THE THE EFFECT OF PRENATAL EXPOSURE TO ARTESUNATE ON DEVELOPING CEREBRUM AND HIPPOCAMPUS IN WISTAR RAT FOETUSES *(RATTUS NORVEGICUS)*

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NIGERIA

OCTOBER, 2016

Declaration

I hereby declare that the work in this dissertation entitled 'The effect of prenatal exposure to artesunate on the developing cerebrum and hippocampus in Wistar rat foetuses' has been carried out by me in the Department of Human Anatomy, Faculty of Medicine, A.B.U, Zaria, under the supervision of Prof. Samuel.S.Adebisi and Dr. Judith.N.Alawa. The information derived from the literatures has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Asma'u Muhammad SHEHU	…….	October, 2016
Name	Signature	Date

Certification

This dissertation entitled 'EFFECT OF PRENATAL EXPOSURE TO ARTESUNATE ON THE DEVELOPING CEREBRUM AND HIPPOCAMPUS IN WISTAR RAT FOETUSES' by Asma'u Muhammad SHEHU meet the regulations governing the award of the degree of Master of Science in Department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dedication

This research work is dedicated to Allah (S.W.A) for giving me the strength and health **to** carry out successfully the research reported in this dissertation. To my parents for their prayers and care, my lovely kids Ahmad and Amal for your inspiration and lastly to my dearest husband Dr. Salihu for your love and support.

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Abstract

Artesunate (AS) is a class of medication known as artemesinin used in the treatment of malaria. The occurrence of*Plasmodium falciparum* malaria during pregnancy exposes the mother and foetus to serious risks. The present study was aimed at evaluating the effect of artesunate during pregnancy with particular reference to the histology and neurochemistry of the cerebrum and hippocampus. Thirty pregnant Wistar rats were divided into three groups and were treated intragastrically on gestation day 9 to 15 with safe or high dose regimen of 4mg/kg/3days + 2mg/kg/4days or 10mg/kg/3days + 4mg/kg/4 days respectively. Control rats were treated with distilled water. Five pups were sacrificed at each time intervals of gestation day (GD) 20, postnatal days (PD) 1, 4, 7, 14, 21, and 28 for morphometrical, histological and immunohistochemical studies on the cerebrum and hippocampus. The brain tissues were dissected, fixed, processed and examined by light microscope. Morphometrically, safe dose showed significantly stunted growth of body particularly in the limbs, tails and brain compared to control. High dose induced marked teratogenic effects characterized by severe embryo loss indicated as resorptions in most dams. However, in the dams that littered there was high incidence of craniofacial and other skeletal malformation. Histologically, the safe dose showed decrease in cell proliferation with marked difference in the layer stratification of the cerebrum and neuronal degeneration characterized by pyknosis, vacuolation, and degeneration of the pyramidal cells compared to the control. High dose showed complete retardation of cerebral and hippocampal development in the few pups. It also showed a severe form of neurodegeneration characterize by cytoplasmic vacuolations, distortion of the pyramidal cell layers, central

chromatolysis, aggregation, clumping and scattered cells. Immunolabelling of neurons specific for the calcium binding protein Calbindin D28k demonstrated fewer primitive cells characterized by less intensity of the stain in the pyramidal cells in treated groups compared to control that showed a strong and positive immunoexpression of Calbindin D28k. These results indicate that artesunate (AS) has a narrow safety margin in rats and could induce teratogenic and neurotoxicological effects in which the mechanism may involve calcium homeostasis in the brain.

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List of Abbreviations

ABC	Avidin Biotin Complex Method
ABU	Ahmadu Bello University
AS	Artesunate
AUCs	Area around the curve
BW	Brain Weight
CA	Cornu ammonis
CB	Calbindin
CBPs	Calcium Binding Proteins
CC	Clumped Cells

CDA	Chroproguanil and dapsone
CDC	Centre for Disease Control
CNS	Central Nervous System
CR	Calretinin
CRL	Crown Rump Length
CSF	Cerebrospinal fluid
CV	Cytoplasmic Vacuolation
CYP	Cytochrome monoxygenase enzyme
DC	Degenerated Cells
DG	Dentate Gyrus
DHA	Dihydroartemesinin
DN	Disintegrated Nuclei
DPC	Degenerated Pyramidal Cell
DPCL	Degenerated Pyramidal Cell layer
EC	Oestrous Cycle
ECG	Electrocardiograph
ED	Embryonic day
FW	Foetal Weight
GABA	Gamma Amino Butyric Acid
GD	Gestational Day
H	Haematoxylin
E	Eosin
IEH	Institute of Environment and Health
LD_{50}	Lethal Dose

ND	Nuclei displacement
NL	Nuclei Loss
PC	Pyramidal Cells
PD	Postnatal Day
PV	Pavalbumin
ROS	Reactive Oxygen Specie
SC	Stellate Cells
SERCA	Sarco Endoplasmic Reticulum
TL	Tail Length
VC	Vacoulation Cell
VP	Vaginal Plug
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria remains one of the world's most significant health problems despite necessary research and control efforts (WHO, 2008). It has continued to be the most devastating human parasitic infection in the world, with about (100 million in Nigeria) at risk of infection (Giles, 1997). It is said to be responsible for over 2 million deaths each year, and a leading cause of death in children in those countries where the disease is endemic. This disease is widespread in tropical and sub-tropical regions (WHO 2010 and Adebisi, 2010).

According to the World Malaria Report, 109 countries were endemic for malaria in 2008, malaria infects between 350 and 500 million people each year, and kills one million (WHO 2006b*;* 2008). The occurrence of malaria during pregnancy exposes the mother and infants to serious risks (WHO, 2010) as pregnancy decreases a woman's immunity to malaria, making her more vulnerable to malaria infection and increasing the possibility of illness, severe anaemia and death of the mother (WHO, 2010). Regarding the unborn child, maternal malaria increases the risk of unplanned abortion, stillbirth, premature delivery low birth weight, infant mortality which is a leading cause of child mortality (WHO, 2003; 2009a, 2010).

Every year at least 30 million pregnancies occur among women in malaria-endemic areas of Africa. Malaria during pregnancy causes up to 10 000 maternal deaths and 200 000 new born deaths each year (WHO 2003; WHO 2009a). Artesunate safety during the first trimester (week 1-12) has not yet been proven (WHO, 2006c). It is therefore imperative that

pregnant women be protected against malaria, and the pregnant woman with malaria receives treatment as soon as possible (WHO, 2002a; 2009a).

Over 40% of the world population lives in endemic areas estimated 500 million clinical cases, and 1-2 million deaths per year, (WHO, 2010). In Africa it accounts for 90% of cases of malaria occurring worldwide. One African child dies of malaria every 30 seconds, responsible for 1 in 4 childhood death and accounts for 10% of Africa's diseases burden, (Agomo *et*., 2007; WHO, 2010). Nigeria accounts for 25% of malaria cases in WHO African region. It is responsible for 50% of all clinic attendance affecting mainly children under the age of 5 years and pregnant women (WHO, 2010). In children an estimated 56 million episodes is under 5 years per year, and is the major causes of poor child development. It causes 25% of infant mortality and 30% of all childhood death, (WHO, 2010). In Nigeria and probably in other parts of West Africa, the predominant parasite is *p. falciparum* (75%), others are *p. malaria* (15%), *p. ovale* (3%,) and *p.vivax* is probably not found in West Africa (James and Gills, 1985); in pregnant women it is up to 15% maternal anaemia, 35% of preventable low birth weight, responsible for 11% of all maternal death and 70% of morbidity in pregnant women, therefore children and pregnant women are most affected (WHO, 2002a; 2009a).

Artesunate is a class of medication known as artemesinin, which are derives from the quinghaosu or sweet wormwood plant *Artemisia annua* (Ngokere *et al*., 2004; Adebisi, 2010). It has been used in China for the treatment of fever over a thousand years (Adebisi, 2010). Artesunate and its active metabolite, dihydroarteesinin (DHA) are blood schizonticides, active against the ring of the malaria parasite of all plasmodium species. It

has an unusually broad activity against asexual parasite, killing all the stages from young rings to schizonts in *p.falcifarum* malaria, artemesinin also kills the gametocytes and hence, ideal for the treatment of severe malaria including cerebral malaria. It is rapidly hydrolysed to the active metabolite dihydroarteesinin (Li *et al*., 2002).

Artemesinin exerts their anti-malarial action via an iron catalyzed decomposition of the Endoperoxide Bridge to produce free radicals. (Meshnick *et al*., 2001) which destroys the parasite possible by alkylation of plasmodia proteins (Asawamahaskda *et al.,* 1994; Bhisutthibhan *et al.,* 1998). The generated free radicals cross the placenta and elaborate a cascade of teratogenic influence (Koch *et al*., 1995). Lou *et al* (2003) has suggested that artesunate induces its developmental toxicity via lipid-peroxidation and by neutralizing the antioxidant defence mechanism of the organism.The effectiveness of AS has been attributed to its rapid and extensive hydrolysis to dihydroartemisinin (DHA) which is threefold to fivefold more active, and toxic, than the parent compound (Li, 2002). DHA rapidly and effectively fights all stages of the parasite life cycle and completely inhibits parasite growth within the shortest exposure time as compared with all other artemisinin and conventional antimalarial drugs (Agomo *et al.,* 2007).

Several recent studies had shown that high doses of artesunate can produce neurotoxicity such as selective damage to the brainstem centers in mice and rats (Nontprasert, *et al*., 1998; Genovese *et al.,* 2000; Nontprasert *et at.,* 2002) gait disturbances, loss of spinal cord and pain-response mechanisms in animals (Genovese *et al.,* 1995; Dayan, 1998; Santiago *et al*., 2008) and various skeletal disorders (Adebesi, 2009; 2010)

Literature abounds with evidences of developmental toxicity of artesunate in laboratory animals (Xu and Zhang, 1996; WHO 2002a; WHO 2002b; Lou *et al*., 2003). In experimental animals, malformations of nervous system mostly occur when the teratogen is administered shortly before or during the closure of neural groove (Singh *et al*., 1972; Ezurumelu and Killackey, 1982).

Plasmodiumfalciparum malaria during pregnancy poses substantial risk to the mother and the foetus. Artesunate is frequently prescribed but their safety during pregnancy is yet to be established and there is lack of published data on this subject. With this background, the present work was aimed to investigate the teratogenic effect of Artesunate at therapeutic and high dosage with the possibility of finding any alterations in the micro architecture and morphometry on the developing brain tissues mainly cerebrum and hippocampus of Wistar rat foetuses following administration of artesunate to pregnant dams.

1.2 Statement of Research Problem.

Malaria is one the most devastating global public health burdens contributing to substantial morbidities, mortalities in many countries in the world including Nigeria and it has been implicated in the increase of maternal mortality as such new drugs like artesunate are discovered (WHO, 2006).

Artesunate is the drug of choice for the treatment of malaria in Nigeria and it is used indiscriminately in pregnancy even without doctor's prescription. Safety during the first trimester ([w has not yet been proven (WHO, 2010).

Adequate data on the teratogenicity and safety of artemesinin in pregnancy are lacking (WHO, 2010) hence further evaluation is necessitated.

1.3 Justification of the Study

Pregnant women and children below 5 years of age are a high risk group in malaria attack and are more likely to develop complications. Birth weight is reduced in pregnant women and poor growth, mental retardation is associated in children, therefore this group need effective and safe antimalarial treatment. Despite the fact that Artesunate is beneficial, cases of toxicity had been reported at pregnancy (WHO, 2010). Artemisinin derivatives have not been evaluated in the first trimester of pregnancy in humans and therefore their use in patients with uncomplicated malaria during this period should be avoided until more information is available (WHO, 2006).

1.4 Aim of the Study

The aim of the study is to evaluate the time course of the teratogenic effects of different doses of prenatal exposure to artesunate on the developing brain tissue mainly the cerebrum and hippocampus in Wistar rat foetuses.

1.5 Specific Objectives

The specific objectives of the study were to:

i. Identify gross morphological and morphometrical changes in the experimental pups following prenatal exposure to artesunate.

ii. Study the histological changes that may occur in the cellular layers of the cerebral cortex and hippocampus following prenatal exposure to artesunate using H&E and Toluidine blue stains.

iii. Evaluate the time-course viability of the expression of calcium binding protein by pyramidal cells of cerebrum and hippocampus through immunolabelling with antibody specific for Calbindin D28k.

1.6 Scope of the Study

The study will be limited to general histomorphological, anatomical, morphometrical and immunohistochemical changes of cells in the cerebrum and hippocampus as a result of exposure to artesunate.

1.7 Significance of the Study

1. Due to paucity of information on the effect of artesunate on the developing brain tissue, this study could provide the useful information on the teratogenic tendencies of artesunate on the developing cerebrum and hippocampus of Wistar rat foetuses.
2. It will also provide knowledge about the actual risk of artesunate during pregnancy which is very essential in order to balance the benefits against the risks.
3. It will also serve as a basis on which further studies could be done on the effect of administration of artesunate on the developing cerebrum and hippocampus.

