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# Maternal Geophagy of Calabash Chalk and the Developing Cerebral Cortex

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## ABSTRACT

Calabash chalk geophagy is a common practice by pregnant women and children. Research has shown adverse maternal cerebral cortical effects following administration, hence, consumption of this chalk may also affect the nervous system developmental processes. This study therefore investigated the effect of maternal geophagy of the chalk on the developing cerebral cortex. Twelve gestating female Wistar rats were divided into two groups of 6 rats each. Animals in control group received placebo distilled water, while the test group animals received 200 mg/kg body weight of calabash chalk suspension orally on days 7-20 of gestation. On day 21, the dams delivered normally, and this marked post-natal day 1 (PND1). The pups were culled to four pups per mother (24 pups per group). On PND4-7 and PND7 respectively, surface righting reflex and cliff avoidance tests were carried out. On PND8 and PND15 respectively, twelve pups each per group were sacrificed after chloroform anaesthesia, and the brains were removed and preserved. Each cerebral cortex was excised and processed for histological study. There was no difference in pups body weights, and cliff avoidance and surface righting tests. The section of the cerebral cortex of PND8 pups in the calabash chalk group showed hyperplasia of cells in the entire cortical layers, while PND15 pups showed less cellular density and size of cells in the cortical layer. There was less Nissl substance staining in both chalk groups, however, the PND15 pups also showed chromatolytic cells compared with control group. In conclusion, maternal calabash chalk geophagy showed gradual cerebral cortical cell death processes which may lead to limitation of its functions.

**Keywords:** Calabash chalk, pups, Nissl staining, cerebral cortex, Wistar rat

## INTRODUCTION

Geophagy is the practice of eating earthy substances, and is a form of the eating disorder, pica. In Nigeria and some other African countries, calabash chalk geophagy is commonly practiced especially by pregnant and postpartum women, as well as children. Reports have shown that pregnant women do this due to their emotional states usually attributed to feelings of misery, homesickness, depression, and alienation (Henry and Kwong 2003; Abrahams et al 2013), and that the chalk could relief these feelings.

Calabash chalk or calabash clay or Calabar stones is also known in some Nigerian languages; nzu in Igbo, and ndom in Efik and Ibibio. It is a mixture of clay and chalk and is commercially available. It may be sold in

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blocks, as large pellets, and in powder forms (Food Standards Agency 2002). Calabash chalk could be naturally occurring, as well as, artificially formulated. This naturally occurring form is chiefly made up of fossilized seashells, while the artificial form may be prepared from clay and mud which may be mixed with other ingredient including sand, wood ash and sometimes salt. The resulting product is moulded and then heated to produce the final product (Food Standards Agency 2002). Calabash chalk is composed of the kaolin clay group, mostly aluminium silicate hydroxide, as well as metals, metalloids and persistent organic pollutants (Campbell 2002; Dean et al 2004; Health Canada 2007; Dooley 2010).

Calabash chalk contain substances such as; iron, aluminium, potassium, titanium, barium, chromium, zinc, manganese, nickel, rubidium, copper, tin, lead, among others (Dean et al 2004), with the metalloid being arsenic (Campbell 2002; Health Canada 2007; Dean et al 2004; Dooley 2010). The organic pollutants as reported include alpha lindane, endrin, endosulphan II and p, pI-dichloro diphenyl dichloroethane (DDD) (Dean et al 2004).

Reports on calabash chalk effects on animal models showed sinusoidal enlargements, fragmented liver parenchyma and depletion of red blood cells (Ekong et. al. 2009; Akpantah et al 2010; Ekong et al 2012a). Ekong et al (2012b and c) reported oedema and haemorrhages in the mucosa of stomach, acanthosis, hyperkeratosis and koilocytic changes in the mucosa of oesophagus, and alteration of growth rate and de-mineralization of femur bone. The calabash chalk has also been indicted in adverse features on the cerebral cortex of dams (Ekong et al 2014). These reports show a disturbing trend that may also affect the developing brain, which has motivated the need for this study on the effect of the chalk on the developing brain.

## MATERIALS AND METHODS

18 mature Wistar rats consisting of 12 females and 6 males were used. The female rats were divided into two groups of control and calabash chalk with each group consisting of 6 rats. The female rats were allowed to mate with the males during the proestrous stage of their oestrous cycle (Marcondes et al 2002).

