chloroform damages the brain, liver and kidneys. It may also cause cancer. Respiratory injuries from chloroform exposure include respiratory depression, pneumonitis and pulmonary edema. Drinking water with chloroform over a long period damages the liver and kidneys. It may also cause cancer. On the other hand, eating food with chloroform in it over a long period damages the liver and kidneys and may cause cancer as well [4]. Body contact with liquid chloroform causes sores and skin irritation. It may also cause cancer. In fact, EPA has classified chloroform as a Group B2, probable human carcinogen [2].

Chloroform, which is toxic to the central nervous system, can cause a person to become unconsciousness and can be fatal at high doses. It has not been found to harm a fetus. Effects noted in humans exposed to chloroform via anesthesia include changes in respiratory rate, cardiac effects, gastrointestinal effects, such as nausea and vomiting, and effects on the liver and kidney [5,2]. In humans, a fatal oral dose of chloroform may be as low as 10 mL (14.8 g), with death due to respiratory or cardiac arrest [5,2]. Inhalation exposures of animals have also resulted in effects on the kidney [5,2].

However, even with all these known negative effects, chloroform is still commonly used as an agent for euthanasia of experimental animals, especially rodents in Africa [6]. However, how much damage can result from brief exposure to chloroform as in when used for animal sacrifice is the subject of this research work. Chloroform can be detected in blood, urine, and body tissues. However, these methods are not very reliable because chloroform is rapidly eliminated from the body, and the tests are not specific for chloroform [2].

Animal sacrifice is a method of inducing humane death in an animal by a method that results in rapid loss of consciousness and death for research purposes [7,8]. The *Oxford English Dictionary* reports that *sacrifice* was first used to refer to killing research animals in 1903 [9]. Humans have been using other vertebrate animal species as models of their anatomy and physiology since the dawn of medicine. Because of the taboos regarding the dissection of humans, physicians in ancient Greece dissected animals for anatomical studies [10]. As the experimentation on animals increased, especially the practice of vivisection, so did criticism and controversy. Objections in the use of animal for

experiment came from two main angles; animal right and applicability of result [11]. Some early scientists believed that animals were inferior to humans and so that results from animals could not be applied to humans [12]. On the other hand, scientists in favor of animal testing held that experiments on animals were necessary to advance medical and biological knowledge. Claude Bernard, who is sometimes known as the "prince of vivisectors" [13] established animal experimentation and sacrifice as part of the standard scientific method [14].

Sacrificing research animals is one of the most challenging tasks in animal studies, and it is imperative to do it as humanely as possible. In the course of research, the fate of almost all experimental animals is to be sacrificed at certain stages of the study, either to gain blood, tissue and other specimens or at humane endpoints to prevent any extension of stress or pain [15].

Sacrificing techniques ultimately cause death by three basic mechanisms; direct depression of neuronal activity necessary for life function, hypoxia and/or physical disruption of brain activity [16]. Common methods of animal sacrifice include; inhalation of anesthesia gas – chloroform, ether, or carbon (IV) oxide, immersion agents, decapitation, injectable barbiturate agents, exsanguinations [17]. Different researchers are of the opinion that particular methods of animal sacrifice like chloroform sedation is more ideal for certain research protocol and not for others. Inhaled anesthetics, beginning with diethyl ether, were first introduced into clinical practice in the 1840s. Since then a wide variety of inhaled agents, including ethers, alkanes, nitrous oxide, cyclopropane, and xenon, have been used to induce unconsciousness, amnesia and immobility [18].

Chloroform sedation has been used to sacrifice animals with apparently no undesirable effects. Chloroform is recommended as more aesthetically acceptable for sacrifice of chickens. Chloroform inhalation remains the most commonly used method of euthanasia of rats in most part of Africa including Nigeria. Some reports however claim that chloroform has significant toxicity, including carcinogenicity, hepato-, and nephrotoxicity [19]. Physical techniques, such as cervical dislocation, which have been assumed to be humane methods for the sacrifice of small rodents, have been shown to have a surprisingly high failure rate [20]. Most physical methods have some limited evidence that they may not immediately abolish brain activity or consciousness [21]. Barbiturates are used extensively and are considered the agents of choice for most animal research but barbiturates reduce cerebral metabolism leading to decreases in cerebral blood flow and intracranial pressure. They also produce respiratory depression and can elicit dose-dependent decreases in blood pressure [19]. Carbon dioxide is recommended for the euthanasia of both rats and mice, considering behavioral criteria. Ether is unsuitable as a sacrifice method as it is dangerous, slow acting and an irritant [22]. This research therefore seeks to shed some light in these areas as regarding rat sacrifice.