1.8 Research Hypothesis

Prenatal exposure to artesunate will result in neurotoxicity particularly to the developing cerebrum and hippocampus of Wistar rat pups.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria Infection

Malaria infection represents a profound global health threat, resulting in substantial morbidity and mortality. In 2012, there were approximately 207 million cases of malaria infection, resulting in an estimated 627,000 deaths (WHO, 2014). Malaria is caused by protozoa of the genus *Plasmodium*, with two species, *Plasmodium falciparum* and *Plasmodium vivax* being responsible for the vast majority of infections. *P. falciparum* infection predominates in Africa (WHO, 2014).

P. falciparum, human infection begins when a female Anopheles mosquito takes a blood meal and *Plasmodium* sporozoites are transferred from the mosquito's saliva into a person's capillary blood. The sporozoites migrate to the liver within hours of the blood meal being taken. The protozoa undergo asexual reproduction inside hepatocytes for approximately six to eight days, at which time merozoites are released into the bloodstream (White *et al.,* 2014). Merozoites released into the bloodstream invade and subsequently reproduce inside red blood cells. Merozoites undergoing asexual reproduction inside red blood cells go through an initial ring stage, followed by subsequent trophozoite and schizont stages (White *et al.*, 2014).

This maturation occurs over approximately 48 hours, after which the schizonts burst to release multiple merozoites into the bloodstream and a portion of the merozoites invading red blood cells do not undergo asexual reproduction, but rather develop into male and female gametocytes (White *et al.,* 2014).

The malaria life cycle is perpetuated when the mosquito bites a human host and inoculates the sporozoites into the human bloodstream. In *P. vivax* and *P. ovale* infections, some of the intra-hepatic sporozoites enter an arrested stage of development called hypnozoite and remain dormant for a period of time before schizogony begins. This delayed primary infection is known as relapse (White *et al*., 2014).

The release of merozoites into the bloodsteam every 48 hours elicits an immune response which generates symptoms of fever, rigors, nausea, and myalgia. Additionally, red blood cells infected with *P. falciparum* merozoites display the capacity to adhere to blood vessel walls, potentially contributing to organ damage due to occlusion; other *Plasmodium* species cannot cause vascular adherence. The spleen acts to remove infected red blood cells, which contributes to the anaemia commonly resulting from malaria infection. Clinically, a case of malaria is considered severe when infection is accompanied by signs of vital organ dysfunction. Complications occurring in severe malaria include cerebral malaria, in which cerebral small blood vessels are packed with Parasitized red blood cells, as well as severe anaemia, impaired consciousness, multiple convulsions, pulmonary oedema, acute kidney injury, acidosis, hypoglycaemia, and respiratory distress (Chen *et al.*, 2014). Even with treatment, the fatality rate of severe malaria is 10 – 20%; left untreated, severe malaria is almost always fatal (WHO, 2010*;* WHO, 2011; White *et al.*, 2014).

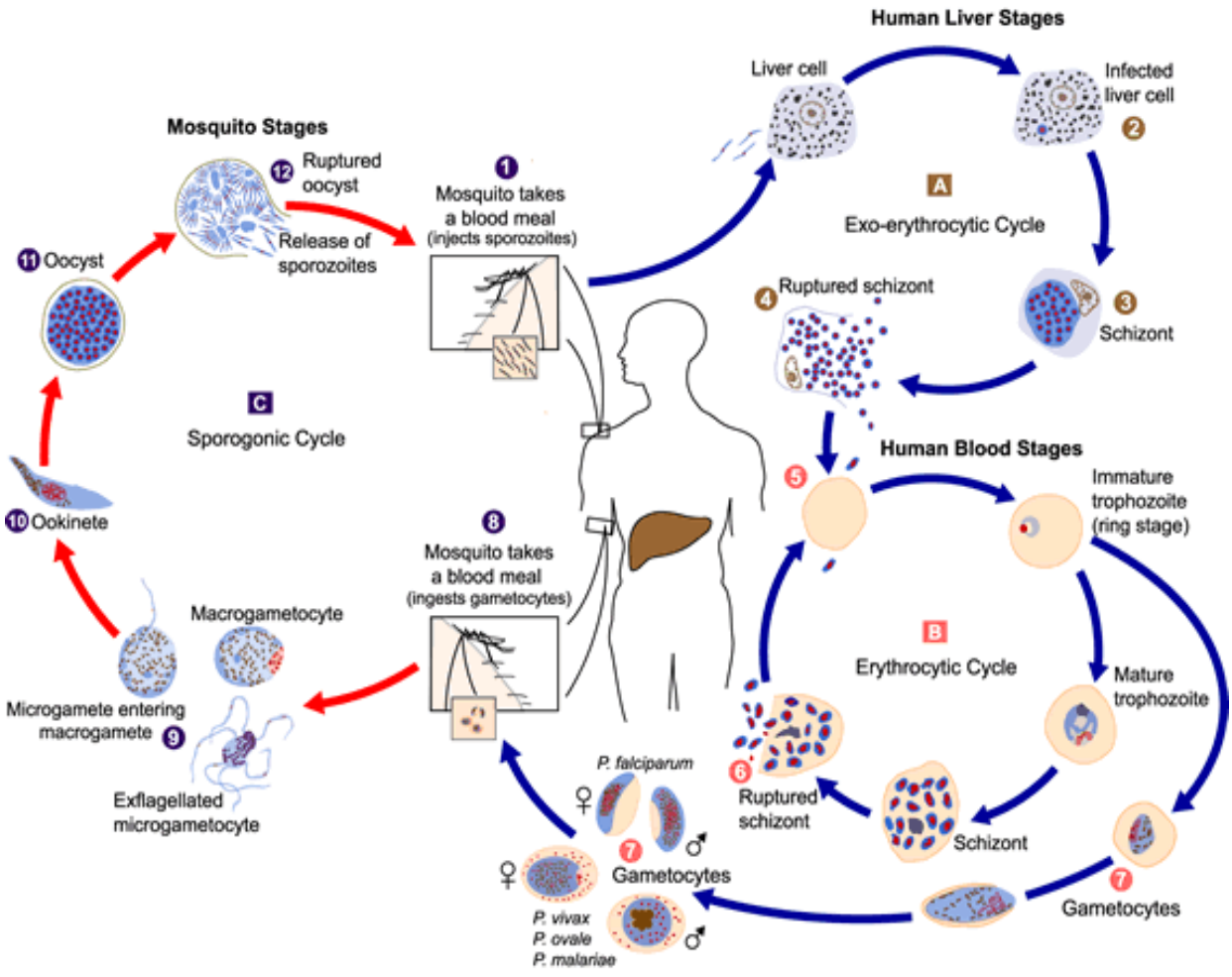


Fig 2.1 Life cycle of *Plasmodium* Species.

(Centres for disease control and prevention (CDC, 2005)

2.1.1Malaria in children

Young children living in high transmission regions but who have not yet acquired a reasonably robust partial protective immunity against malaria are at substantial risk of experiencing malaria-related morbidity and mortality. WHO estimates that in 2012 approximately 73% of deaths worldwide due to malaria occurred in African children under five years of age. Even among children treated for and surviving severe malaria, (Malaguarnera, 2002) long term sequelae, including neurological damage from cerebral malaria, may be observed (Crawley *et al.*, 2010). Approximately 3 – 15% of children surviving cerebral malaria display neurological deficits, including paralysis of one side of the body, cerebral palsy, deafness, cortical blindness, and impaired learning; these neurological sequelae are of varying duration (White et al., 2014). A child with a malaria infection can receive antimalarial treatment before progression to severe malaria can occur (Crawley *et al.*, 2010; WHO, 2014).

2.1.2 Malaria in Pregnant Women

Schantz-Dunn (2009) reported that malaria infection during pregnancy represents a substantial global health problem, with malaria being responsible for approximately 10,000 maternal deaths every year compared to their non-pregnant counterparts, pregnant women are three times more likely to develop severe disease from a malaria infection. Furthermore, in high transmission areas, pregnant women experience higher rates of *falciparum* parasitemia and severe malaria-related anaemia than their non-pregnant counterparts. This severe anaemia in pregnancy increases the risk of development of congestive heart failure and haemorrhage during delivery. In these regions, placental malaria infection, characterized by *P. falciparum* sequestration in placental tissue, also represents a

significant health threat. Placental malaria can increase the risk of miscarriage, intrauterine growth restriction, preterm birth, and low birth weights (Schantz-Dunn, 2009; Desai *et al*., 2007). Low birth weights, in turn, increase the risk of neonatal death, with malaria infection in pregnancy being responsible for an estimated 100,000 neonatal deaths each year (Desai, 2007; Shulman et *al.*, 2002).

2.2 Development of the Central Nervous System (CNS)

2.2.1 Pre and postnatal stage

This stage is characterized by extensive growth of the brain. In rats, neuronal cell populations are produced up to postnatal day 21 in the rat cerebellum around half of all the cell population is produced in the postnatal period (Krinkle and Eisenbrandt, 1994). According to Bayer and Altman (1995), central nervous cellular proliferation and differentiation continues after birth in many species**.**

2.2.2 Impact of neuroteratogen during neurogenesis

According to Bayer and Altman (1995), Bayer *et al*. (1993), loss of precursor neurons due to a neuroteratogen assault will not be compensated for. The surviving precursor neurons produce only the programmed number of neurons during a programmed time window. Should a massive injury causing an entire loss of a certain neuronal population would have further consequences on cell migration, settlement and differentiation of neuronal cell population which have been produced or will be produced after the injury (Bayer and Altman, 1995; Bayer *et a*l., 1993).

Loss of neurons reduced the overall volume of the neuronal population but does not change the density of the structure; this means that cell numbers are primarily observed as change in the size of structures (Rodier, 1998; Rodier and Gramann, 1979). Halls and Das (1978) administered a neuroteratogen agent in rats at gestational day 14 and 15 observed a thinness of the cerebral cortex and a reduction of cortical neurons to about 41% of the controls. Despite, the thinness of the cortex, the six cortical neuronal layers was well stained. This observation is in accordance with the observations in human infants with mental retardation, where the brain may be small but with no abnormal cytoarchitecture patterns (Suzuki, 1980). Size reduction without an obvious histomorphological recognizable change in the normal cytoarchitecture led Rodier and Gramann (1979) to suggest the use of 'simple morphometry' as a viable tool for evaluating cell loss in the developing brain.

As evidenced from the paragraph above, neurons in the central nervous system are generated according to precise time schedule (Singh, 2003; Moore and Persaud, 1998; Bayer and Altman, 1995).

2.2.3 Impact during migration and settling of neurons

Once a presumptive neuron turns off cell division in its germinal matrix, it migrates to its predetermined location in the brain parenchyma and settles (Rakic, 1971). In the cerebellum for instance, neuroblasts of the neuroepithelium migrate to form a second germinal layer, the external granular layer that produces billions of granular cells and interneurons of the cerebellar cortex. The cerebellar cortex is a good site to demonstrate one important pattern of neuronal migration. Neuronal migration is characterized by an astrocyte guided translocation of nerve cells from their primary or secondary germinal

layers to their adult locations (Rakic, 1971; Sieviers *et al*., 1994). The physical contact between glial cell migrating neurons is mediated by adhesion molecule, which thus facilitates migration of neurons. These cell-cell contacts have been considered as a putative site for neurotoxicant action (Lopachin and Aschner, 1993; Reuhl, 1994).

Bayer *et al*., (1993), assumed that the granular cell ectopias in the cerebellar cortex after x-irradiation were caused by the effect of the neuroteratogen during migration, killing many precursor cells in the external layer. This resulted in a break in the normal steady stream of new granular cells settling in their proper locations. Once an ectopia has been produced by abnormal migration, it becomes a permanent anatomical feature of the brain (Bayer *et al*., 1993). However, small ectopias are less reliable as a morphological marker for early injury during brain development as there are also reports of a spontaneous occurrence or a disappearance of ectopic cell overtime in the brain (Schmel and Rakic, 1979).

2.2.4 Impact on the development of neurotransmitter systems

During neonatal brain growth spurt, a rapid development of all major neurotransmitter systems is observed, especially those that are particularly implicated in the fast transmission of information in the neural networks (IEH, 1996). The process of noradrenergic synaptogenesis is observed as early as gestational day (GD) 14 in rats and reaches adult values in density and distribution at post-gestational day (PD) 14 in the neocortex. Mesencephalic dopaminergic nuclei are shown to give rise to fibre projections at GD in the diencephalons, achieve adult topology at PD 12 (neocortex and adult densities at PD 35. Sprouting of serotoninergic raphe nuclei axons begins at GD 15 in the pons and adult levels of terminal field densities are noted at PD 21 in the cortex, hippocampus, septal

regions and brainstems (Broening and Silkker, 1998). Another neurotransmitter system that is important in developmental neurotransmission is less direct and more a modulatory one, influencing the excitatory and inhibitory actions of other neurotransmitters (IEH, 1960; Jett, 1998). So damage may have serious consequences.

2.2.5 Immature CNS areas in the postnatal life of mammals

Development of CNS in mammals does not end at birth; it continues through the postnatal life (Mohamed *et al*, 2015). Various morphological and physiological changes occur also in the human brain during infancy and adolescence. The rate of developmental changes are very high in the infancy, whereas adolescence represents one of the **most** dynamic events of growth and development in the human as a second surge of synaptogenesis still occurs. In these stages, developmental injuries and infections can result in several lasting CNS problems such as mental retardation, hydrocephalus and epilepsy. Neurotoxic agents including drugs and chemicals affect all steps of CNS development (Bernocchi *et al*., 2015).