Two 'non-salted' blocks of calabash chalk was purchased from a local market in Calabar, Nigeria. They were chopped into small pieces and grounded into powder with the aid of a manually operated grinder. 40 g of the calabash chalk was weighed and dissolved in 1000 ml of distilled water in a glass jar. Since the calabash chalk is partially miscible with water, it was administered as suspension, stirred prior to the administration. 200 mg/kg of the calabash chalk suspension and equivalent quantity of distilled water were respectively administered orally to the gestating rats on days 7-20 of gestation.

All the dams in the two groups were allowed to full term on day 21, and the day of birth was called post-natal day one (PND1) (Hotchkiss et al 2007). The pups were temporarily separated from their mothers, examined for gross malformations and their body weights measured and immediately re-united with their mothers. Their body weights were subsequently measured weekly. The pups were culled to four pups per mother (24 pups per group).

Developmental behaviour

Surface righting reflex activity and cliff avoidance test were carried out with all the pups on PND4-7. For the surface righting reflex activity, each pup was placed in a supine position on a flat board that was 50 cm above the ground, and was allowed to upturn itself, and the time recorded. Each rat was tested in two trials per day. This was carried out on PND4-7.

For the cliff avoidance test, each pup was placed on the edge of a flat board that was 50 cm above the ground, with the forepaws and nose over the edge. The time required to completely turn back and move away from the edge was recorded. Each rat was tested in one trial per day. The number of rats with successful responses within 30 seconds was recorded. They were tested on PND7.

On PND8 and 15, twelve pups each per group were sacrificed by chloroform anaesthesia, and the brains were then excised, weighed and preserved in 10% buffered formalin for histological study. Thereafter the cerebral cortex were excised and routinely processed for general histomorphology using Haematoxylin and eosin (H & E) and Nissl substances using cresyl fast violet, staining methods. Cellular populations were determined with ImageJ™ software (version 1.77c, National Institutes of Health, USA). Briefly, live images (at the predetermined area) of the sections were captured by the ImageJ™ software through the light microscope at ×160 and ×400 magnifications. They were converted to 8-bit images and threshold to 210 at the scale of 1 µm while ensuring that the scale was in the global mode. Microscopic scale was then set for camera binning of 1 × 1 at ×40 objectives. Nuclei of the cells were then quantified at this magnification.

Student-t test using Primer™ software was applied for the statistical analysis. All results were presented as Mean ± Standard error of Mean, and regarded as significant at $p<0.05$.

## RESULTS

At birth (PND1), there was no significant difference ($p<0.05$) in body weights between the pups whose mothers were treated with 200 mg/kg body weight of calabash chalk suspension compared to the control group. Also, at PND 8 (one week) and 15 (two weeks), there was no significant difference in the

body weights of the pups in the calabash chalk group compared with the control group (Table 1).

Table 1: Mean body weights of the pups in the experimental groups

| | Pups PND1 (g ± sem) n = 24 P = 0.642 T = 0.469 | Pups PND8 (g ± sem) n = 24 P = 0.103 t = 1.661 | Pups PND15 (g ± sem) n = 12 P = 0.094 T = 1.707 |
|---|---|---|---|
| Control | 5.44 ± 0.15 | 10.76 ± 0.37 | 19.28 ± 0.44 |
| 200 mg/kg of calabash chalk | 5.35 ± 0.12 NS | 9.74 ± 0.49 NS | 17.81 ± 0.74 NS |

Sem = Standard error of mean; NS - Not significantly different from control group (1) at p<0.05; P - Probability level; t - t-ratio; PND - Post natal day

Developmental processes

There was no difference in the days of ear opening, incisor teeth appearance, fur appearance and eye opening between the calabash chalk group and the control group (Table 2).

Table 2: Developmental processes in the pups in days

| Groups | Ear opening (PND) | Incisor teeth appearance (PND) | Fur appearance (PND) | Eye opening (PND) |
|---|---|---|---|---|
| Control | 3 | 5 | 8 | 14 |
| 200 mg/kg of calabash chalk | 3 | 5 | 8 | 14 |