The cerebellum and hippocampus are essential parts of the hindbrain and forebrain respectively. The cerebellum is responsible for a number of functions including motor skills such as balance, coordination, and posture [23]. The hippocampus on the other hand is part of the limbic system located in the brain's medial temporal lobe, near the center of the brain. It comprises primarily of pyramidal cells. The hippocampus plays important roles in memory, including the formation of new memories about experienced events (episodic or autobiographical memory) [24], the detection of new events, places and stimuli [25]. The hippocampus also encodes emotional context from the amygdala and helps in retrieving declarative memories and spatial relationships.

2. MATERIALS AND METHODS

2.1 Materials

Male Wistar rats, weighing scale, wire gauze cages, animal feeds, distilled water, drinking cans, feed plates, hand gloves, tap water, dissecting set, chloroform, Bouin’s fluid, refrigerator, cotton wool, measuring cylinder, conical flask, tags and markers, inhalation chamber, rotary microtome, glass slides, microscope, rubber dish, methylated spirit, stopwatch, specimen bottles, xylene, haematoxylin and eosin, alcohol and paraffin wax.

2.2 Experimental Animals

Twenty (20) adult male Wistar rats weighing 160-250 g were used in this study. They were purchased from the animal house of the Department of Anatomy, University of Port

Harcourt. They were housed in standard cages and left to acclimatize for fourteen days under natural condition in the animal house of the Department of Anatomy, Madonna University, Elele before the commencement of the experiment. The animals were housed in well ventilated cages and kept under healthy natural environmental condition and 12 hour light/dark cycle. The cages contained saw dust with high urine absorbing capacity. The animals were fed with rat chow and water *ad libitum*. The rats were separated into four groups of five animals each.

2.3 Experimental Design

The rats were randomly divided into four groups (A, B, C and D) of five rats each. After two weeks of acclimatization, Group A rats which served as the control group were sacrificed using cervical dislocation, Group B rats were sedated for one day, Group C rats were sedated once a day for two days while Group D rats were sedated once a day for three days with chloroform.

2.4 Collection of Tissue

At the end of the experiment, the brains of the rats were harvested. The brains of four rats from each group were homogenized for antioxidant studies while the brain of one rat from each group was fixed in Bouin's fluid for histological studies using H&E (haematoxylin and eosin).

2.5 Anti-oxidant Assay

Tissue homogenate of the brain samples from four rats from each group was taken for anti-oxidant tests to examine the activities of the following anti-oxidant parameters: Superoxide dismutase (SOD), Catalase (CAT) and Malondialdehyde (MDA).

Brain samples were collected, Phosphate buffer solution was prepared and poured into the specimen bottle containing each brain sample, The sample was homogenized using a homogenizing machine. The sample was centrifuged with cold centrifuge at 10,000 rpm and the supernatant was collected. The rest of the assay was done using the supernatants.

2.6 Determination of Oxidative Stress Parameters

The protein concentrations of the samples were determined by the Biuret method as described by Gornall et al. [26] which has been modified by the addition of potassium tartarate to prevent precipitation of copper ions (Cu^{2+}) as cuprous oxide.

The level of SOD activity was determined by the method of Misra and Fridovich [27]. The ability of SOD to inhibit the auto-oxidation of epinephrine (adrenaline) at pH of 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide (O_2^-) radical generated by the xanthine oxidase reaction caused by the oxidation of adrenaline to adrenochrome and the yield of adrenochrome produced per O_2^- increased with increasing pH [27] and with increasing concentration of adrenalin. One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenalin.

Catalase activity was determined according to the method of Sinha [28]. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570-610 nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture. Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation. The MDA level was calculated according to the method of [29]. Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 105 M-1Cm-1.

