Chronic consumption of calabash chalk diet induces depression, cognitive impairment and hepatotoxicity in mice

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ABSTRACT

Background: Calabash chalk (CC) is a geophagic material consumed in many parts of the world for pleasure, as a remedy for hyperemesis gravidarum, sialorrhea, and for foetal bone development. Several potential harmful elements are reported to be present in CC.

Objectives: This study evaluated the effect of CC diet on neurobehavioural indices, brain and liver histomorphological changes in mice.

Methods: One hundred and twelve mice were randomly distributed into four major groups of twenty-eight mice per group. Each group was subdivided into four groups of seven mice each and fed with either standard animal feed (control), 10%, 20% or 40% CC diet (treatment groups) for thirty days. Weights of mice were monitored at interval of ten days. On the 31st day, mice from the control and various treatment groups were subjected to either the forced swim test, tail suspension test, three-chamber approach or novel object recognition tests. Twenty-four hours post neurobehavioural tests, animals were sacrificed, their brains and livers isolated, preserved in 10% buffered formalin and used for histological assays.

Results: There was a significant increase ($p<0.05$) in the duration of immobility in the forced swim and tail suspension tests in mice fed with higher concentrations of CC diet. A decrease in contact time spent on the novel object and with the unfamiliar mouse was observed in mice fed with 20 and 40% CC diet. While no cytotoxicity of neuronal and glial cells was observed in this study, hepatotoxicity was noticed in the livers of CC fed mice.

Conclusion: In this study, chronic consumption CC diet produced features characteristic of depression, cognitive dysfunction and hepatic damage in mice.

Key words: Calabash chalk, depression, histomorphology, brain, liver

La consommation chronique d'un régime à base de craie de calebasse induit la dépression, des troubles cognitifs et l'hépatotoxicité chez la souris

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RÉSUME

La craie de calebasse (CC) est un matériau géophagique consommé dans de nombreuses régions du monde pour le plaisir, comme remède à l'hyperemesis gravidarum, à la sialorrhée et pour le développement osseux du fœtus. Plusieurs éléments potentiellement nocifs sont signalés comme étant présents dans la CC.

Objectifs : Cette étude a évalué l'effet du régime CC sur les indices neurocomportementaux, les changements histomorphologiques du cerveau et du foie chez la souris.

Méthodes : Cent douze souris ont été réparties au hasard en quatre groupes principaux de vingt-huit souris par groupe. Chaque groupe a été subdivisé en quatre groupes de sept souris chacun et nourri avec une alimentation animale standard (témoin), ou avec un régime CC à 10%, 20% ou 40% (groupes de traitement) pendant trente jours. Le poids des souris a été contrôlé à intervalles de dix jours. Le 31e jour, les souris du groupe témoin et des différents groupes de traitement ont été soumises à des tests de nage forcée, de suspension de la queue, d'approche à trois chambres ou de reconnaissance de nouveaux objets. Vingt-quatre heures après les tests neurocomportementaux, les animaux ont été sacrifiés, leur cerveau et leur foie ont été isolés, conservés dans du formol tamponné à 10% et utilisés pour des analyses histologiques.

Résultats : On a constaté une augmentation significative ($p<0,05$) de la durée d'immobilité dans les tests de nage forcée et de suspension de la queue chez les souris nourries avec des concentrations plus élevées de régime CC. Une diminution du temps de contact avec l'objet nouveau et avec la souris non familière a été observée chez les souris nourries avec 20 et 40 % de régime CC. Bien qu'aucune cytotoxicité des cellules neuronales et gliales n'ait été observée dans cette étude, une hépatotoxicité a été constatée dans le foie des souris nourries au CC.

Conclusion : Dans cette étude, la consommation chronique du régime CC a produit des caractéristiques de dépression, de dysfonctionnement cognitif et de dommages hépatiques chez les souris.