Bernocchi *et al*., (2015) also reported that as a model of postnatal developing CNS areas, the cerebellum and hippocampal dentate gyrus (DG) are brain regions particularly vulnerable in the developing animal as well as in new-born human because injury may alter physiology and cause neuropsychiatric disorders. In the rat or mouse, both areas are later-developing brain regions with important neurodevelopmental changes, especially in the first three weeks of postnatal life when multiple interrelated processes occur in maturing neuroarchitecture. These range from cell proliferation, migration and differentiation to synaptogenesis and synaptic connectivity. In the postnatal developing hippocampus, cell

migration and differentiation continue to be very active and critical in the steps of the histogenesis of rodent hippocampal formation (Bernocchi *et al*., 2015). The pyramidal cells and large interneurons arise during the prenatal life, dentate gyrus (DG) granule cells originate at embryonic day (ED) 16 and continue to proliferate during postnatal development until they migrate to their final destination. Therefore, cell migration and synaptogenesis are postnatal events of all cell types in the hippocampus.

2.3 Hippocampus

2.3.1 Morphology and formation of the hippocampus

Anatomically, the hippocampus is an elaboration of the edge of the cerebral cortex (Gray, 2000; Chris, 2008). In rodent, the hippocampus, a horseshoe shape paired structure, with one hippocampus located in the left brain hemisphere and the other in the right hemisphere (Gross, 1993). The hippocampus, the dentate gyrus and the subiculum collectively make up the hippocampal formation, which is said to be part of the limbic system as shown in Figure 2.2. The hippocampus and the dentate gyrus belong to the allocortex and has simplified laminar pattern compared with the neocortex.

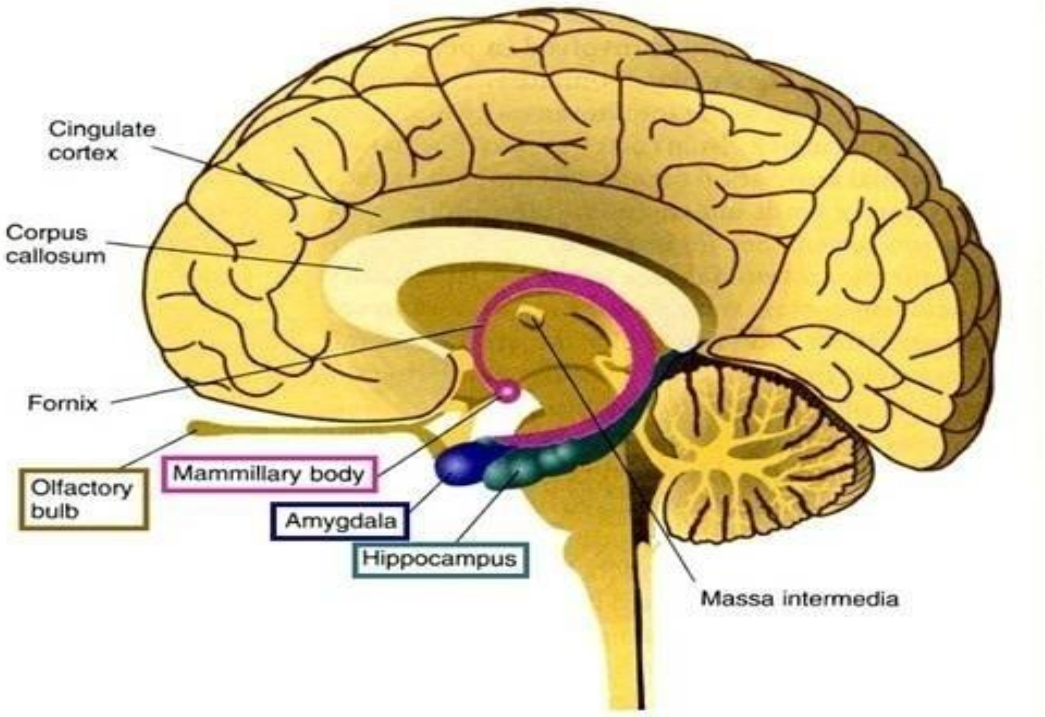


Figure 2.2:Hippocampus and associated structures of the limbic system (Chris, 2008).

2.3.2 The cells and layers of hippocampus

The hippocampus is composed of multiple subfields. Though terminology varies among authors, the terms most frequently used are dentate gyrus and the cornu ammonis literally "Amun's horns", abbreviated CA. The dentate gyrus contains the fascia dentata and the hilus, while CA is differentiated into fields CA1, CA2, and CA3 (Chris, 2008). The horse shoe appearance of the hippocampus is caused by cell density differentials and the existence of varying degrees of neuronal fibres as shown in Figure 2.4. The CA regions are also structured depth-wise in clearly defined strata (Hiroaki, 2007; Philippe, 2007; Annabelle, 2009).

The alveus is the deepest layer and contains the axons from pyramidal neurons, passing on towards the fimbria/fornix, one of the major outputs of the hippocampus. Stratum oriens is the next layer superficial to the alveus. The cell bodies of inhibitory basket cells and horizontal trilaminar cells, named for their axons innervating the three layers-the oriens, pyramidal, and radiatum are located in this stratum (Annabelle, 2009). The basal dendrites of pyramidal neurons are also found here, where they receive input from other pyramidal cells, septal fibres and commissural fibres from the contra lateral hippocampus usually recurrent connections, especially in CA3 and CA2 regions (Annabelle, 2009).

Stratum pyramidale contains the cell bodies of the pyramidal neurons, which are the principal excitatory neurons of the hippocampus. This stratum tends to be one of the more visible strata to the naked eye. In region CA3, this stratum contains synapses from the mossy fibres that course through stratum lucidum. This stratum also contains the cell

bodies of many interneuron, including axo-axonic cells, bistratified cells, and radial trilaminar cells (Chris, 2008).

Stratum lucidum is one of the thinnest strata in the hippocampus and only found in the CA3 region. Mossy fibres from the dentate gyrus granule cells course through this stratum in CA3, though synapses from these fibres can be found in stratum pyramidale (Chris, 2008).

Stratum radiatum like stratum oriens, contains septal and commissural fibres. It also contains Schaffer collateral fibres, which are the projection forward from CA3 to CA1. Some interneurons that can be found in more superficial layers can also be found here, including basket cells, bistratified cells, and radial trilaminar cells. Stratum lacunosum is a thin stratum that too contains Schaffer collateral fibres, but it also contains perforant path fibres from the superficial layers of entorhinal cortex. Due to its small size, it is often grouped together with stratum moleculare into a single stratum called stratum lacunosum-moleculare (Chris, 2008). Stratum moleculare is the most superficial stratum in the hippocampus. Here the perforant path fibres form synapses onto the distal, apical dendrites of pyramidal cells.

The hippocampal sulcus or fissure is a cell-free region that separates the CA1 field from the dentate gyrus. The phase of recorded theta rhythm varies systematically through the strata; therefore, the fissure is often used as a fixed reference point for recording EEG as it is easily identifiable (Chris, 2008).

2.3.3 The dentate gyrus

The dentate gyrus is composed of a similar series of strata namely; Polymorphic layer is the deepest layer of the dentate gyrus and is often considered a separate subfield. This layer contains many interneurons, and the axons of the dentate granule cells pass through this stratum on their way to CA3 (Annabelle, 2009). Stratum granulosum contains the cell bodies of the dentate granule cells.

Stratum moleculare, outer third is where both commissural fibres from the contralateral dentate gyrus run and form synapses as well as where inputs from the medial septum terminate on the proximal dendrites of the granule cells. Stratum moleculare, external two thirdsare the deepest of the strata, sitting just superficial to the hippocampal fissure across from stratum moleculare in the CA fields. The perforant path fibres run through this stratum, making excitatory synapses with the distal apical dendrites of granule cells (Gross, 1993; Chris, 2008).

2.3.4 Functions of hippocampus

The hippocampus is believed to play important role in several functions of the body. The hippocampus is the part of the brain that is involved in memory formation, organization and storage. Hippocampus is a limbic system structure that is particularly important in forming new memories and connecting emotions and senses, such as smell and sound, to memories. The hippocampus acts as a memory indexer by sending memories out to the appropriate part of the cerebral hemisphere for long-term storage and retrieving them when necessary (Gross, 1993; Chris, 2008).

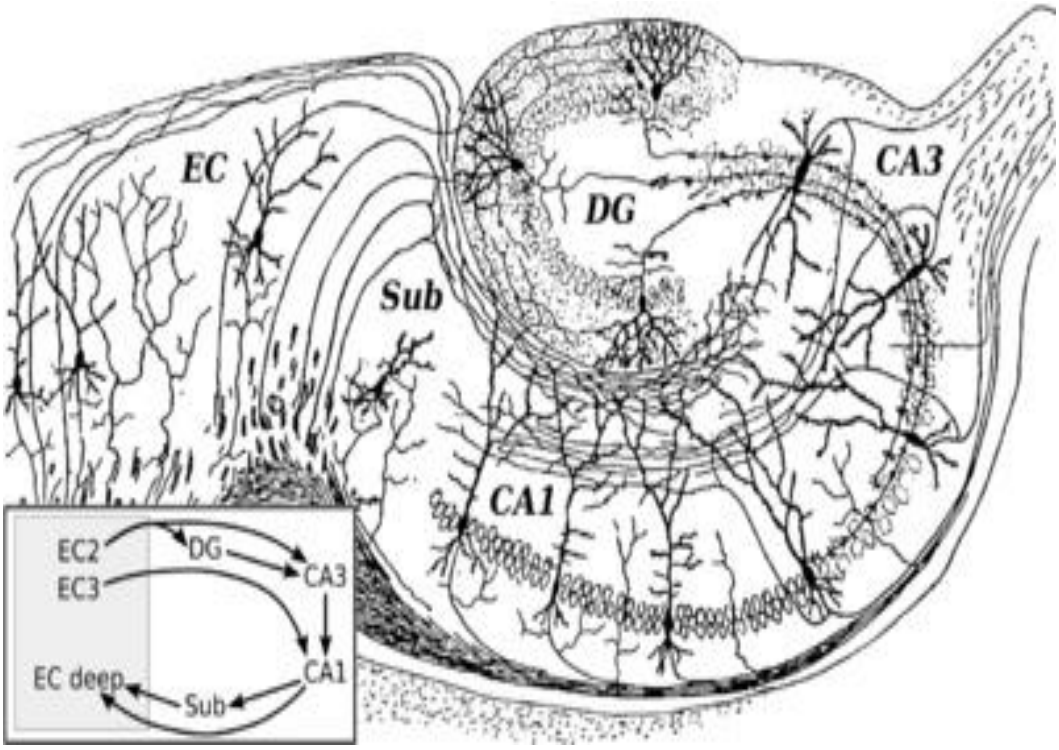


Figure 2.3 Basic circuit of the hippocampus (Kursk, 2011).

2.4 Cerebrum

2.4.1 Morphology of the cerebrum

The cerebrum comprises a large portion of the brain. It lies in front or on top of the brainstem and in humans is the largest and best-developed of the five major divisions of the brain. The cerebral cortex is the largest part of the brain; it is formed by grey matter and cerebral hemisphere on the surface as shown in Figure 2.4. The cortex is convoluted with projections called sulci and gyri. The cerebral cortex is about 1.5 - 4mm thick, and contains nerve cells, fibres, neuroglia and blood vessels. The cerebral hemisphere is supplied by cerebral branches of polygon of Willis i.e. the anterior, middle and posterior cerebral arteries (Kandel *et al.*, 2000).