Table 1. Showing experimental design

Groups	Number of rats	Method of sacrifice	No. of days of sedation
A	5	Cervical dislocation	-
B	5	Chloroform inhalation	1
C	5	Chloroform inhalation	2
D	5	Chloroform inhalation	3

2.7 Histological Studies

The brain tissue specimens were collected from one rat from each group immediately after the rats were sacrificed and fixed in Bouin's fluid for histological studies using H&E (haematoxylin and eosin) preservation, and to impact a rigid consistency to the tissue. The tissue was allowed to stand for forty-eight hours before tissue processing was carried out. The tissue was passed slowly through ascending concentration of alcohol; it was then infiltrated with xylene twice for an hour each. The tissue was then infiltrated in molten paraffin wax at a constant temperature of 56-60°c in four changes for about four hours. The tissue was then taken for embedding; molten paraffin was poured in an embedding mould and the tissue was then placed immediately with a forceps in the mould with the surface facing downwards. Solid sections were cut at 10 µm until it got to the cerebellum. The section was immersed in a water bath keeping the temperature between 50-55°c, the water was then dried off and the slide was put in an incubator at 37-40°c, so that the section was completely fixed on the slide and dry. The slides were dipped into a staining rack and allowed to dewax in two changes of xylene for 2 minutes; the tissues were hydrated by passing them through descending grades of alcohol for 2 minutes each. They were dropped in water for 2 minutes and stained with Erlich Haematoxylin for 30 minutes. They were then washed in slow running tap water and differentiated in 1% acid alcohol for 1 second. They were then blued in Scott's tap water for 10 minutes, counterstained with 1% eosin for 10 minutes, washed in running water, dehydrated in ascending changes of alcohol for 2 minutes each and finally cleared with two changes of xylene for 2 minutes each then mounted with DPX.

2.8 Statistical Analysis

The result of the antioxidant studies was analyzed using Microsoft Excel version 2010 and e-ANOVA statistical tool. Results were presented as Mean ± Standard deviation of 4 rats in each group.

3. RESULTS

3.1 Recovery Time

The Group C rats which were sedated once a day for two days recovered on average time of 6.5 minutes on the first day and the brains were harvested on the second day after sedation. The Group D rats which were sedated once a day for three days recovered on average time of 7.3 minutes on the first day, 10.4 minutes on the second day and the brains were harvested on the third day after sedation.

3.2 Antioxidant Studies

The results are presented as Mean ± Standard deviation of four (4) animals. The results are considered significant (*) at 95% confidence (P≤0.05). (**) indicates high statistical significance at P≤0.05.

The results of the antioxidant studies presented in Table 2 indicated that there was increase in malondialdehyde (MDA) levels in the experimental groups (B & C & D) compared to the control. However, only groups B & C were statistically significant. Increase in MDA levels is an indication of rising lipid peroxidation which is an indicator of oxidative stress.

Superoxide dismutase (SOD) levels were significantly increased across the experimental groups compared to the control. Catalyse (CAT) levels increased across the experimental groups B & D compared to the control but decreased in group C animals.

Table 2. Average recovery time of groups C and D rats following chloroform sedation

Groups	Average recovery time for day one (mins)	Average recovery time for day two (mins)
C	6.5	-
D	7.3	10.4

Table 3. Showing the antioxidant results

Group	MDA	SOD	CAT
A	0.23±0.07	0.27±0.01	2.58±0.66
B	0.39±0.19*	0.61±0.16**	3.28±1.00*
C	0.56±0.32**	0.45±0.20*	2.40±0.56
D	0.26±0.03	0.87±0.08**	6.23±0.80**

3.3 Histopathological Studies

The histological slides are presented in Plates 1 to 8. Plates 1 to 4 show slides of rat cerebellum while plates 5 to 8 show slides of the hippocampus.