Mots-clés : Craie de calebasse, dépression, histomorphologie, cerveau, foie

INTRODUCTION:

Geophagia or geophagy, the deliberate consumption or crave for eating earth, soil, chalk or clay is a common practice in various parts of the world including Nigeria. It is common in pregnant women, lactating mothers and children. Geophagia is associated with religious beliefs, medicinal, dietary and aesthetic considerations and is practised by people of all races, sex and age groups, though it seems to be more widespread in the tropics.[1-5] Calabash chalk, one of the most commonly consumed geophagic substances is also known as Calabar stone, calabash clay; la craie (French speaking communities), mabele (Congolese), shile (Ghanaians), nzu and eko respectively by the Igbo and Bini speaking communities in Nigeria. In Cameroon, it is more popularly called Calabar chalk; named after Calabar, a neighboring city located in the southern part of Nigeria famous for the mining and sale of geophagic clays.[2,5,6]

Calabash chalk is readily available in the open markets in commercial quantities in the salted form as chunks/blocks, in the non-salted from as pellets and in the powdered form.[3,7] Calabash chalk occurs naturally as a material consisting of fossilized sea shells or made artificially from a mixture of clay, wood, ash and salt, moulded and baked in the oven. The most common constituent of calabash chalk is aluminum silicate hydroxide, thought to be from kaolin clay.[2,3,6,7] Other constituents include lead, aluminum, potassium, manganese, titanium, nickel, cadmium, arsenic, silicone, calcium, zinc, chromium, iron and pollutants of organic nature such as alpha lindane, endrin, endosulfan and dichlorodiphenyl dichloroethane.[2,6-8]

In Nigeria, calabash chalk is more commonly consumed by pregnant women especially in the South Eastern part of the country. In Cameroon, it is used more commonly in the second and third trimesters and amongst primigravidas.[3,8,9,10] Reasons adduced for its use include its ability to prevent diarrhoea, nausea and vomiting, reduce dyspepsia, excessive production of saliva and aid foetal skin and bone development. It also has a pleasurable taste for pregnant women who sometimes crave for it, and it is used to achieve wrinkle free skin.[3,9-10]

Evaluation of the effects of calabash chalk in preclinical studies include induction of oedema and haemorrhage in the stomachs and hyperkeratosis in oesophageal tissues of rodents;[11] reduction in maternal weight gain, inhibition of uterine implantation, reduction in number of foetuses formed in utero and histomorphological changes in cerebellum of rats.[12,13] While Ekong *et al*[14] demonstrated a reduction in bone density and mineralization of bones of young rodents, hypertrophy of foetal cerebral cortex was reported by Ekanem and colleagues.[14,15] Studies have documented hypochromic anaemic potential,[6] anxiogenic and analgesic effects[16] of calabash chalk. The effects on inflammation, endometrial hyperplasia and metaplasia,[17] impaired locomotion and social behaviour,[16] altered cerebral cortical changes and neuronal death have also been demonstrated.[18]

The presence of high concentrations of lead, other heavy metals, organic pollutants and potentially harmful elements make consumption of calabash chalk potentially toxic. The deleterious effects of heavy metals, especially on physiological functions have been proven worldwide; for example exposure to heavy metal contamination have been implicated in the pathophysiology of several disease conditions such as mood disorders, anxiety, depression, schizophrenia and cognitive impairment.[19-24] Other studies have established a causal relationship between high concentration of heavy metals in various body fluids and urine with the development of depressive disorders, schizophrenia and maternal depression.[25-27]

Though studies have been carried out on some neurobehavioural indices, the effects of calabash chalk on depression and cognitive function are not documented in the scientific literature. In this study we seek to evaluate the effect of calabash chalk incorporated diet on depression, cognition and histomorphology of the brain and liver cells of female, non-gestating mice.

MATERIALS AND METHODS

Animals

One hundred and twelve female Swiss mice aged 7-8 weeks, weighing 22-27g obtained from the Central Animal House of Igbinedion University, Okada, Edo State, Nigeria were used for this study. They were kept in polypropylene cages in the animal house of Igbinedion University, Okada and maintained under room temperature (26-28^{o}C), natural lighting conditions and adequate ventilation. The animals had unlimited access to animal feed (Top Feed^{R}, Benin City, Nigeria) and clean water. Adequate hygiene was maintained through daily cleaning of cages. Handling of the animals was done according to standard protocols for the use of laboratory animals of the Public Health Service Policy on Humane Care and Use of Laboratory Animals, National Institutes of Health, USA.[28] Ethical approval was obtained from the Ethics Committee, Faculty of Pharmacy, University of

Benin, Nigeria (EC/FP/021/07).

Experimental protocol

One hundred and twelve mice were randomly assigned into four major groups of twenty-eight mice per group (I-IV). Animals in these 4 groups were further divided into 4 sub-groups (A-D) of seven mice per group.