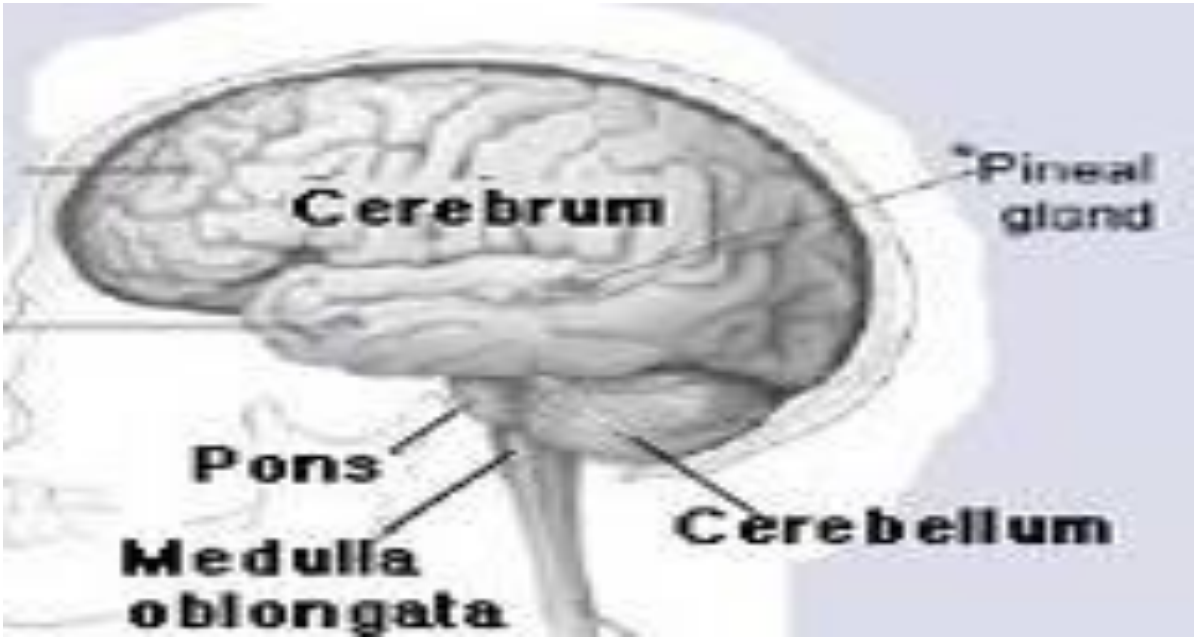


Figure 2.4 The structure of the brain showing the cerebrum (Kandel *et al.,* 2000)

2.4.2 Cellular layers of the cerebral cortex

The neurons of the cerebral cortex vary in size, shapes, lengths, branching patterns and orientation of their processes. These neuronal cells are named based on their specific characteristics feature which demarcates them from each other. The neurons include the followings:

The pyramidal cells: They are most abundant cervical neurons in the cortex. All others are referred to as non-pyramidal neurons. About 2/3 (two third) of the cerebral cortex cells are pyramidal cells. Their cell bodies are triangular in shape; apex is directed towards the cortex. Large dendrites arise from the apex while others arise from basal angles. The axis arises from the base of the pyramidal cells. (Kandel *et al.*, 2000). The process of pyramidal cells extends vertically through the entire thickness of the cortex and established numerous synapses. The axons of pyramidal cells may terminate in different ways. They may travel to the other region like the basal ganglia, the brain stem or the spinal cord. Also they may give collateral fibres that terminate within the cortex and may be short and terminate within the same area in the cortex (Kandel *et al.*, 2000).

The stellate cells: They are small and multipolar and form 1/3 (one third) of the total population of neurons of the cortex under low magnification. They look like granular cells. They are therefore being termed granular neurons (cells) by earlier workers. Stellates cell are of various types depending on their bodies and on the pattern of their ramification

processes. Their axons are short and end within the cortex and their processes extend chiefly in radical direction within the cortex in some case horizontally. Some Stellates cells may be uniform rather than Stellates, with one process arising at either end. Stellates neurons are classified as spiny and non-spiny neuron. The spiny Stellates cells use glutamate as neurotransmitter, while in most non-spiny Stellates cells it is Gamma Amino Butyric Acid (GABA).The neurons of the cerebral cortex are grouped into six main layers, from outside to inside namely as shown in Figure 2.7, according to Kandel *et al*., (2000); Shipp (2007) and Saladin (2010).

Layer I: The molecular layer, which contains few scattered neurons and consists mainly of extensions of apical dendritic tufts of pyramidal neurons and horizontally-oriented axons, as well as glial cells. Some Cajal-Retzius and spiny stellate cells can be found in the molecular layer. Inputs to the apical tufts are thought to be crucial for the ‘‘feedback’’ interactions in the cerebral cortex involved in associative learning and attention (Saladin, 2010). While it was once thought that the input to layer I came from the cortex itself,it is now realized that layer I across the cerebral cortex mantle receives substantial input from ‘‘matrix’’ or M-type thalamus cells in contrast to ‘‘core’’ or C-type that go to layer IV.

Layer II, The external granular layer, contains small pyramidal neurons and numerous stellate neurons.

Layer III, The external pyramidal layer, contains predominantly small and medium-size pyramidal neurons, as well as non-pyramidal neurons with vertically-oriented intracortical

axons. Layers I through III are the main target of interhemispheric corticocortical afferents, and layer III is the principal source of corticocortical efferents (Shipp, 2007).

Layer IV, The internal granular layer, contains different types of stellate and pyramidal neurons, and is the main target of thalamocortical afferents from thalamus type C neurons as well as intra-hemispheric corticocortical afferents.

Layer V, The internal pyramidal layer, contains large pyramidal neurons such as the Betz cells in the primary motor cortex. It is the principal source of subcortical efferents, as such there are large pyramidal cells which give rise to axons leaving the cortex and running down through the basal ganglia, the brain stem and the spinal cord.

Layer VI, The polymorphic or multiform layer contains few large pyramidal neurons and many small spindle-like pyramidal and multiform neurons. Layer VI sends efferent fibres to the thalamus, establishing a very precise reciprocal interconnection between the cortex and the thalamus. These connections are both excitatory and inhibitory. Neurons send excitatory fibres to neurons in the thalamus and also from collateral to other ones via the thalamic reticular nucleus that inhibit these thalamus neurons or ones adjacent to them. Since the inhibitory output is reduced by cholinergic input to the cerebral cortex, this provides the brainstem with adjustable "gain control for the relay of lemnsical inputs (Kandel *et al.,* 2000).



Figure 2.5 The cellular layers of cerebral cortex (Kandel *et al.*, 2000).

2.4.3 Variations of cortical structure

The structure of the cerebral cortex shows considerable variations in the region, both in terms of thickness and in prominence of the various laminae. These variations are as follow

I. In the agranular cortex, the external and internal granular Laminae are inconspicuous. This type of cortex is referred to as precentral gyrus (area 4) and is therefore to be of typical area.

II. In the granular cortex, the granular cortex is highly developed while the pyramidal and ganglion layer are poorly developed or absent. This type of cortex is mostly present in sensory area including the post-central gyrus (Kandel *et al.,* 2000).

2.4.4 Functions associated with the cerebrum

The cerebrum is the most highly developed part of the human brain and is responsible for determining intelligence**,** motor function,planning and organization**, t**ouch sensation,thinking, perceiving, producing and understanding language. Most information processing occurs in the cerebral cortex. The cerebral cortex is divided into lobes of which each has a specific function (Kandel *et al.,* 2000).

Motor function: The cerebrum directs the conscious or volitional motor functions of the body. These functions originate within the primary motor cortex and other frontal lobe motor areas where actions are planned. Upper motor neurons in the primary motor cortex send their axons to the brainstem and spinal cord to synapse on the lower motor neurons, which innervate the muscles. Damage to motor areas of cortex can lead to certain types of motor neuron disease (Shipp, 2007). This kind of damage results in loss of muscular power and precision rather than total paralysis. The motor area of classical description is located in the precentral gyrus on the superior lateral surface of the hemisphere and in the anterior part of the precentral lobule; the medial surface. This corresponds to area 4 of Brodman and possibly part of area 6, which lies in the pre-central gyrus, the specific region in the area responsible for movement of specific part of the body (Kandel *et al.,* 2000).

Sensory processing: The primary sensory areas of the cerebral cortex receive and process visual, auditory, somato-sensory, gustatory, and olfactory information. Together with association cortical areas, these brain regions synthesize sensory information into our perceptions of the world around us (Kandel *et al.,* 2000).

Olfaction: The olfactory bulb in most vertebrates is the most anterior portion of the cerebrum, and makes up a relatively large proportion of the telencephalon. However, in humans, this part of the brain is much smaller, and lies underneath the frontal lobe. The olfactory sensory system is unique in the sense that neurons in the olfactory bulb send their axons directly to the olfactory cortex, rather than to the thalamus first. Damage to the olfactory bulb results in loss of sense of smell (Kandel *et al*., 2000).

2. 5 Artesunate (AS)

Artemisia annua belongs to the class of flowering plants Magnoliopsida. It is an annual shrub of 50-150 cm in height. It has fern-like leaves, yellow flowers, and a camphor-like scent. Artemisia annua is also known as Sweet Wormwood, Sweet Annie, Sweet Sagewort, Annual Wormwood, or "*Qing Hao"* (ancient Chinese name meaning "green herb") (De Ridder *et al*., 2008).

The Artemisia annua grows in mild climates in tropical areas at higher altitudes and is mainly widespread in China and Vietnam. But most Artemesia annua is nowadays commercially grown on small properties and larger plantations, due to high demand and a quite short dry-leaf and artemisinin yield. Artemisia annua is also cultivated in among other East Africa, the United States, India and Brazil (De Ridder *et al*., 2008; MMV, 2009). Artesunate as an antimalarial agent is used primarily in treatment of malaria, which is thought to be one of the oldest infectious diseases evolving around 10,000 years ago.

Artesunate is a drug used to treat malaria, especially chloroquine resistance malaria in Nigeria (Woodrow *et al*., 2005; Agomo *et al*., 2007). It is a semi-synthetic derivative of artemesinin, the active compound of Chinese herb artemesinin annua which consist of the sodium succinyl salt of dihydroartemesinin (Ittarat., *et al*.1999). It is highly effective against multi-drug resistance strains of plasmodium hence its increasingly wide usage for the treatment and management of malaria (Hien and White, 1993; Batty*et al.*, 1996; Price *et al.*,1998; Van Agtmeal *et al.*, 1999; Agomo *et al*., 2007) with good tolerability and lack of significant adverse side effects (Looareesuwan, 1994).

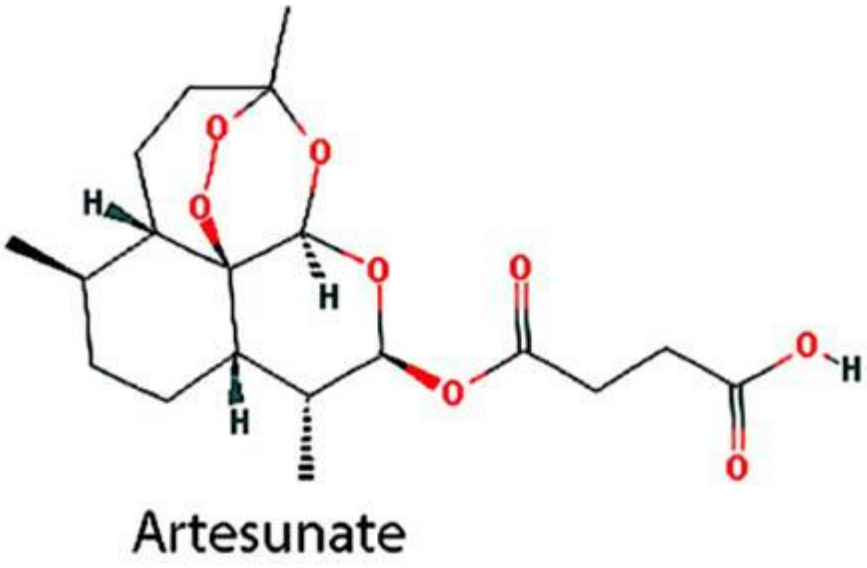


Figure 2.6 Chemical structure of artesunate

Artesunate is an antimalarial agent. It is a water-soluble hemisuccinate derivative of dihydroartemisini, a sesquiterpene lactone isolated from Artemisia annua. Artesunate and its active metabolite dihydroartemisinin are potent blood schizonticides, active against the ring stage of the parasite. Artesunate is ideal for the treatment of severe malaria, including cerebral malaria. The arteminisinin derivatives are also advantageous because they prevent parasites from maturing to the more pathological stages and more, offer the broadest antimalarial action against all range of development stages of plasmodium falciparum. Artemisnin compound and, to a less extent, chloroquine prevents ring stage from maturing, hastening their clearance, and preventing end-organ pathology that would otherwise occur if cytoadherence progressed unchecked. On the other hand, antimalarial drugs exerting maximum effect emax, that is, the proportion of total plasmodia killed per treatment, reduce total parasite number 10 to 10,000 fold per cycle (Moris *et al*., 2014)

2.5.1Formulation

Artesunate is formulated either as tables, in a gel enclosed in gelatine for rectal administration (recto-cap) or as dry powder of artesunic acid for injection, supplied with an ampoule of 5 percent sodium bicarbonate. The powder is dissolved in the sodium

bicarbonate to form sodium Artesunate, and then diluted in 5 percent dextrose or normal saline for intravenous or intramuscular injection. Oral formulation is probably hydrolysed completely before entering the systemic circulation (Adebisi, 2010).

2.6 Pharmacokinetics and Properties of Artesunate

2.6.1 Absorption of artesunate

Oral formulation is probably hydrolysed completely before entering the systemic circulation. artesunate is rapidly absorbed from the gastrointestinal tract upon oral administration, and they exhibit a short half-life in plasma. When applied as intramuscular injection, absorption is more protracted and leads to plasma levels that are sustained for longer periods of time; repeated administration by this route also leads to accumulation and higher plasma levels. This property may be important in terms of the risk assessment regarding the potential neurotoxicity of artemisinins and for all application modes (Li *et al*., 1999; Svensson *et al*., 1999).

2.6.2 Tissue and organ distribution of artesunate

In the early Chinese studies organ distribution of artemisinin has been estimated by radioactivity determination or by thin-layer chromatography. The resulting pattern 1 hour after oral administration of rats showed highest levels in liver, followed by brain, plasma, and lung, with kidneys, muscle, heart and spleen at somewhat lower levels of exposure. In pregnant mice treated with 3H-artemisinin radioactivity was also detected in the fetuses.