PND - Post natal day

Table 3: Surface righting reflex and cliff avoidance tests in the experimental groups

| | Surface righting reflex (seconds) | | | | Cliff avoidance (seconds) |
|---|---|---|---|---|---|
| Groups | PND4 n = 24 P =0 .384 t = 1.03 | PND5 n = 24 P = 0.000 t = 6.75 | PND6 n = 24 P = 0.215 t = 1.52 | PND7 n = 24 P = 0.894 t = 0.20 | PND7 n = 24 P = 0.118 t = 2.01 |
| 1 (Control) | 3.53 ± 0.62 | 1.99 ± 0.15 | 1.78 ± 0.26 | 1.83 ± 0.23 | 9.20 ± 1.51 |
| 2 (200 mg/kg of calabash chalk) | 2.59 ± 0.25 NS | 1.65 ± 0.08* | 2.04 ± 0.12 NS | 1.86 ± 0.18 NS | 7.85 ± 1.12 NS |

Results are presented as mean ± standard error of mean; * - Significantly different from control group (1) at p<0.05; NS - Not significantly different from control group (1) at p<0.05; PND - Post natal day; P - Probability level; t - t-ratio

Behavioural studies

There were no difference in surface righting reflex on PND 4, 6 and 7. On PND 5, the calabash chalk group spent a significantly (p<0.05) less time surface righting compared to the control group. There was also no significant difference between the calabash chalk group and the control group in cliff avoidance test (Table 3).

Histomorphology of the cerebral cortex

The section of the cerebral cortex of PND8 pups in the control group showed six cortical layers; outer marginal zone, cortical, subcortical, intermediate, subventricular and ventricular layers. The cortical layers were thick, and dominated with large-size cells. In the marginal layer, there were small sparsely dense cells. The cortical layer consisted of a dense cellular population, while the subcortical layer was less populated. Layers 4-6 which were less distinct showed dense cellular population (Figure 1). The section of the cerebral cortex of PND15 pups of the control group presented thick cortical layers; marginal zone, cortical, subcortical, intermediate, and subventricular, layers. The marginal zone showed small sparsely dense cells. The cortical plate had a dense population of pyramidal-shaped cells with other cell types appearing fewer in density. The subcortical plate blended imperceptibly with the intermediate layer through to the subventricular layers. The cells were less dense, except at the subventricular layer where there were dense (Figure 2).

The section of the cerebral cortex of PND8 pups in the calabash chalk group showed hyperplasia of cells in the entire cortical layers. Layers 3-6 were indistinguishable compared to the control (Figure 1). The section of the cerebral cortex of PND15 pups showed less density and size of cells in the cortical layer, which blended imperceptibly with the subcortical plate (Figure 2).

The section of the cerebral cortex in the PND8 pups of the control group showed deeply stained Nissl substances

in the entire layers (Figure 3). In PND15 pups of the control group, the Nissl substances were also deep staining (Figure 3). In the calabash chalk group, the PND8 pups showed less deep staining Nissl substances in the entire layers of the cerebral cortex (Figure 3). In the PND15 pups, the section of the cerebral cortex also showed some of the neurons exhibiting less Nissl substance staining with some showing chromatolysis compared with control group (Figure 3). There was differences in the cortical cellular population of the PND8 and 15 pups in the calabash chalk group compared to their respective control groups, however, these differences were not significant.

## DISCUSSION

This study was to investigate the effect of maternal administration of the kaolin-base calabash chalk on the histomorphology of the developing pup's cerebral cortex. The result of this study showed that from birth (PND1) until 2 weeks of age (PND15), there was no difference in the body weights of the growing pups between the test and control groups. This indicates that the chalk or its constituents may not affect the birth weight and the growth rate of the pups. Kaolin, which is the basal constituent of calabash chalk is reported to result in low birth weights of pups (Patterson and Staszak 1977). This previous study is at variance with the present study probably due to the dosage of the chalk, or the interaction of its constituents with one another. However, Trckova et al (2009) reported that stoppage of kaolin ingestion restores body weight in young piglets. This may be a reason the growth of the pups were not affected, since the dams were only exposed to the chalk prior to their birth.

Some normal developmental processes of animals such as; ear opening, incisor teeth appearance, fur appearance and eye opening as reported in this study showed no difference between the test and control groups, which indicates that the presence of the chalk or its constituents may not have influenced these developmental processes. Reports have shown that some drugs do not affect these developmental processes (Drago et al 1999; Thullier et al 2002). However, for any physical development of rodents to be disturbed, it requires extremely high doses of prenatal drug treatment (Tucker 1985), which may not have been the case in this study.