Mice in Group 1A were fed with standard animal feed for 30 days, while mice in Groups 1B-D were fed with 10%, 20% and 40% calabash chalk diet respectively for thirty days. Animals in Groups 2A-D, 3A-D and 4A-D received similar diets to those in Groups 1A-D for 30 days. The weights of all the animals were measured at interval of 10 days for the duration of the study.

On the 31st day, while mice in Group IA-D were subjected to the tail suspension test (TST), those in Group 2A-D were used for the forced swim test (FST). Animals in Groups 3A-D and 4A-D were used for the three-chamber social interaction test and novel object recognition (NOR) tests respectively.

Twenty-four hours post-neurobehavioural studies, animals were humanely sacrificed via cervical dislocation, the brains and livers were isolated, quicky transferred to 10% buffered formalin solutions and used for histological assays.

Preparation of experimental diet

Blocks of salted calabash chalk procured from a local market in Awka, Anambra State, Nigeria were pulverized to obtain a slightly coarse powder which was kept in dry and air-tight bottles from which low, medium and high calabash chalk diets were prepared. Ten percent calabash chalk diet was prepared by mixing 1 g of calabash chalk with 9 g of rodent chow (low dose). Twenty percent calabash chalk diet was obtained by mixing 2 g of calabash chalk with 8 g of rodent chow (medium dose), while forty percent (high dose) calabash chalk diet comprised 4 g of calabash chalk with 6 g of animal feed.[8]

Tail suspension test

The TST was used to evaluate depression like behaviour in animals based on the fact that a mouse suspended by the tail alternates between periods of activity and immobility (despair). Briefly each mouse was suspended from the edge of a shelf 60 cm above a table top via an adhesive tape placed approximately 1cm from the tip of the tail top for six minutes. Periods of immobility were recorded by trained observers blinded to treatment.[29]

Forced swim test

In the FST, animals were placed in a transparent open cylinder (diameter 10 cm, height 25 cm) containing water at a depth of 20 cm and at temperature of 25^{O}C for six minutes.

Periods of immobility in the last four minutes of the experiment was cumulatively recorded by trained observers unaware of treatment. After each test, the water was changed, mice were properly dried and were returned to their home cages. A mouse was judged to be immobile when it made movements only necessary to keep its head above water. [30]

Three chamber sociability test

The three-chambered sociability and social recognition memory task was employed to assess social cognition in this study. This test which assesses cognition in the form of general sociability and interest in social novelty in rodent models of central nervous system disorders is based on the fact that rodents normally prefer to spend more time with another rodent (sociability) and will tend to investigate a novel intruder more than a familiar one - social novelty.[31-34]

In the habituation (adaptation) phase, each mouse was placed in the middle chamber with two pencil cups in the middle of the left and right chambers and allowed to adapt for 5 minutes. Thereafter, another 2 identical pencil cups were placed again in the left and right chambers, one containing another mouse, stranger 1 and a second empty cup and the mouse was left to explore for 10 minutes. Direct contact, i.e., time spent sniffing each pencil cup, snouting 3-5 cm around each cup, the time spent in each chamber, and the number of entries into each chamber were recorded by trained observers unaware of treatment (social affiliation phase). One hour later, a second mouse (stranger 2) was placed in the empty cup and mice allowed to explore for 5 minutes. Periods of direct contact were again recorded by trained observers unaware of treatment (social novelty/preference phase). Stranger 1 and 2 used were habituated to being enclosed in pencil cups in the three-chamber apparatus for 10 minutes daily on two consecutive days prior to the experiment. The 3 chambers were cleaned with 70% alcohol after the test for each animal.

Novel object recognition test

The NOR test is based on the spontaneous exploration of environment, with premise that animals spend more time exploring a novel object than a familiar one. In phase

I, the animals were habituated for 5 minutes in the open field apparatus. Twenty-four hours later in phase II, two identical objects were placed at the end of the open field apparatus 10 cm from the left and right walls and mice were allowed to explore for 10 minutes. Time spent sniffing, licking or climbing each object was recorded by observers unaware of treatment. In phase 3, 24 hours after phase II, one of the identical objects was replaced with a novel object and time spent sniffing, licking or climbing each object was recorded by observers unaware of treatment. The apparatus was cleaned with 70% alcohol after the test for each animal to prevent olfactory clues.[35,36]

Histological assays

The fixed specimens of the brains and liver were processed and serial sections of 4 µm thickness were cut using a microtome, these were subsequently processed using the haematoxylin and eosin staining method and histological changes were observed with the aid of a microscope.[37]

Statistical analysis

The results were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Dunnet's post hoc test using GraphPad Prism version 8.0 A difference was considered significant at $p < 0.05$. The results are presented as mean ± standard error of mean (SEM).