Shortly after drug administration, the levels in the intestine were markedly higher than in any other organs; brain levels were second, followed by the concentrations in kidney and liver. One hour after the injection, the levels were markedly reduced and were more similar between organs; artesunate concentrations in brain and fat seemed to be declining at a lower rate than other organs (Zhao and Song, 1989).

Zhao and Song, (1989) reported that in tissues collected, the total measured tissues was in the small intestine and its content. Lesser amounts of radioactivity were distributed in other tissues. Six hours after the injection, high levels of radioactivity were still present in the intestine, colon, and in their contents with slow loss; however, drug distribution was significantly increased in other tissues such as kidney, spleen, liver, heart, adrenals, blood, muscle, colon, and colon content. Study on patients with severe malaria, artesunate was determined in the cerebrospinal fluid (CSF) at different times after an intravenous dose of 120 mg artesunate (Zhao and Song, 1989).

2.6.3 Metabolism of Artesunate

Artemisinin and its ether derivatives are converted by cytochrome P450 enzymes, while artesunate - as an ester of artenimol – is hydrolyzed by esterases; thus, artesunate tends to disappear very fast from circulation, while the metabolism of the other compounds is less rapid, especially when administered parenterally. Since, however, artenimol is as active as, or even more so than, the different parent compounds, there is no loss of antimalarial activity upon the metabolic conversion to artenimol. Artemisinins do induce their own metabolism. Metabolites are mainly excreted as glucuronides (Riamet *et al.,* 2005). The metabolism of artemisinin in human liver microsomes is primarily mediated by cytochrome

P-450 monooxygenase enzyme (CYP) 2B6, with a secondary contribution by CYP3A4 in individuals with low CYP2B6 expression. The contribution of CYP2A6 to artemisinin metabolism is likely of minor importance. There is a large body of evidence suggesting that artemisinin influences the CYP activity, which could result in drug-drug interactions. An induction of activity by artemisinin was reported for CYP2A5, CYP2A6, CYP2B1, CYP2B6, CYP2B10, CYP2C19, and CYP3A4. In addition, artemisinin activates the constitutive androstane receptor and pregnane X receptor, which explains the upregulation of CYP2B6 and CYP3A4. The data regarding CYP1A2 are contradictory, whereas artemisinin inhibits CYP2D6. Artemisinin leads to an autoinduction of drug metabolism, which reduces its own bioavailability. The metabolism of artemisinins involves the formation of dihydroartemisinin (Riamet *et al.,* 2005).

2.6.4Elimination of artesunate

After oral administration of artesunate the radioactivity was excreted in the urine and feaces (China Cooperative Research Group, 1982a). There are no further, recent specific studies investigating the excretion of artemisinins and their metabolites very little artesunate was found to be present in urine, faeces and bile. However, biliary excretion in rats has been quantified for total radioactivity after dosing the animals with 14C-artenimol (Maggs *et al*., 1997). Artesunate was excreted into the intestines via bile ductules, this suggests the higher hepatic biotransformation of artesunate is similar to metabolic profiles of other artemisinin compounds. The biliary metabolites of artesunate in rats were predominantly from the hydrolysis pathway first to DHA, followed by glucuronylation and other conjugated products from multiple oxidative pathways for both compounds (artesunate and dihydroartemesinin). Two peaks of radioactivity were detected in blood and plasma and the

most of radioactivity was excreted into the intestines, showing a potential enterohepatic circulation of artesunate (Maggs *et al*., 1997).

2.7 Mechanism of Action of Artesunate

The structural determinant of the activity of artemisinins is the Endoperoxide Bridge which is a specific feature of this type of compounds. It has been suggested that the parasiticidal activity starts with the reaction of artemisinins with haem iron, leading to the generation of activated oxygen species, such as oxygen radicals, or of a C-centred radical of artemisinin itself, which are then further producing lethal damage to the parasite (Meshnick *et al*., 1993; Olliaro *et al.*, 2001a). Chemically, such a redox-cycling reaction between artemisinin and ferrous/ferric ions has (Sibmooh *et al.*, 2001) also been demonstrated to increase oxidative stress in *P. falciparum*-infected erythrocytes, but not in uninfected cells and depleting in consequence intra erythrocytic levels of antioxidants, especially glutathione (Ittarat *et al.*, 2003). The reductive scission of the endoperoxide bond may be catalyzed by ferrous ion in the form of haemin, produced by metabolic breakdown of haemoglobin in the food vacuole of the parasite, and binding affinities of artemisinins with ferrous haem have indeed been found to be correlated to theirrespective antimalarial activities (Paitayatat *et al.,* 1997). plasmodial proteins under conditions where no reaction occurred with erythrocyte proteins (Asawamahasakda *et al*., 1994).

schizontocidal activity of artemisinins suggested an interference with haemoglobin catabolism in the food vacuole of the parasite, inhibiting haemozoin formation and increasing haemozoin breakdown, both resulting in an accumulation of toxic haem, as well as in the generation of membrane damage and alkylation of parasite proteins by the free

radicals produced by the catalytic action of haem upon the artemisinin endoperoxide (Pandey *et al.*, 1999).

These effects might effectively be brought about through the alkylation of haem by artemisinin(Kannan *et al.*, 2005; Robert *et al.*, 2005). they also have an inhibitoryaction on parasite proteases, a possibility corroborated by the recent finding that protease inhibitors used in HIVtreatment have also some anti-malarial effect (Senior, 2005). On the other hand, various more recent investigations have provided evidence that neither haemozoin formation can be considered as target of the antimalarial activity of artemisinins (Haynes *et al.*, 2003), nor that the presence of haem is necessary for the schistosomicidal activity (Parapini *et al.*, 2004). As a very specific target for the activity of artemisinins, the sarco/endoplasmatic reticulum (SERCA) has recently been suggested (Eckstein-Ludwig *et al.*, 2003). The only plasmodial enzyme of the SERCA-type, PfATP6, was clearly inhibited. (Jung *et al.*, Jambou, 2005). Also, a proteomic approach to the investigation of cellular changes elicited by artemisinins has been suggested (Cooper and Carucci, 2003). Treatment showed clearly an upregulation of membrane-associated calcium-binding protein in the parasite (besides the upregulation of a number of stress-related heatshock proteins, a proteinase and two proteins related to protein metabolism) and may thus point to the involvement of changed calcium homoeostasis in the parasite (Makanga *et al.*, 2005). Furthermore, other targets for the schizonticidal activity of artemesinin have been shown to reduce the erythrocyte membrane fluidity which is increased upon infection with *P. falciparum* (Sibmooh *et al.*, 2000), to inhibit endocytosis (Hoppe *et al.*, 2004) and to damage the inhibition of PfATP6 by different artemisinin derivatives (Eckstein-Ludwig *et al.*, 2003) mitochondrial electron transport chain (Li *et al.*, 2005b).

2.8 Reproductive Toxicity of Artesunate

Published information on reproductive toxicity of artemisinin derivatives has been scarce, but it has been generally acknowledged that artemisinins have a distinctive embryotoxicity exhibiting a very steep dose-response relationship (WHO, 2005a). Longo et al, (2006) reported that doses are presented as fractions and application was either in the first 6 days of pregnancy, or on days 7 to 12, or 13 to 19, respectively. Dosing in the very early stage had no effect on the development of the foetuses, while treatment at the later stages resulted in complete embryonic loss. When treatment was administered between pregnancy days 6 and 15 and about 50% loss of embryos was observed at the lowest dose, while at the higher doses total loss was observed again. Umbilical hernia was the only malformation detected in 6.1% of the foetuses, when 14 mg/kg was administered on days 6 to 8 of pregnancy (Longo *et al.*, 2006).

Longo *et al.*, (2006) showed a large embryonic loss at relatively modest doses. In mice, 100% resorptions were observed at a dose of 21.4 mg/kg, while in rats and rabbits >90% resorptions were seen at 10.7 and 2.7 mg/kg, respectively. It was agreed that artesunate and all the other artemesinin analogues are developmental toxicants in the rat and rabbit. The dose-response relationships for the compounds are unusually steep. The effects are dominated by embryo-lethality and late resorptions and a few instances of morphological abnormalities, all under circumstances not related to maternal toxicity. All the artemesinin analogues have been associated with embryo-lethality over a narrow dose range. General experience has shown that morphological abnormalities and embryo-lethality are part of a continuous spectrum. A small change in dose or other factors may favour the occurrence of

one over the other. There have been minor indications in some experiments of pre-implantation loss and possibly of some change in sperm counts, but neither has been systematically studied. The main effect of embryo-lethality, as seen in many embryo toxicity studies, is not necessarily predictive of the same effect in humans. If there were a similar action in pregnant women it might become apparent during the first trimester of pregnancy (Longo *et al.*, 2006).

The mechanism of the developmental toxicity in animals is not known. It is not clear whether the initial site of action is in the mother, the foetus, or the placenta. In addition to the predominant embryo-lethality, there are instances of effects on development of the cardiovascular system, the axial skeleton, and the limbs, but these do not suggest the nature of the toxic action. There is decreased sensitivity to the effect on foetal growth or survival following dosing in later stages of pregnancy.

Clark *et al.,* (2004) reported in a recent study of the effects of artesunate, as well as of the combination of artesunate with chlorproguanil and dapsone (CDA), on the embryonal development and on teratogenic effects have been investigated in rats and rabbits In rats, artesunate alone was administered orally in doses of 6, 10 and 16.7 mg/kg between days 6 to 17 of pregnancy, the effects observed in the foetuses consisted of a low incidence of cardiovascular defects (ventricular septal and vessel defects) and of a syndrome of skeletal defects, especially with long bone shortening and/or bending. He also showed that embryotoxic effects are apparent as reduced numbers of foetuses and litters at the high dose.

Rabbits exhibited an analogous pattern of cardiovascular and skeletal defects. Cardiovascular defects were especially prominent in artesunate alone treated litters, White *et al.,* (2005) reported that the mechanism of these teratogenic effects has been investigated and results have been established that the most sensitive period for teratogenic effects of artemisinins in rats is between days 10 to 11 of pregnancy artesunate was shown to produce a marked reduction in the numbers of embryonic erythroid cells around 18 - 24 hours after a single dose, with the effect persisting for several days. It was furthermore shown that embryonic erythroid cells were more sensitive to the cytotoxic and lytic action of artesunate than adult erythrocytes (Laffan *et al*., 2005).

Laffan *et al.*, (2005) suggested that, an inhibitory action on the development of early erythroid precursor cells, leading to anaemia and hypoxia, and resulting finally in damage to growing tissues and embryonic death. Attention should be drawn in this respect also to the anti-angiogenic effects ascribed to artemisinins which might have an additional negative influence on the development of the cardiovascular system. (Longo *et al.,* 2006) studied rat embryos in culture Embryos at gestation day 9.5- 11.5 were excised and treated *in vitro* with artenimol after which embryos were observed for heartbeat, yolk sac circulation, morphological abnormalities, number of somites and various other parameters. The primary event leading to developmental damage was considered to consist of a specific toxicity to red blood cells during yolk sac haematopoiesis, while at higher concentrations also angiogenesis seemed to be affected.

Tissue damage and effects on embryo morphology and malformations were attributed to these effects. Longo *et al*., (2006) reported that the primary targets are the embryonic

erythroid precursor cells forming in the blood islands of the yolk sac. It is furthermore suggested, that the mechanistic basis of this damage to erythroid precursor cells is the same as, the mechanism for the schizonticidal activity of artemisinins. haemoglobin is actively synthesized in these cells, which are deficient in antioxidant defence possibilities, and consequently production of reactive oxygen species and increased oxidative stress may damage specifically these cells. The conditions in the embryo culture *invitro* would point to the inhibition of the SERCA-ATPase with consequences for an impaired calcium-ion homoeostasis and cell death (Laffan *et al*., 2005).

2.9 Neurotoxicity of Artesunate

Brewer *et al.,* (1994a) reported that intramuscular injection of arteether at a dose of 20 mg/kg/ in dogs showed a syndrome of progressive clinical neurological defects in conjunction with cardio-respiratory collapse and death was noted, while no such effects were seen to occur at the lower dose of 10 mg/kg.

These findings were subsequently investigation in dogs given ECG and histopathology of the brain, lesions observed in the brains of both rats and dogs consisted of scattered neuronal degeneration and necrosis, characterized by swelling and rounding of nerve cell bodies, increased eosinophilia, vacuolization of cytoplasm with loss of Nissl substance (central chromatolysis), swelling and fading of nuclei, and separation and clumping of fibrillar and granular components of nucleoli; similar effects were seen in the rat brains (Brewer *et al.,* 1994a; Brewer *et al.,* 1994b).

Studies in mice, rats and monkeys, and the histopathological lesions in the brainstem have been observed either in the presence or the absence of clinically manifest neurotoxicity (Genovese *et al.,* 1995; Petras *et al.,* 1997; Kamchonwongpaisan *et al*., 1997; Genovese *et al.,* 1998a; Nontprasert *et al.,* Genovese *et al.,* 1998b; Dayan, 1998; Genovese *et al.,* 1999; Petras *et al.,* 2000; 2002), neurotoxicity a class effect of artemisinins, was dependent not only on the dose applied but also on the mode of application and the physical-chemical properties of the derivative tested.