Simple reflexes and indicators of motor development are used to assess damage by toxins or chemical agents on developing animals (Pellis et al 1991). In this study, no difference was recorded in surface righting reflex and cliff avoidance tests, an indication that the reflex activities of the growing pups were not affected by the calabash chalk administration. The difference seen in PND5 may be inconsequential. Some drugs have been reported as not having any effect, as seen by Ferguson et al (2005) who earlier reported that prenatal folate treatment showed no effect on righting reflex. Prenatal stress is reported to result in slower reflex activities (Drago et al 1999), however, this may not be the case as this effect was not observed in the present study.

The PND8 (one week old) pups in the chalk

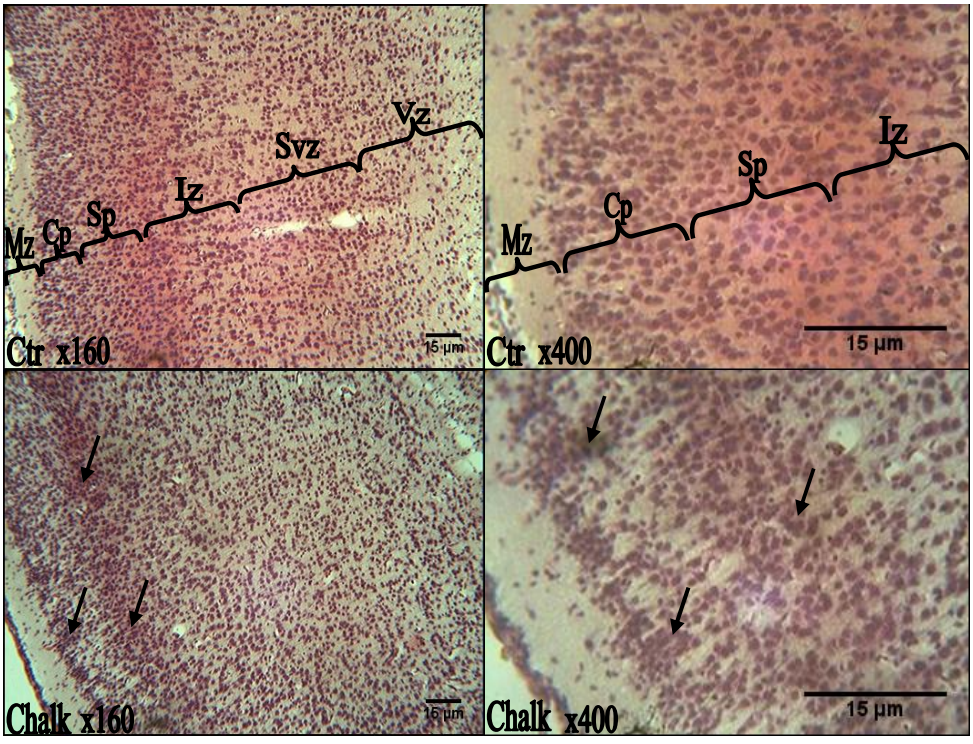


FIGURE 1: Photomicrographs of sections of the cerebral cortex of PND8 (one-week-old) pups of the control and the chalk group (H & E):
Ctr - Photomicrograph of the section of the cerebral cortex of the Control group showing six cortical layers: Mz (marginal zone), Cp (cortical plate), Sp (subcortical plate), Iz (intermediate plate), SVz (subventricular zone), and Vz (ventricular zone) (Mag. ×160, ×400).
Chalk - Photomicrograph of the section of the cerebral cortex of the Chalk group whose mothers received 200 mg/kg of calabash chalk suspension showed hyperplasia of cells (arrows) (Mag. ×160, ×400) compared with the Control group.

group whose mothers received 200 mg/kg of calabash chalk showed cellular hyperplasia in the cerebral cortex. The presence of cellular hyperplasia indicates that the calabash chalk or its constituents may have crossed the blood-placental membrane to initiate trauma to this brain area. Maturing neurons are not known to proliferate when traumatized especially in the cerebral cortex, thus, glial cells may have been involved in the high cellular proliferation. Astrocytes and microglia are known to proliferate when the brain tissue is traumatized with chemical agents and/or infections (Roumier et al 2008; Tambuyzer et al 2009), hence, astrocytes and microglia may be the likely cells involved in the cellular hyperplasia.