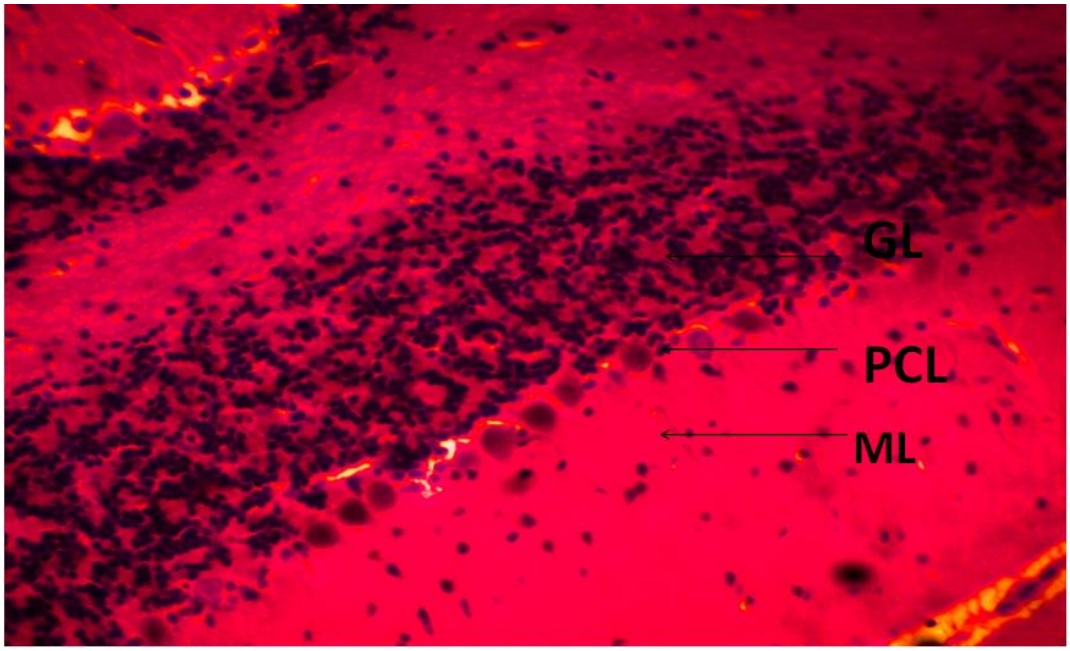

Plate 1. Representative photomicrograph of rat cerebellum in the control group A sacrificed by cervical dislocation, showing normal cerebellar cortical architecture; molecular layer (ML), Purkinje cell layer (PCL) and granular layer (GL). The cells appear normal and the cytoarchitectural orientation is preserved

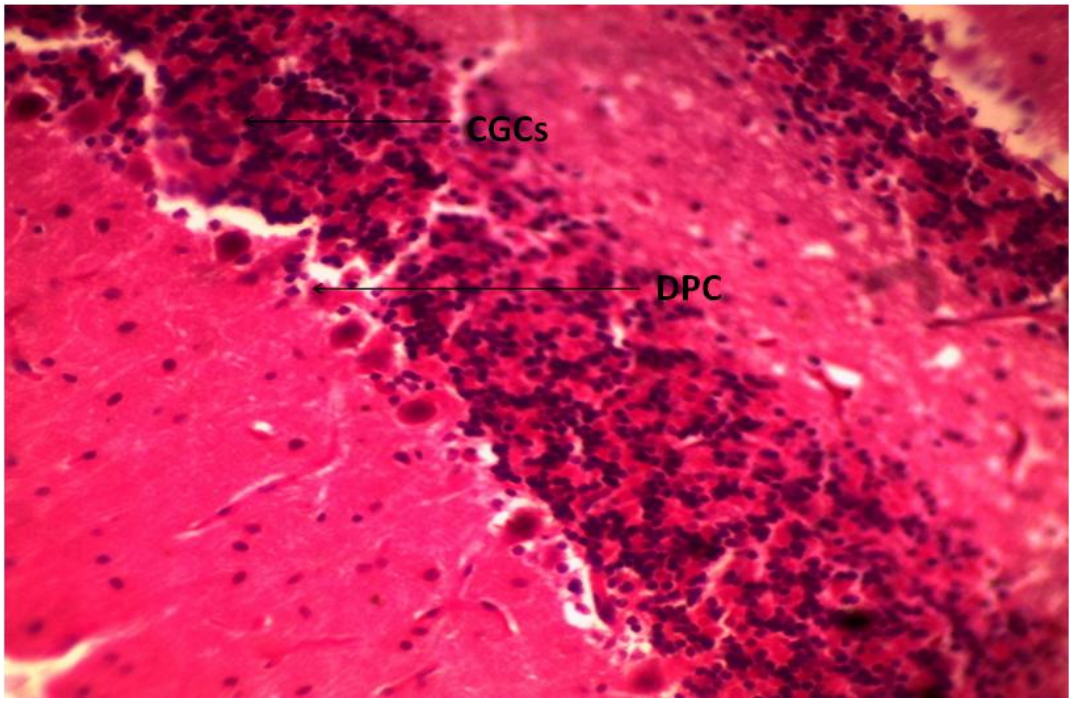

Plate 2. (a) Representative photomicrograph of rat cerebellum in group B showing degenerating purkinje cells (DPC) and congestive granular cells (CGCs) X500