Studies in mice investigated the aspects of compound differences as well as differences due to the application route between artesunate and Artemether. Mortality was higher for artemether in the high dose group compared with artesunate at the same dose; no significant effects on body weight were observed at the lowest dose, while differences in body weight development were apparent between artemether and artesunate at the higher doses. Olfactory responses, estimates as the time needed to find food, were significantly impaired in the artemether- but not in the artesunate-treated groups. Nontprasert *et al.,* (1998) also reported that neurological parameter more than artesunate and intramuscular injection was more toxic than oral application Nontprasert *et al.,* (2000) suggested that route and formulation of artemether and artesunate on mortality and neurotoxicity on the brains of dogs treated intramuscularly showed neuropathic lesions with dose-dependently increased incidence and severity, while no such lesions were observed in orally treated animals oral dosing has the contrary effect, explainable by autoinduction of first-pass metabolism through the enteric route of application in contrast to the parenteral route. arteether produced exceedingly higher levels of neuronal damage in brainstem nuclei than did artesunate (Nontprasert *et al.,* 2000).

Behavioural performance tests showed significant deficits only for the arteether-treated animals since the auditory, vestibular and motor nuclei of the brain stem proved especially vulnerable to the effects of artemisinins, their involvement in the expression of this neurotoxicity has been investigated, and the respective regional damage was also used to investigate eventual neurotoxic effects in humans. Rats were trained in a complex radial-arm maze task with auditory stimuli performance, deteriorated significantly pointing to a neurotoxic defect in auditory processing (Genovese *et al.*, 2000).

Histopathology showed that treated rats showed marked damage (necrosis, chromatolysis, gliosis) to neurons in the nucleus trapezoideus (Genovese *et al.*, 2001). But not only the administration route and the physical-chemical properties determine the kinetic neurotoxicity but also the formulation. Toxicokinetic profiles were different with the oil formulation and the cremophor formulation, animals receiving the substance in the oil formulation had a higher level of neurotoxic damage with significantly more severe grading's than the cremophor formulation. This is because of the induction of the neurotoxic damage in the brain stem is not dependent on Cmax values reached, but on a sustained exposure the level.

Several neuronal and glial cells (foetal rat primary neuronal cells, foetal rat secondary astrocytes, and transformed neuroblastoma cell lines derived from rats and mice), artemisinin derivatives were shown to damage only neuronal cells in a concentration and time-depend.

Neuronotoxic activity was as a release of lactate dehydrogenase into the culture medium or as inhibition was observed to be highest for artenimol and artesunate (Wesche *et al*., 1999). The proliferation of undifferentiated neuroblastoma and glioma cells and the inhibition of neurite outgrowth from neuroblastoma cell were obtained, and it was confirmed that the endoperoxide bridge of artemisinin derivatives was a necessary component for this toxicity. Artenimol was demonstrated to be of higher toxicity than artemether or arteether. It was suggested that reactions with cytoskeletal proteins, like crosslinking reactions, might form the mechanistic basis of this specific neurotoxicity (Fishwick *et al*., 1995). In the presence of an exogenous metabolic activation system from liver able to convert artemether and arteether into the primary metabolite artenimol, neuronal toxicity has been shown also to increase for these compounds (McLean and Ward, 1998).

The involvement of oxidative stress in this neuronal toxicity has been made plausible not only by the necessity for an intact endoperoxide structure, but also by the findings of enhanced toxicity of artemisinins in the presence of haemin or iron, and of the respective protective effects of ascorbic acid and glutathione (Smith *et al.*, 1997; Smith *et al.*, 1998; Smith *et al.*, 2001). The specific sensitivity of brain stem neuronal cells was explained by their less active antioxidant system (Schmuck *et al*., 2002).

The pharmacokinetic properties and the distribution of these compounds into the CNS have to be considered for an estimate of the potential magnitude of any problem in humans. From nonclinical studies it has been estimated that brain levels of artemether and arteether should amount to only about 10 % of the respective plasma concentrations; the lesser lipophilicity of artesunate and artenimol considered to be more neurotoxic than artemether and arteether should lead to even lower brain Influence on the number of neurofilaments

and inhibition of mitochondrial function in primary brain stem neuronal cells, by artemisinin (Schmuck *et al.*, 2002) concentrations the blood-brain barrier is compromised, such as in cerebral malaria. A small clinical study in 6 patients with cerebral or severe *P. falciparum* malaria treated with an injection of 120 mg artesunate (2.0 - 2.86 mg/kg) showed that, although no artesunate could be detected in cerebrospinal fluid, concentrations of artenimol were measurable and increasing with time to produce neurotoxicity *in vitro* (Davis et al., 2003). In accordance with this, clinical studies have generally failed to detect CNS disturbances related to artemisinin administration (Van Vught *et al.*, 2000; Kissinger *et al.*, 2000; Toovey and Jamieson, 2004).

2.10 Calbindin D28K (CB)

Calbindin is a member of the EF-hand family of calcium binding proteins (CBPs) and it has a low molecular weight. It is a neuronal immunohistochemical marker of specific cell populations in the central nervous system (CNS) (Celio *et al.*, 1996). Calbindin (CB) is able to bind calcium with a fast association rate (Nagerl *et al.*, 2000). Calbindin seems to be involved in the modulation of specific calcium-dependent activities of developing neurons, such as neurite elongation and spine formations inside the neuronal cytosol might exert a protective role against the overfull calcium influx, which may cause degenerative processes (Maskey *et al.*, 2010).

Calcium plays a complex role as an important moderator of a number of vital physiological processes like neuronal excitability, axonal transport, synthesis and release of neurotransmitters, membrane permeability, and enzyme activities (Yew *et al.*, 1997). Neuronal calcium might play a role in neuron survival as well as programmed cell death

and pathological neuronal degeneration with disturbance in calcium regulation leading to lethal effects (Krebs, 1998). Therefore, efficient regulation of intracellular free calcium concentration is crucial for neuronal function and survival, which can be achieved by an active uptake mechanism of the cell's internal membrane structures such as mitochondria and endoplasmic reticulum as well as calcium binding proteins (CaBPs), considering the importance of calcium homeostasis in numerous processes for cell viability (Krebs, 1998).

A neuroprotective role with a sole function as a cytoplasmic calcium buffer has been postulated for calcium binding proteins, the impairment of which leads to the failure of buffering of the intracellular calcium and neuronal death (Heizmann *et al.*, 1992). A calcium binding protein characterizes specific neuronal types in the CNS (Baimbridge, 1992) among which PV, CB and CR are considered to be excellent neuronal markers for a subpopulation of the hippocampal neurons (Celio *et al*., 1990). Loss of CaBPs has been linked with impaired Calcium homeostasis and is related with both neuronal and behavioural deficits (Airaksinen *et al.*, 1997). Hence, dysregulation of calcium homeostasis could contribute to impaired memory processes of the hippocampus.

Physiological calcium entry into neurons regulates normal neuronal development, metabolism, and ageing and is involved in the control of synaptic transmission (Ghosh *et al.*, 1995). Hyrc *et al.*, (1997) reported that elevated calcium levels will leads to neuronal degeneration.

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2.11 Ooestrous Cycle of the Rat

Female rat shows a 4-day ooestrous cycle (Freeman, 1988). A cycle consists of prooestrous, oestrous, and dioestrous phases (Ebling and Highman, 1978; Freeman., 1988).

Vaginal smears are widely used to identify these phases of the oestrous cycle (Ebling and Highman, 1978). Observation of the cell population in the vaginal smear is the most popular and reliable for identifying the different phases (Freeman, 1988). The populations of the different cell types are controlled by circulating oestrogen, as oestrogen causes a proliferation of vaginal epithelial cells in the prooestrous. For mating purposes, a prooestrous rat should be transferred to the cage of a male rat and left overnight and the vaginal smear checked the following morning to ensure that successful copulation has occurred (Freeman, 1988). According to Ebling and Highman, 1978 and Freeman, 1988, ovulation in the female rat begins at about midnight and is indicative of the receptivity period of the female rat to the male rat.

Nucleated cells are the major population in the smear at the prooestrous phase. In the early oestrous, the vaginal smear is full of markedly swollen cornified cells lacking nuclei, whereas the at dioestrous 1 and dioestrous 2, numerous leukocytes appear in addition to the nucleated cells and shrinking cornified cells (Freeman, 1988; Ebling and Highman, 1978).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

Forty-five (45) Wistar rats were used in the investigation. They included 30 virgin female Wistar rats with mean weight of (120g-160g) and 15 male Wistar rats with mean weight of (180g-240g). The rats were obtained from the animal house of the Department of Human Anatomy and the Department of Pharmacology, Ahmadu Bello University, Zaria. The rats were allowed to adapt to the new environment and for further breeding. The rats were kept in 11 plastic cages with 8 cages housing 5 female rats each and three cages housing 5 male rats each for a period of two weeks under standard laboratory conditions. The rats were fed on rat pellets (Sander Agro Feeds Limited, Ibadan). Clean tap water was also made available for drinking in plastic water bottles, with stainless steel nozzles. The room was kept clean and well ventilated. Assessment of the weight was done at an interval of three days before and during the experiment. They were subsequently grouped and mated and a total of seventy-two (72) Wistar rats from the breed were used for the study.

3.1.2 Drug and reagents

Artesunate (mekophor chemical pharmaceuticals, vietnam, 11002CX), Bouin's fluid, kit for immunohistochemical staining of calbinbin primitive neuron (Abcam USA, ab11426), Toluidine blue, heamatoxylin and eosin stain, xylene, paraffin wax, Animal feed, Chloroform.

3.1.3 Equipment

Vernier caliper, Beakers, Specimen bottles, digital weighing balance, scale vein, syringes, pipette, glass slides, digital microscope, dissecting tray and dissecting kit, Cannula, Cotton wool.

3.2Methods

3.2.1 Determination of oestrous cycle

Collection of the vaginal smears from the 30 female rats was done using a small plastic pipette. Small amount of distilled water was put in to the pipette and then inserted into the rat's vagina which is gently rubbed against the posterior wall of the vagina to remove some of its contents. The cells were sucked up into the vagina while the suspended cells were put into a clean grease free microscope slide. Assessments of the cells population were done with the aid of a Celesterone Digital Light Microscope and photomicrographs were also taken (Marcondes *et al.,* 2002 and Adebisi, 2009).

3.2.2 Mating of rats

Rats that were in the proestrous phase were mated with a male rat in a ratio of 2 females to 1 male (flagel, *et al.*, 2002). Positive mating was confirmed by examination of the vaginal plug early in the morning of the day after mating. A clean plastic pipette and tap water was used, the vaginal lumen was flushed and content aspirated. It was then immediately transferred to a clean glass slide and examined under celesterone digital light microscope. The presence of spermatozoa or a brownish plug obtained the following morning confirmed coitus, and the sperm positive day was designated as day zero of gestation.

3.2.3Confirmation of mating and pregnancy

Pregnancy in the rats was confirmed again by examining the vaginal cytology from each rat after 2 days using a plastic pipette and distilled water which was rubbed against the posterior wall of the vagina and the content aspirated. The smears that remain in the diestrous phase of the estrous cycle implied that the rat's regular 4-day cycle has been altered as a result of pregnancy (Marcondes *et al.*, 2002 and Adebisi, 2009).

3.3 Experimental Design and Procedure

Pregnant rats were subsequently divided into 3 Groups: Control Group A and Experimental Groups B and C with 10 pregnant rats in each group. The drug Artesunate (powder for oral suspension) was then dissolved in distilled water to a concentration of 2mg/ml and the bottle was then shaken well. The rats were administered equivalent volumes of the dosages based on the individual maternal body weights. Each experimental rat was administered artesunate by orogastric intubationper kg of body weight for a period of 7 days from 9^{th} to 15^{th} day of gestation to target the period of brain development (Bayer and Altman, 1995, Mesembe *et al*, 2004). Group A served as the control group and as such received equal volume of distilled water in ml/kg per kg of body weight, while group B and C were treated with 4mg/kg/3days + 2mg/kg/4days and 10mg/kg/3days + 4mg/kg/4 days respectively (WHO,2010). The remaining pregnant rats from both the experimental groups and the control group were allowed to litter their young ones normally. A random selection of 5 litters from each group was done on the postnatal days (PD) PD 1, PD 4, PD 7, PD14, PD 21, and PD 28 day. The Pups were humanely sacrificed following chloroform inhalation and the developing brain removed.

Table 3.1 Animal Grouping and treatment with Artesunate

Experimental Groups	Treatment (Dosage/kg body weight/ frequency)	
A (Control)	1ml/kg/3 days	1 ml/kg/4 days
B (Therapeutic dose)	4 mg/kg/3 days	2 mg/kg/4 days
C (High dose)	8 mg/kg /3 days	4 mg/kg/4 days

Group A (control) received equal volume of distilled water.