RESULTS

Effects of calabash chalk diet on the weight of mice

There was a significant reduction ($p<0.05$) in the mean body weights of mice fed with 20% and 40% calabash chalk diet by the 10th day of the study. By the 20th and 30th day, weight of mice fed with all 3 doses of calabash chalk diet were significantly lower ($p < 0.05$) than those of the control group. Data is presented in Table 1.

Table 1: Effect of calabash chalk diet on the weight of mice

Treatment	Day 1	Day 10	Day 20	Day 30
Control	21.17±0.40	23.33±0.49	27.00±0.37	30.50±056
10% C. chalk	21.50±0.34	23.00±0.51	24.50±0.85*	26.56±0.50***
20% C. chalk	20.83±0.47	21.33±0.33*	24.83±0.48*	26.17±0.16***
40% C. chalk	21.17±0.31	22.33±0.42*	25.50±0.43*	27.67±0.76**

Values are expressed as Mean ± S.E.M. n=7 mice. *p<0.05, **p<0.01, ***p<0.001 compared to the control group.

Effect of consumption of C. chalk diet on duration of immobility in the TST and FST in mice

Consumption of C. chalk diet resulted in a significant increase ($p<0.05$) in the duration of immobility in the TST at and 20% and 40% dose level, while in the FST, mice fed with 40% calabash chalk diet showed a significant increase ($p<0.05$) in the duration of immobility compared to the control group. Results are shown in Fig 1

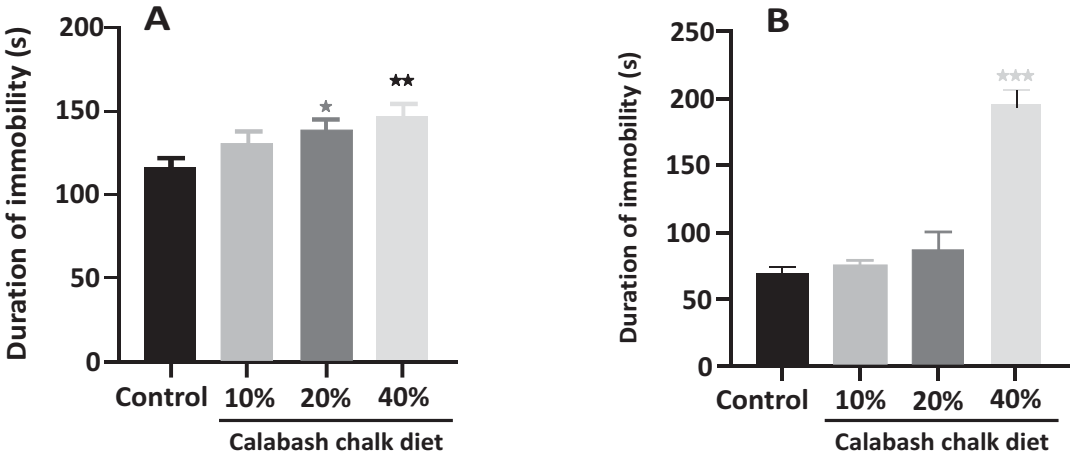

Fig. 1 Duration of immobility of mice in the TST (A) and FST (B) following 30-day consumption of standard and calabash chalk diet. *p<0.05, **p<0.01, ***p<0.001 compared to the control group. n=7 per group.

Effect of consumption of calabash chalk diet on the duration of contact with unfamiliar mouse/object in the three chamber sociability and novel object recognition tests

Consumption of calabash chalk diet resulted in a significant (p<0.05) reduction in the number and duration of contacts of mice with the stranger mice in the three-chamber social interaction test in medium and high dose levels of calabash chalk diet. The number and duration of direct contact with the novel object in the NOR test was significantly reduced in the 20% and 40% calabash chalk diet compared to the control group. Data is presented in Fig 2.