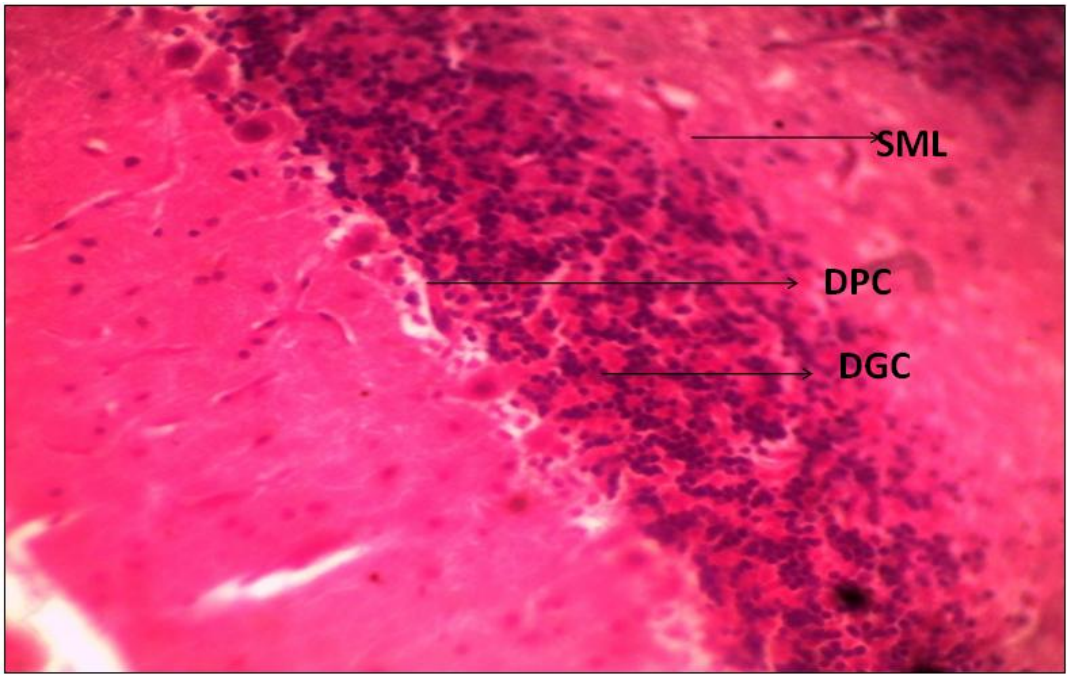

Plate 3. (a) Representative photomicrograph of rat cerebellum in group C showing scanty molecular layer (SML), dying purkinje cells (DPC) and dying granular cells (DGC). X500

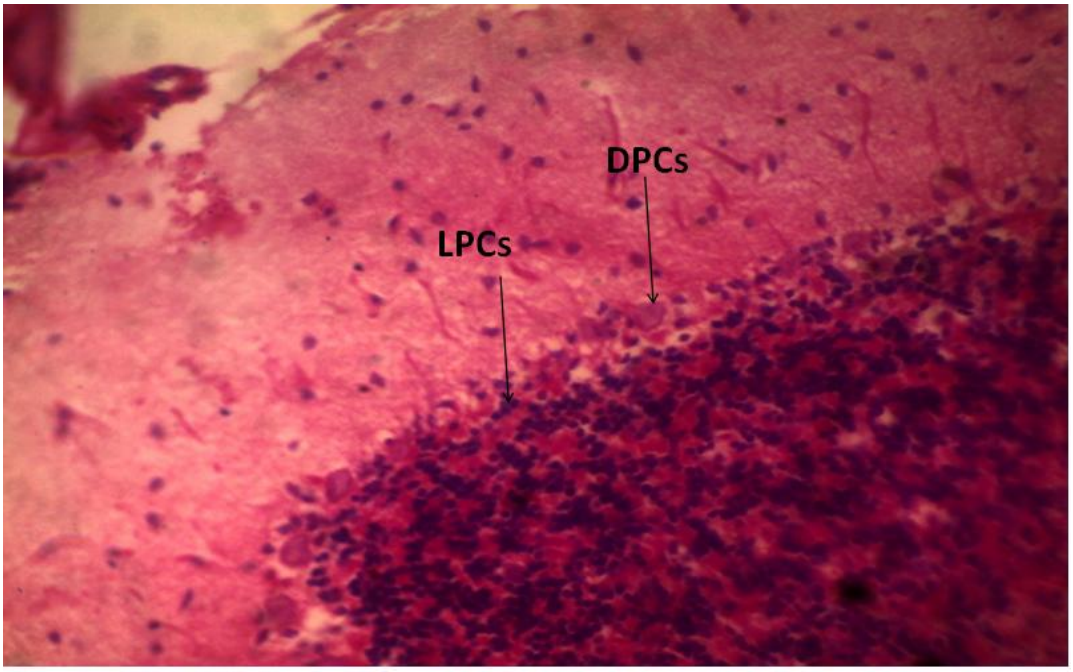

Plate 4. (a) Representative photomicrograph of rat cerebellum in group D showing many dead purkinje cells (DPCs) and areas of lost and sparsely distributed purkinje cells (LPCs) with more gap between the Purkinje cell layer and the adjoining granule and molecular layers respectively X500