3.4Retrieval of Foetal Tissue

One rat from each group was sacrificed on the 20th day of gestation (GD) by chloroform inhalation method and the foetuses retrieved through maternal abdominal incision. The abdominal wall was opened and the full extent of both uterine horns exposed promptly. Records of litter size and number of resorped site were noted. Retrieved foetuses were blotted dry and weighed with a digital weighing balance (VIC 303).

The remaining pregnant rats from both the experimental groups and the control group were allowed to litter their young ones normally. A random selection of 5 litters from each group was done at each time interval of postnatal days (PD) PD 1, PD 4, PD 7, PD14, PD 21, and PD 28 days. The litters were weighed using a digital weighing balance (VIC 303) and morphometric measurement was conducted on the brain and body which includes forelimb, hind limb and tail length, brain width, and brain length and brain weight.

The litters were then anaesthetized in a chloroform chamber and the head was dissected through incision along the dorsal aspect, the decapitated heads of the litters were then fixed in Bouin's fluid. Brains were dissected using a razor blade and scalpel, the retrieved brains were blotted dry to remove any adhesions; weighed and linear measurements were done on the brain using a Verniercalliper.

3.5 Morphometrical Assessment

The following linear measurements were done on the body and brain of the neonates using a Verniercalliper.

- Foetal weight (g)

- Brain weight (g)
- Tail length (cm)
- Forelimb length (cm)
- Hind limb length (cm)
- Brain Length (cm)
- Brain width (cm)

3.6 Tissue Processing

The tissues were fixed in Bouin's fluid which and were processed and stained using Haematoxylin and Eosin for general morphology and normal cell bodies and Toluidine Blue stain for nuclear materials and Nissl substances.

3.6.1 Haematoxylin and Eosin Staining Method

Tissue slices of 3 to 4cm thick of the kidneys, liver, stomach, heart, spleen, ovaries, uterus and testes were cut and fixed in labelled containers containing 10% formalin while the brain was fixed in Bouin's fluid. They were then dehydrated for 2 hours in each of the following ascending grades of alcohol: 85%, 90% and 100% v/v. The dehydrated tissues were then cleared in toluene for 2 hours, after which the tissue slices were embedded in paraffin wax and left to cool. The blocks were trimmed on the microtome at 5 microns. The ribbon of sections was dehydrated in xylene and rehydrated in the following grades of alcohol: 100%, 90% and 70% v/v and subsequently infiltrated with molten paraffin wax. They were then stained in Haematoxylin for about 5 minutes, differentiated in 1% acid alcohol, blued in Scotch's tap water and stained with Eosin for 3 minutes. They were later

rinsed, dehydrated in ascending grades of alcohol: 70%, 90% and 100% v/v, and finally cleared in a pox. Sections of the tissues were viewed using a digital microscope (LEICA DM 750, Germany) and photomicrographs were taken at ×100 and ×400 magnifications (Auwioro *et al.*, 2010).

3.6.2 Special stain using Toluidine Blue Method

The prepared serial sections of the brain tissues were de-waxed then transferred to 95% alcohol. The slides were put in alcoholic colophonium solution for 5 minute (10g colophonium in 105ml 95% alcohol. The slides were then transferred to two changes of 95% alcohol each for 3 minutes, followed by staining in toluidine blue 0.1% for 30 seconds, and then were differentiated in a mixture for 10% analene & 95% alcohol. It was cleared in different changes of Caiput oil, and finally mounted in Canada balsam. Finally, the sections of tissues were viewed using a digital microscope (LEICA DM 750, Germany) and photomicrographs were taken.

3.7 Immunohistochemical Analysis

3.7.1 Peroxidase linked avidin-biotin complex (ABC) method

Tissues were routinely processed through a graded series of alcohols, cleared in xylene and embedded in paraffin. 5μm-thick sections were obtained and processed for immunohistochemical staining which is used for localization of an intracellular calcium binding protein CAL D28K. The staining was carried out by the peroxidase linked avidin-biotin complex (ABC) method. Blocking of endogenous peroxidase activity was carried out with 0.08% hydrogen peroxidase (H_2O_2) in methanol for 5 minutes in order to the block unspecific binding (Sternberger,1986). Sections were incubated with (1:10) normal goat

serum in 0.1 M PBS, pH 7.2 then sections was incubated for 16-20 h at 4 °C in mouse anti-calbindin IgG (Abcam USA, ab1146). The antibody was diluted to 1:500 in PBS containing 0.25% sodium azide and 2.5% bovine serum albumin. Sections were then incubated in biotinylated sheep anti-mouse IgG (Abcam USA, ab11246) followed with streptavidin horseradish peroxidase (therm scientific), both at a dilution of 1:50 in PBS, for 1 h at room temperature. Sections were washed in PBS for 30 minutes. Incubated sections were then immersed in glucose oxidase-DAB-nickel ammonium sulphate (GDN) substrate (Shu *et al.*, 1988) for 10 minutes, washed in distilled water and counterstained with hematoxylin for 3 minutes and cover slip using DPX mountant. Sections were examined with a (DM 750 LEICA, Germany) digital microscope and photomicrographs were taken.

3.8 Statistical Analysis

Data obtained from the morphological and morphometrical analysis were presented as Mean ± Standard error of the mean (SEM.). For statistical evaluation of data, one-way analysis of variance (ANOVA) was used to compare the level of difference between and within the groups followed by Dunnett post- hoc test using SPSS software version 20. Values of $p \leq 0.05$ were considered statistically significant. Charts were produced using Microsoft$^{(R)}$ Excel 2007 for Windows.

CHAPTER FOUR

4.0RESULTS

4.1Vaginal Smear Cytology

The results from the investigation of the oestrous cycle of the 30 female rats are presented in plate's I-V. The proestrous cytology (plate I) consisted of predominately nucleated cells, while the oestrous cytology (plate II) consisted of rhomboidal shaped anucleated cornified cells. The metestrous cytology (Plate III) shows a characteristic of numerous populations of nucleated cells (N), cornified cells (C) leukocyte (L). The diestrous phase (plate IV) was characterized by a thin smear that consisted of few leucocytes, few nucleated cells and few shrinking anucleated cornified cells, while (plate V) shows the presence of a brownish plug obtained the following morning confirmed coitus, and the sperm positive day was designated as day zero of gestation and (plate VI) showed the diestrous phase of the oestrous cycle implied that the rat's regular 4-day cycle has been altered as a result of pregnancy.

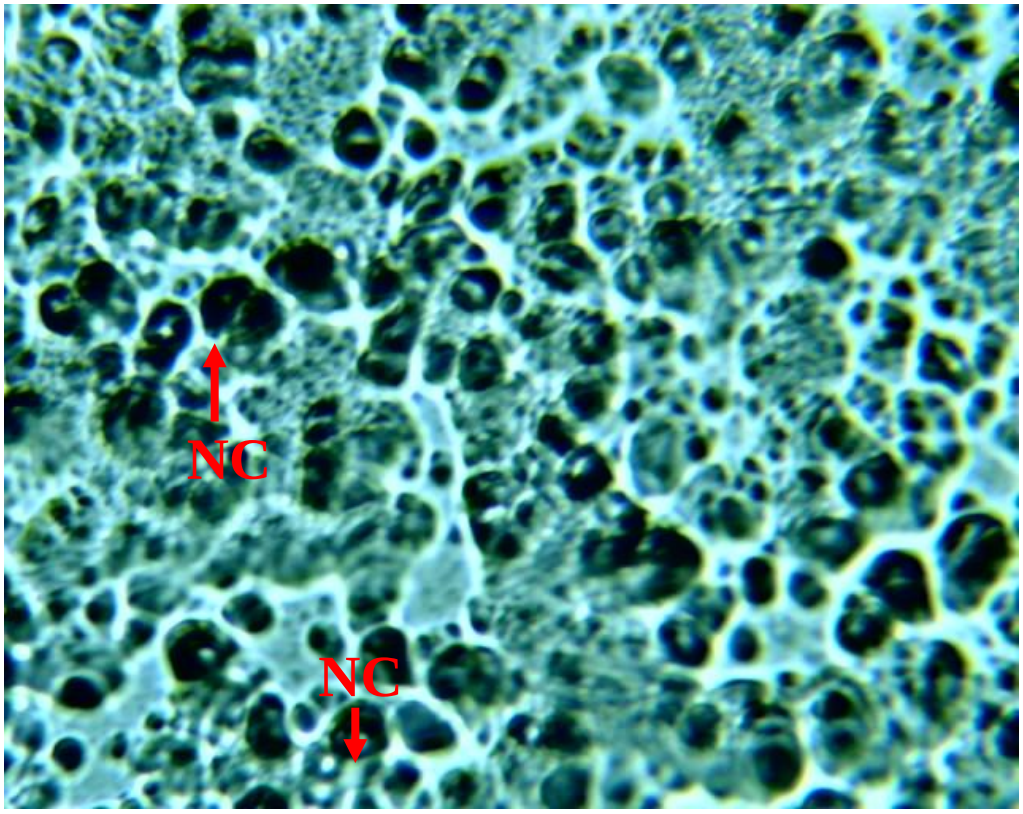


PLATE I: A photomicrograph of the late proestrous smear showing numerous Round nucleated cells (NC) (Mag×250)



PLATE II: A photomicrograph of oestrous smear showing rhomboidal shaped anucleated cornified cell (C) (Mag×250)

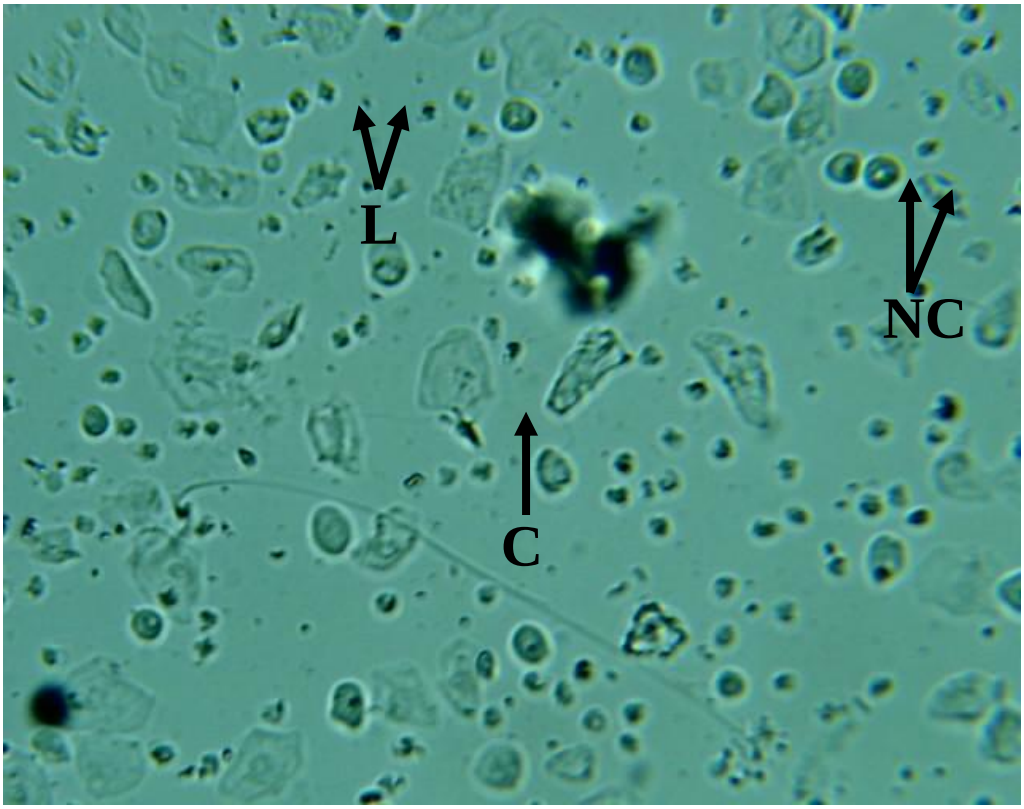


PLATE III: Photomicrograph of metestrous smear showing a characteristic numerous population of nucleated cells (NC), cornified cells (C) leukocyte (L), (Mag×250).