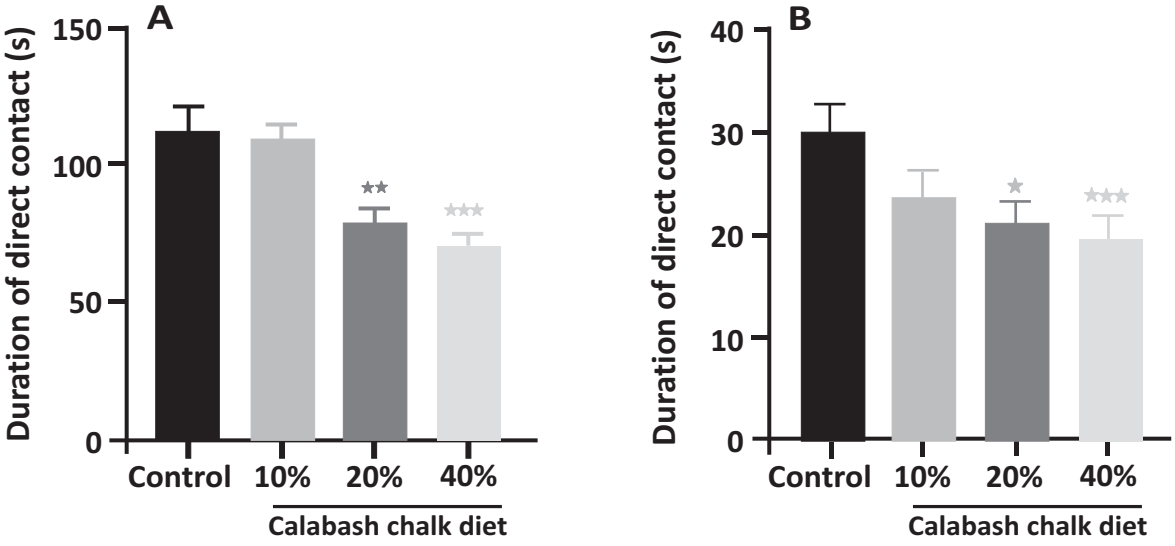

Fig. 2 Duration of direct contact of mice with unfamiliar mice in the three-chamber social interaction apparatus (A) and the novel object in the NOR test (B) following daily consumption of calabash chalk diet for 30 days. *p<0.05,**p<0.01 compared to the control group, n=7.

Effect of consumption of calabash chalk diet on the liver, hippocampus and cerebral cortex of mice

In the livers of mice in the control group, there were no morphological changes in the hepatocytes. Ballooning degeneration and steatosis indicative of hepatic injury were noticeable the groups fed with 10, 20, and 40% calabash chalk (Fig. 3).