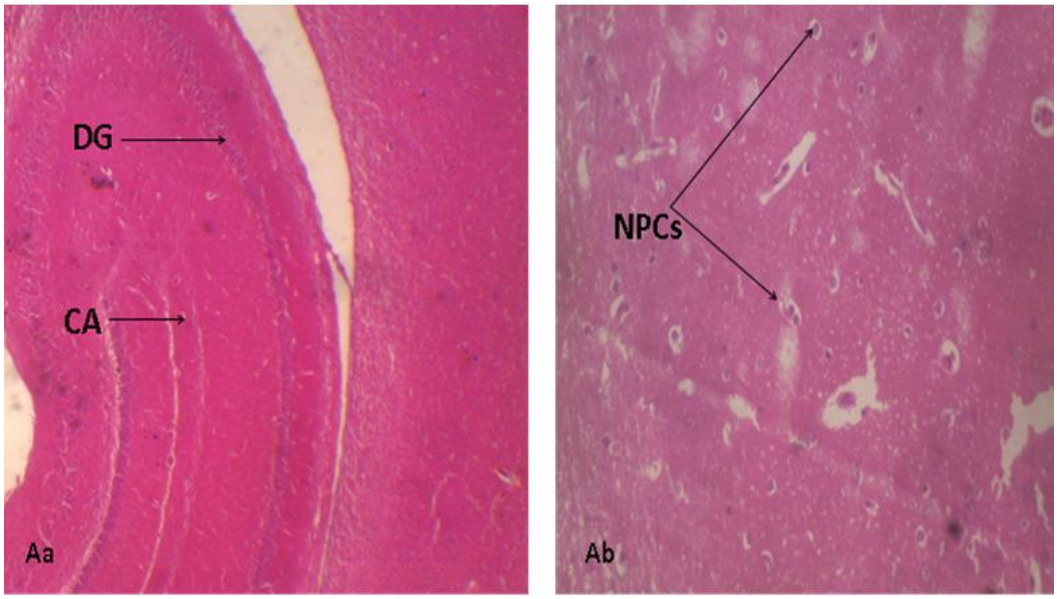

Plate 5. Representative photomicrograph of rat hippocampus in group A (control) in lower (Aa) and higher (Ab) magnifications. Aa shows the dentate gyrus (DG) and Cornu ammonis (CA) layers of the hippocampus (X125). Ab shows normal pyramidal cells (NPCs) X500