It was reported that lead and arsenic can cross the blood-placental membrane (Goyer 1990; Zadorozhnaja et al 2000; Vahter 2009). As these two elements are constituents of calabash chalk, they alone or with other constituents may have stimulated the proliferation of cells in this brain area of the study. Proliferation of cells is usually a mechanism by a tissue to cope with trauma, be it physical or through chemical changes (Kumar et al 2005). Thus, the invasion of the brain area by the calabash chalk or its constituents could pose a threat to the integrity of the brain.

In the two weeks old pups, there was reduction in the pyramidal neuron size and population in the cerebral cortex of the chalk group. Reduction in neuronal sizes may represent an early sign of pyknosis (Kafa et al 2008). Pyknosis itself represents an irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis (Kumar et al 2005; Kroemer et al 2009), which may indicate a degeneration of the cells of the cerebral cortex. As degeneration is a gradual process, this may be a reason the developmental and reflex activities were not affected.

Nissl substance was less deeply demonstrated in the cerebral cortex of the one- and two-weeks old pups of the chalk group, with chromatolysis also observed in the two-week pup's cerebral cortex. This is in line with the report of the histomorphology study. The Nissl substances are large granular bodies found in neurons (Kiernan 2009). These granules are the rough endoplasmic reticulum and are the site of protein synthesis (Young and Heath 2000; Snell 2001; Singh 2002; Kiernan 2009). This staining method is useful in localizing the perikaryon because of its presence in the soma and dendrites of neurons (Snell 2001). Thus, Nissl susbtances are important indices in tracing neuronal population (Young and Heath 2000; Singh 2002).

Nissl substances show changes under various physiological and pathological conditions, hence in injured neurons, disappearance of the Nissl substances indicates chromatolysis (Singh 2002; Lowe and Cox 1992). Chemicals including drugs, toxins and lack of oxygen, cause alterations in the distribution pattern of Nissl bodies, which thereby influenced their metabolic activities (Macsween and Whaley 1992: Bancroft and

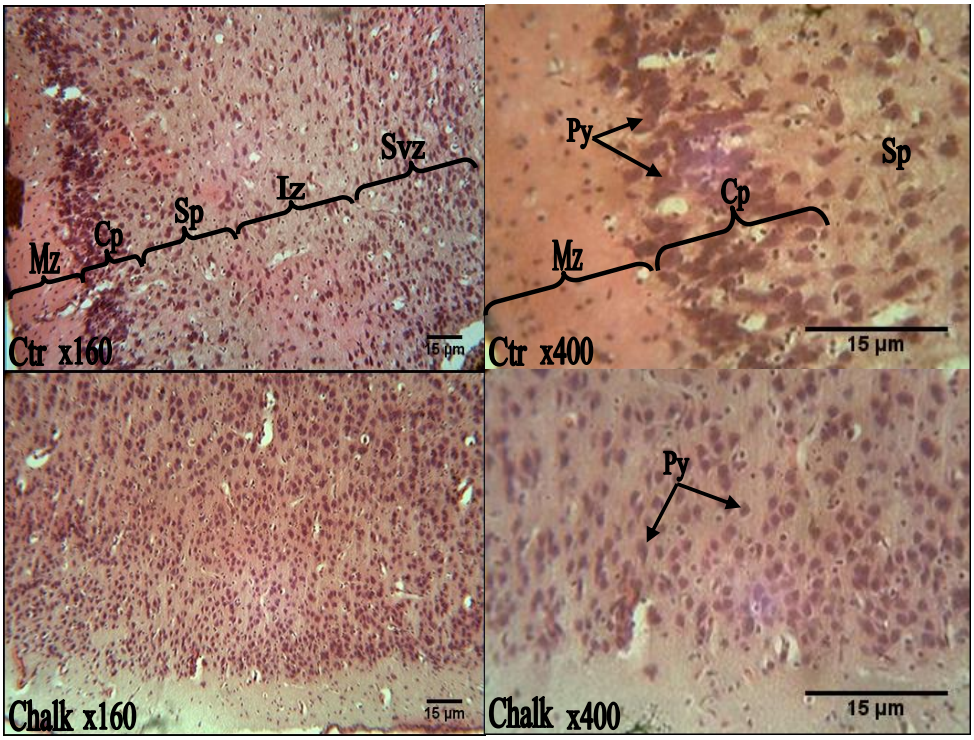


FIGURE 2: Photomicrographs of sections of the cerebral cortex of PND15 (2 weeks old) pups of the control and the chalk group (H & E):

Ctr - Photomicrograph of the section of the cerebral cortex of the Control group showing the cortical layers: Mz (marginal zone), Cp (cortical plate), Sp (subcortical plate), Iz (intermediate plate), SVz (subventricular zone). Found in the cortical and subcortical plates were several pyramidal (Py) neurons (Mag. ×160, ×400).