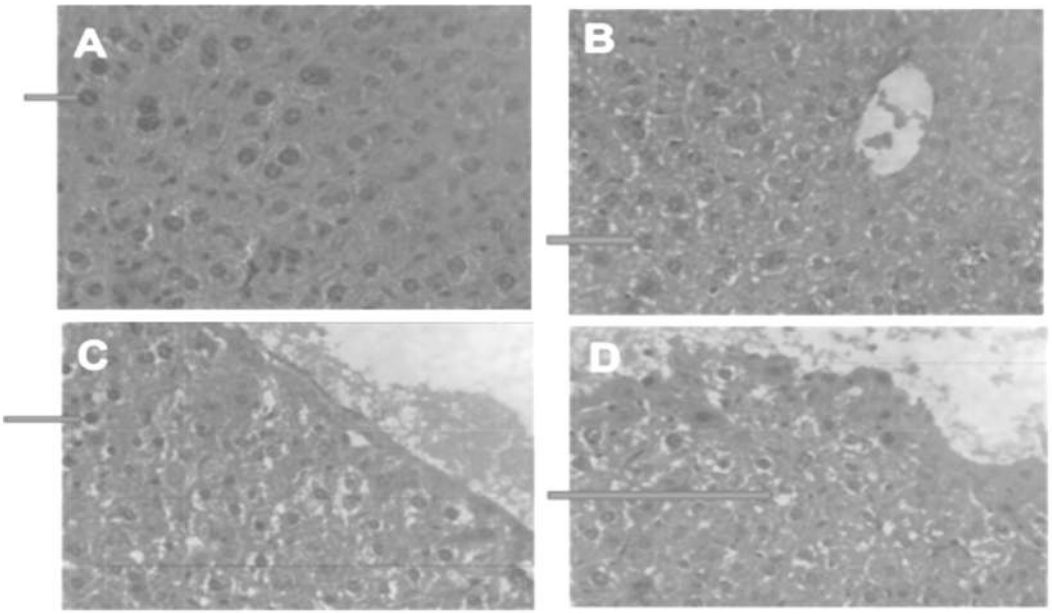

Fig. 3: Representative photomicrograph of the livers of mice fed with standard diet (control, A), 10% calabash chalk diet (B), 20% calabash chalk diet (C) and 40% calabash chalk diet (D) for 30 days (Haematoxylin and Eosin staining, 400× magnification). In Group 1, hepatocytes appear normal with no signs of degeneration while in Groups 2 - 4, ballooning degeneration and steatosis of hepatocytes indicative of hepatic damage are noticeable.

Features of ballooning degeneration and steatosis and Periodic acid-Schiff (PAS) positivity were noticeable in the histological evaluation of the liver of mice fed with calabash chalk diet (Fig. 4).

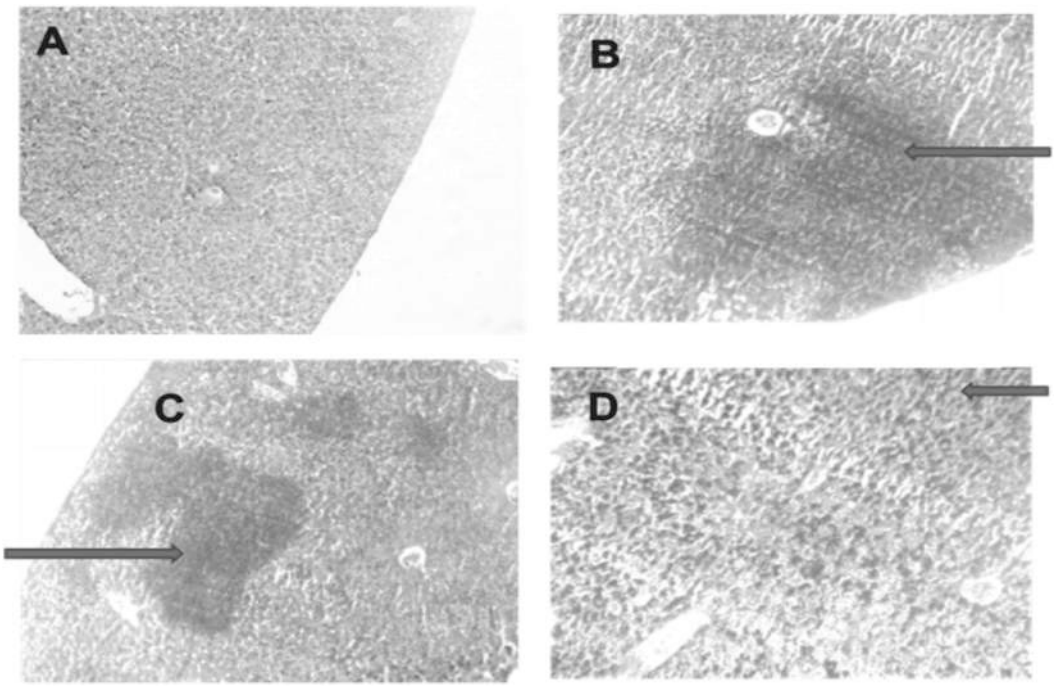

Fig. 4: Representative photomicrograph of histochemistry of the liver of mice showing PAS positivity. Standard diet (control, A), 10% calabash chalk diet (B), 20% calabash chalk diet (C) and 40% calabash chalk diet (D) [PAS staining, 400× magnification]. No PAS positivity seen in cells of A, while PAS positive keloid materials deposited in the Kupffer cells and portal macrophages are evident in Groups B-D.

In the histomorphological evaluation of the hippocampus and cerebral cortex of mice fed with calabash chalk diet, no injury to the cyto-architecture of the neurons and glial cells was observed (Fig. 5 and 6).