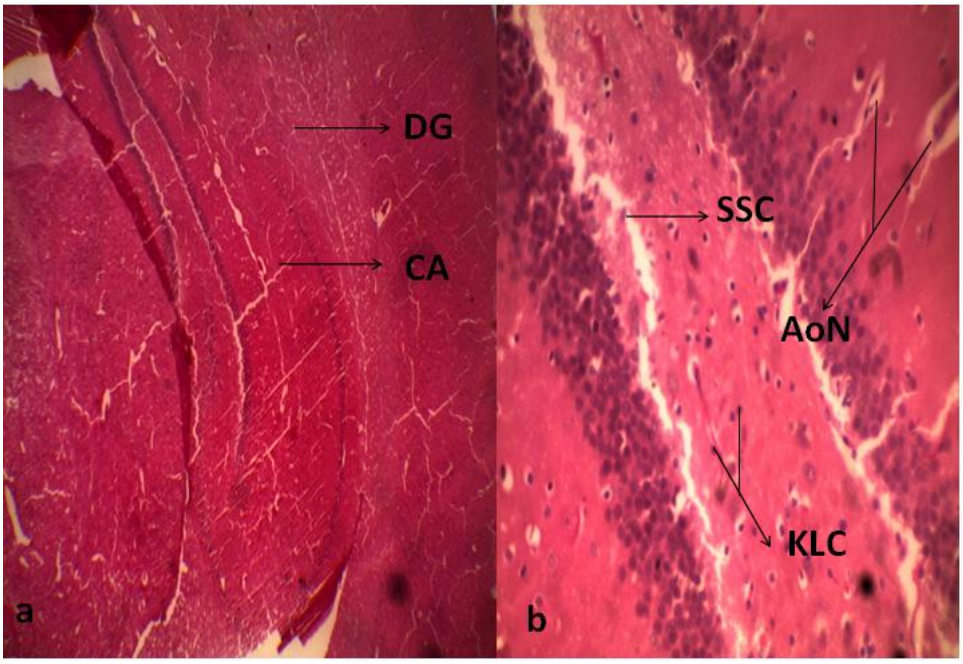

Plate 6. (a) Representative photomicrograph of rat hippocampus in Group B. The lower magnification (X125) shows the dentage gyrus and cornu ammonis layers. (b) (higher magnification) showing areas of necrosis (AoN) and numerous karyolytic cells (KLCs) in the stratum oriens layer; and sequestrated stellate cells (SSCs) (X500)

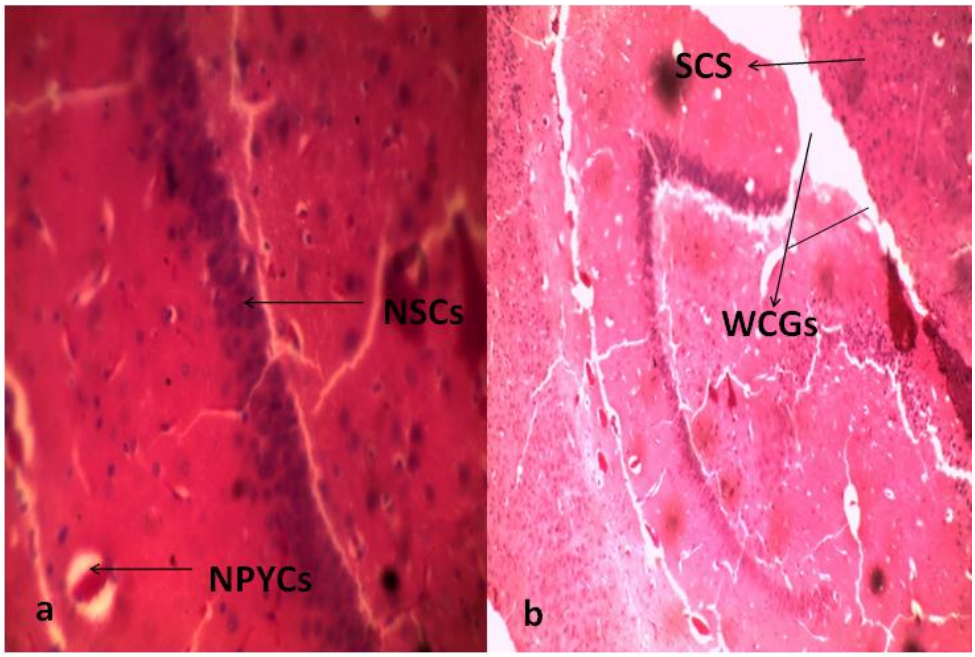

Plate 7. Representative photomicrograph of rat hippocampus in group C. (a) (Lower magnification) showing normal satellite cells (NSCs) and normal pyramidal cells (NPYCs). The hippocampal layers are also relatively normal although the satellite cells appear sequestrated(X125). (b) (Higher magnification) showing shrunken cells (SCs) and wide cellular gaps (WCGs) (X500)