Chalk - Photomicrograph of the section of the cerebral cortex of the Chalk group whose mothers received 200 mg/kg of calabash chalk suspension showed less population and size of the pyramidal cells in the cortical plate (Mag. ×160, ×400) compared with the Control group.

Gamble 2007).

It is now known that the Nissl stain do not stain only for neurons, but also for the glia (Hamidi et al 2004). Hence, a higher distribution of Nissl stains also represents the presence of glia. As Nissl substances denote protein synthesizing ability of the neuron, chromatolysis may result in the loss of function of the neurons, and since protein is the working molecules of the cells, this may ultimately result in cell death. This may be a mechanism through which calabash chalk affects the cerebral cortex.

In conclusion, maternal calabash chalk geophagy showed gradual cerebral cortical cell death processes which may lead to limitation of its functions.

**Conflict Of Interest**

None declared

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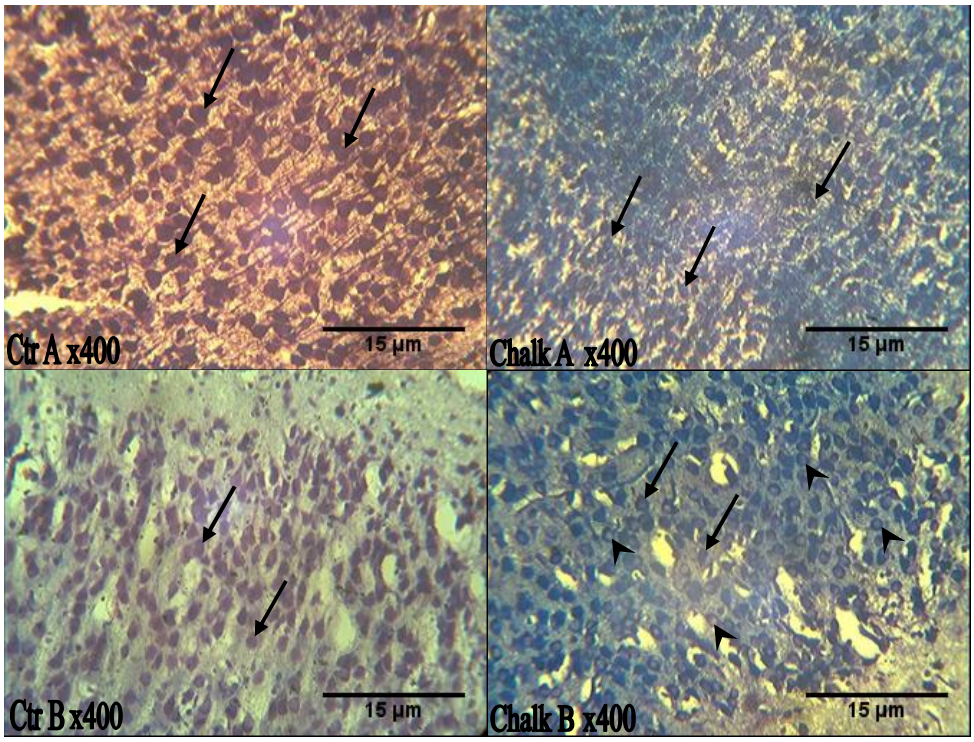


FIGURE 3: Photomicrographs of sections of the cerebral cortex of PND8 (one-week-old) and PND15 (2 weeks old) pups of the control and the chalk group (Cresyl Fast Violet):
Ctr - Photomicrographs of the sections of the cerebral cortex of the Control group on PND8 (Ctr A) and PND15 (Ctr B) showing deeply stained Nissl substances (arrows) throughout the entire layers (Mag. ×400).
Chalk - Photomicrographs of the sections of the cerebral cortex of the Chalk group on PND8 (Chalk A) and PND15 (Chalk B) showed some of the cells exhibiting less Nissl substance staining (arrows), with some showing chromatolysis (arrow heads) compared with the Control group (Mag. ×400).

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