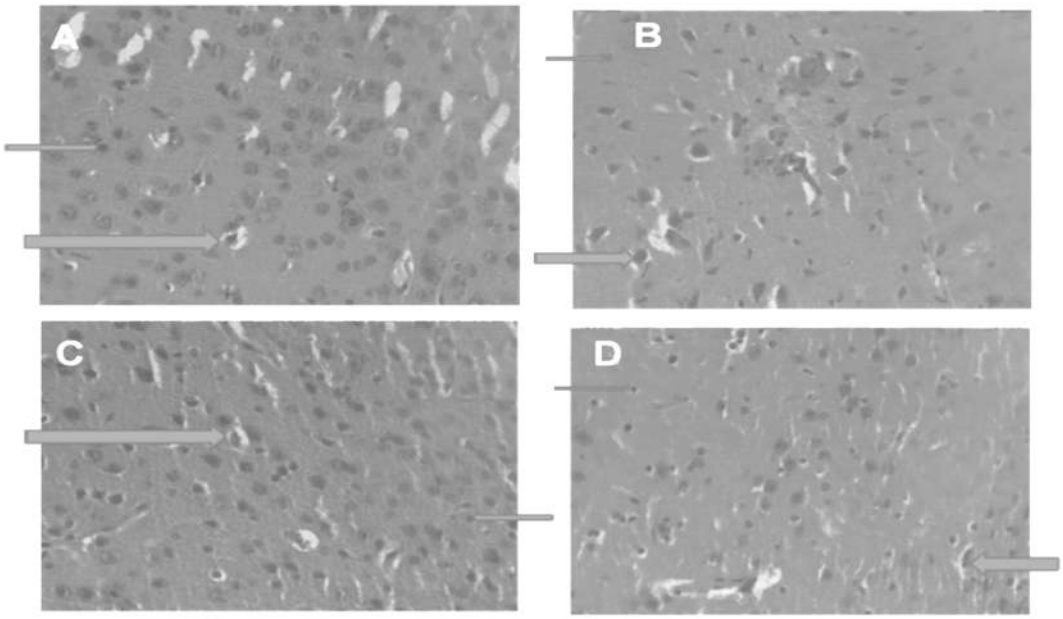

Fig. 5: Representative photomicrograph of the hippocampus of mice on standard diet (control, A), 10% calabash chalk diet (B), 20% calabash chalk diet (C) and 40% calabash chalk diet (D) for 30 days (Haematoxylin and Eosin staining, 400× magnification). No features of injury are visible in the neurones and glia cells.

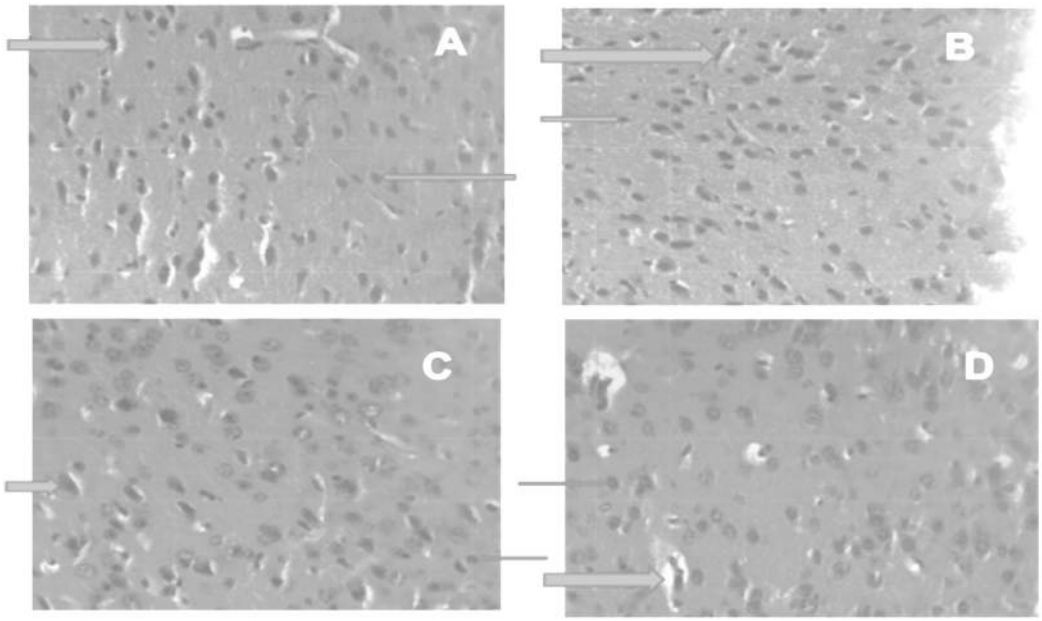

Fig. 6. Representative photomicrograph of the cerebral cortex of mice on standard diet (control, A), 10% calabash chalk diet (B), 20% calabash chalk diet (C) and 40% calabash chalk diet (D) for 30 days (Haematoxylin and Eosin staining, 400× magnification). No features of injury are visible in the neurones and glia cells and the cerebral cortex.