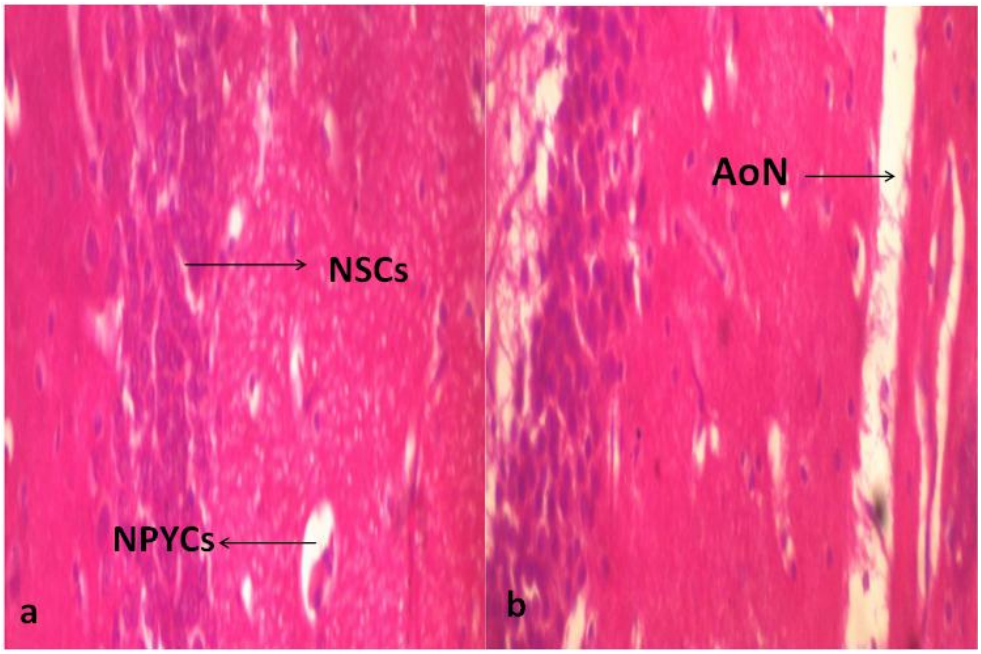

Plate 8. (a) Representative photomicrograph of rat hippocampus in group D. a (Lower magnification) shows normal satellite cells (NSCs) and normal pyramidal cells (NPYCs). b (Higher magnification) showing a prominent area of necrosis (AoN). The satellite cells also appear relatively congestive (X500)

4. DISCUSSION

There are many methods used for animal sacrifice and chloroform sedation is one of the leading methods, especially in Nigeria. Antioxidant studies carried out on the animals showed that they were under oxidative stress following sedation with chloroform even for just a few minutes. This was obvious as malondialdehyde (MDA) levels were increased in the experimental groups compared to the control. This is in line with the works of Gornall et al. [26] which shows that oxidative stress leads to a rise in MDA levels in rats. Furthermore, the antioxidant enzymes which are responsible for mopping up free radicals generated by lipid peroxidation (SOD and CAT) were analyzed. Superoxide dismutase (SOD) levels were significantly increased across the experimental groups compared to the control. SOD levels are reported to deplete under oxidative stress as they engage to combat the free radicals [26]. However, the result of our studies shows a contrary trend. This could be an eye-opener to the fact that SOD may not always deplete in all cases of oxidative stress. Catalyse (CAT) levels increased in experimental groups B & D but decreased in group C animals compared to the control.

The fact that these animals were under oxidative stress shows that chloroform sedation is not a safe method for rat sacrifice especially when studying brain tissues. Oxidative stress has been associated with many chronic diseases some of which are incurable: Cancer, diabetes, high blood pressure [30]. The micrographs of the cerebella of the rats in groups B, C and D (Plates 2, 3, 4) show increasing signs of cell death respectively and the nuclear outline of the purkinje cells were faint compared to the control (Plate 1). There were signs of nuclear death, the basket cells were also atrophied and more sparsely distorted in the molecular layer. A closer look at the granular cells in the granular layer revealed several degrees of cell death. This together with the results of the antioxidant studies gives a strong indication of cerebellar impairment which may lead to motor dysfunction. Health conditions associated with cerebellar dysfunction include digestive disturbances [31], liver enlargement and tenderness [32], necrosis [31], jaundice [32]. The micrographs of hippocampi of rats in groups B, C and D (Plates 6, 7 & 8) showing increasing signs of shrunken and dead cells compared to the control. They also showed numerous karyolytic cells in the stratum oriens layer; and sequestrated stellate cells. There were also signs of microgial cell infiltration and laminar necrosis. Damage to the hippocampus may result to anterograde amnesia [33], Alzheimer's disease [34], medial temporal lobe epilepsy [35].

5. CONCLUSION

The result of this research shows that chloroform inhalation has detrimental effects on the Purkinje cells of the cerebellum, and the granular and pyramidal cells of the hippocampus. Therefore, chloroform sedation is not ideal for experimental purposes as a method of rat sacrifice, especially when studying rat brain.

6. RECOMMENDATION

- It is recommended that chloroform sedation should not be used as a method of sacrifice when carrying out rat brain studies.
- It is recommended that further research be done on other methods of sacrifice to ascertain their humaneness and safety for brain studies.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Ethical approval was gotten from the Research Ethics Committee of the Faculty of Basic Medical Sciences, Madonna University Nigeria following the submission of the research proposal (Mau/FBMS/ANA/18/0035).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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