DISCUSSION

This study evaluated the effect of consumption of calabash chalk diet on depression, social interaction, cognition and histomorphology of the brain and liver in mice. Higher doses of calabash chalk diet increased duration of immobility in the TST and FST, and attenuated social interaction and cognition. In addition, there was a reduction in mean body weight and altered histomorphology of the liver, but not of cerebral cortex or hippocampus of mice.

The tail suspension and forced swim tests are used to evaluate the antidepressant and depressogenic potential of compounds in acute animal models of depression.[38] Compounds with antidepressant effects reduce the duration of immobility in both tests,[29,39-40] while agents which increase the duration of immobility are devoid of antidepressant effect or may induce depressive state.[38] In this study, the highest dose of calabash chalk increased the period of immobility in both the FST and TST while the middle dose and highest doses increased immobility in only the TST; this is indicative of depressogenic activity.

The present study reveals a decrease in exploratory behaviour in the social interaction test in mice fed with calabash chalk diet when compared to the control group. Many behavioural elements contribute to normal social behaviour, including sensory perception, stress, anxiety, depression, exploratory activity and cognition. The three-chamber sociability test has been used to evaluate social interaction in animals.[32-34,41] The impairment in social interaction observed in mice suggested a possible dysfunction in the working memory of the mice.[41,42] Hence to test our hypothesis, we investigated the working memory using the NOR test. There was a significant reduction in the duration and number of contacts with the novel object, suggesting a dysfunction in the working memory of the mice fed with calabash chalk diet. Cognitive abilities have been shown to determine social competence and influence social interaction.[43] The results from our study are similar to previous work by Owhorji *et al*,[8] who reported an impairment in locomotor activity and social behaviour in mice fed with calabash chalk diet.

Change in body weight is an important index when evaluating the relative toxic effect of compounds as reduction in body weight can be indicative of toxicity.[44] The mean body weights of mice fed with calabash chalk diet were significantly reduced, compared to mice fed with standard rodent diet for 30 days.

In this study, there were no gross structural abnormality of the hippocampus and cerebral cortex of mice fed with calabash chalk diet. Ekong *et al* reported hypertrophy and vacuolation of the pyramidal cells in the cerebral cortex of gestating rats.[18] The disparity in these two studies could be due to the doses used and possible hormonal changes resulting from pregnancy. While Ekong *et al* administered suspension of calabash chalk orally to gestating rats, in our study, the calabash chalk was incorporated in the diet, fed to non-gestating mice. During pregnancy, remobilization of stored lead occurs with migration of lead from the stores in skeletal muscles to the blood. As lead crosses the blood brain barrier, high levels of the metal in blood stream would result in higher levels in the brain and consequent neurotoxicity.[10,45]

Histomorphological changes were observed in the liver of all the groups fed with calabash chalk diet compared to mice fed with standard rodent diet. The liver is the principal organ for the detoxification of noxious exogenous substances in the body, but can also be made susceptible to damage when overwhelmed with exogenous toxicants.[46] The liver showed ballooning degeneration and steatosis of the hepatocytes. Hepatocyte ballooning is an indication of special form of livers degeneration closely related to hypertrophy and hyperplasia, causing membrane damage to cytoskeleton and loss of cell shapes. It has been recognized as one of the criteria for the diagnosis of steatohepatitis[47,48]. Other studies have also demonstrated hepatotoxicity effects of calabash chalk manifested as hyperplasia and hypertrophy of liver cells, sinusoidal enlargement and parenchymal fragmentation.[49, 50]

Histochemistry of the hepatocytes revealed the presence of PAS positive keloid materials deposited in the Kupffer cells and portal macrophages. These materials demonstrate the presence of weakened and improper functioning basement membrane components, which have been implicated in fibrotic hepatocyte.[51] Elevated basement membrane component in liver fibrosis has been demonstrated in other studies.[52,53]

CONCLUSION

The present study suggests that chronic consumption calabash chalk diet produces features indicative of depression, cognitive dysfunction and hepatic damage in mice.

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