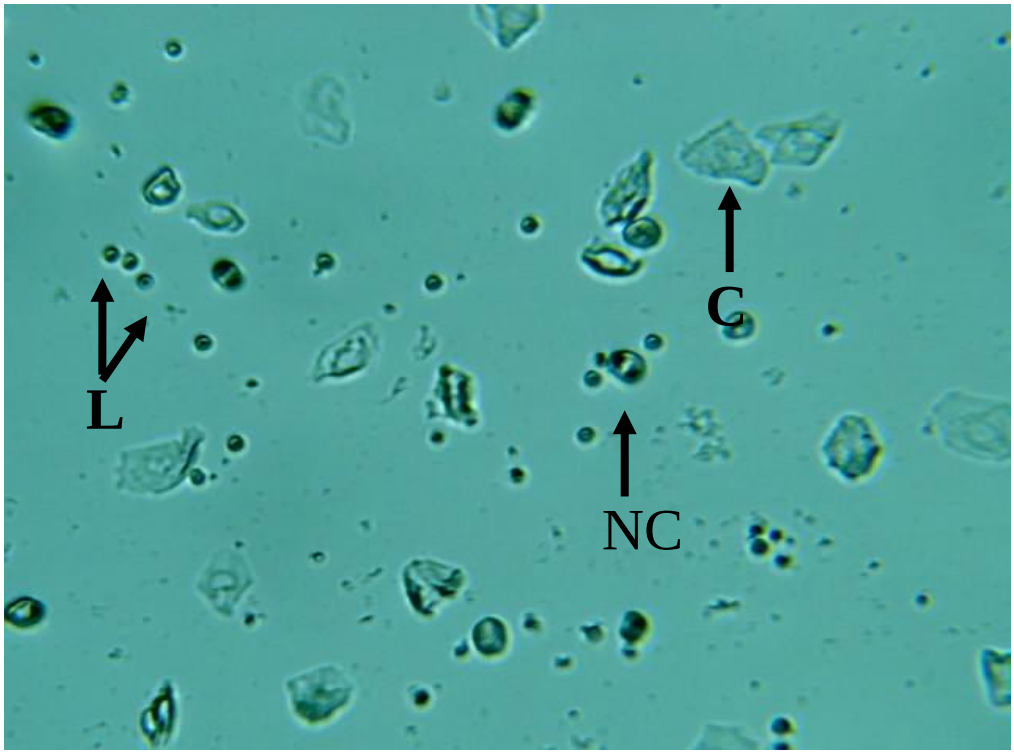


PLATE IV: Photomicrograph of Diestrous smear showing a characteristic thin smear with a scanty population of nucleated cells (NC), cornified cells (C) and Leukocyte (L) (Mag×250).

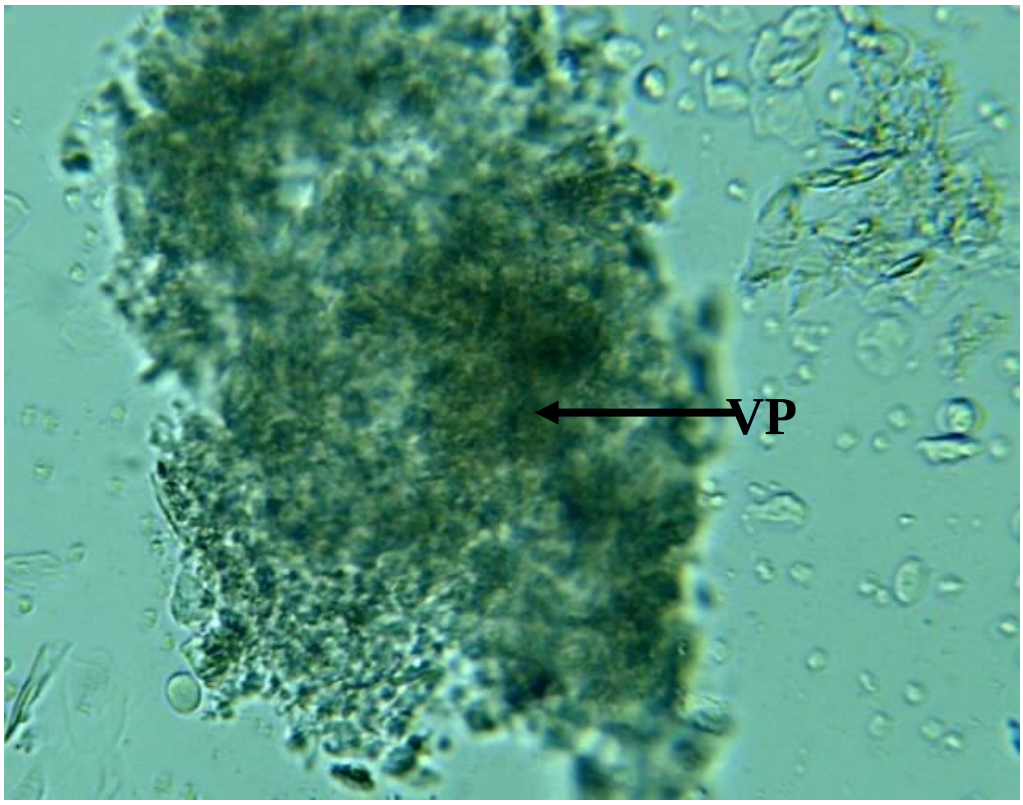


PLATE V: Photomicrograph of the estrous smear showing a vaginal plug (VP) in a pregnant Wistar rat (Mag×250).

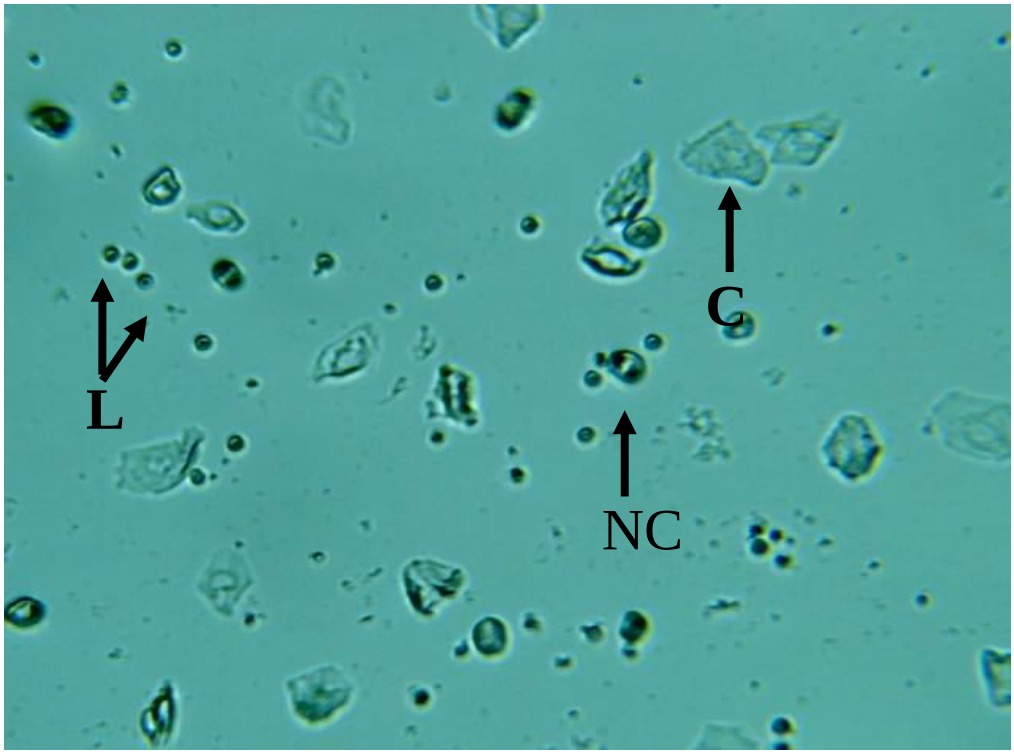


PLATE VI: Photomicrograph of Diestrous smear showing a characteristic thin smear with a scanty population of nucleated cells (N), cornified cells (C) and leukocyte (L) confirms pregnancy (Mag×250).

4.2 Morphological Studies

4.2.1 Gross anatomy of pups

The results on the morphology of the pups in the control group (Group A) showed a normal body structure with no visible physical change and no anomaly. Some pups in (Group B) showed a mild dark spot on the neck region (cervico-thoracic) with no major gross anomaly. Pups in the high dose group (Group C) showed a degree of gross anomaly, there was an incidence of craniofacial malformations especially around the face. The eyes were closed which signifies blindness and there was a prominent dark spot on the face and both limbs which signifies haemorrhage. The limbs and tail looks shorter when compared with the control pups.



PLATE VII: Group A pup of postnatal day1 showing normal body structure without any gross anomaly**.**



PLATE VIII: Group B pup of postnatal day1 showing mild dark spot (DS) on the cervical region (haemorrhage).



PLATE IX: Group C pup of postnatal day1 shows an incidence of craniofacial malformation with prominent dark spot on the limbs (haemorrhage).

4.1.2 Number of litter/litter size

There was a reduction in the number of litters delivered in the artesunate treated animals when compared with control groups. The number of litters delivered differs significantly in the groups. In the control (Group A) the litter number was between ten to nine (10-9). In (Group B) that was administered artesunate 4mg/kg for three days and 2mg/kg 4days, the number of litters delivered ranges from five to seven (5-7) while in (Group C) that received high dose of the drug reduced to two (2) with severe embryonic loss also known as resorption.

Table 4.1:Effect of Artesunate on the mean Litter size

Treatment	N	Mean Litter Number	Embryonic Loss
Control Group	10	9.10±0.18	_____
Therapeutic Dose	10	5.80±0.33***	+++
High Dose	10	2.00±0.00***	+++++

Values are presented as mean ± S.E.M., *** = $p<0.001$ compared to control – One-way ANOVA followed by Dunnett's post hoc test, n= Number of pregnant rat in a group =10

4.2 Morphometrical Analysis Results

4.2.1 Foetal Parameters

4.2.1.1 Foetal Weight

There was a significant change (reduction) in the foetal weight (g) in group B pups at postnatal days PD1 and PD 21 (4.78 ± 0.1 6 and 14.33 ± 0.16) and in group C at PD1 (4.54±0.17) respectively when compared to the control groups. There was also a significant ($p<0.05$) increase at PD 7 (11.10 ± 0.20) when compared to the control group (9.97 ± 0.24). Changes observed in GD 20, PD 4, PD 14 and PD 28 were statistically non-significant.

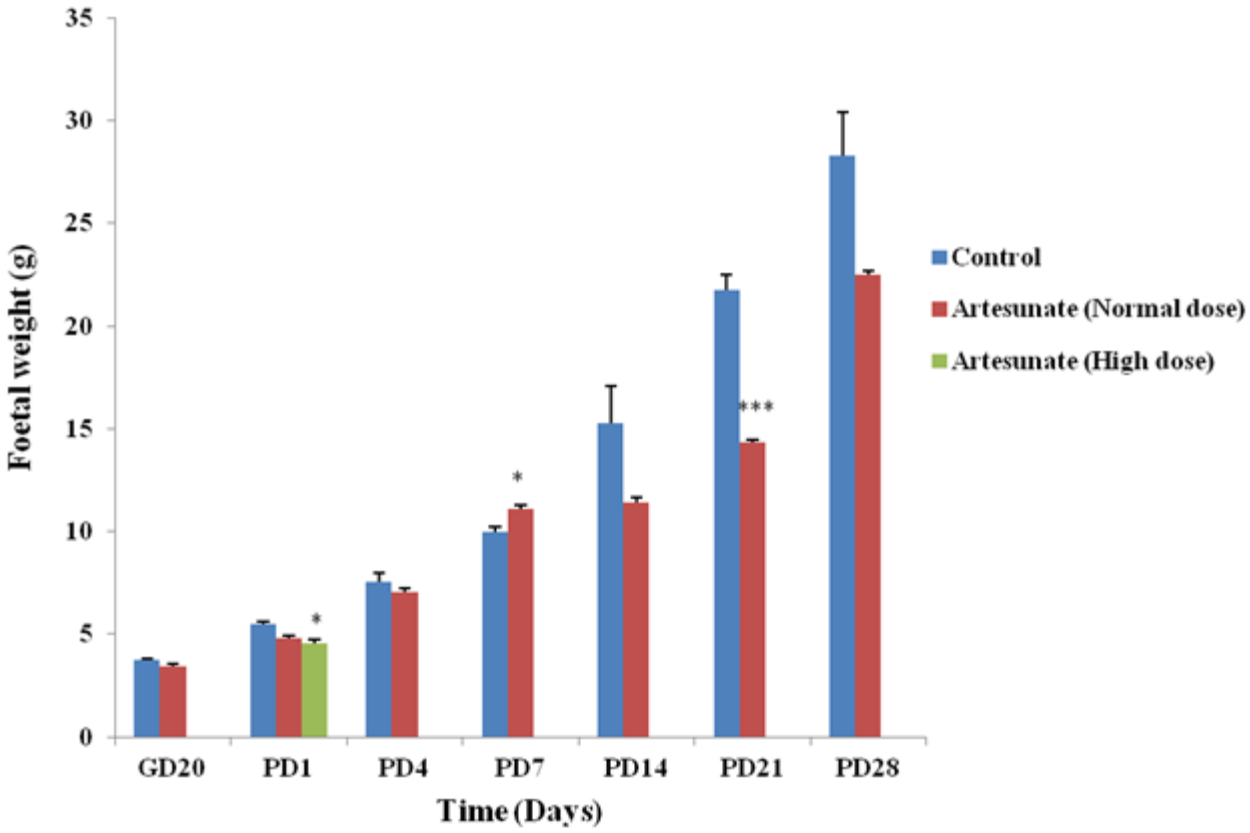


Figure 4.1: Effect of Artesunate on Foetal weight, * = $p<0.05$, ***= $p<0.001$ – One-way ANOVA followed by Dunnett post hoc test, n= 5, GD = gestation day, PD = postnatal day.

4.2.1.2 Brain Weight

The Brain weight (g) of pups in group B at PD 7 (0.45 ± 0.01), PD 14 (0.55 ± 0.01), PD 21 (0.75 ± 0.01) PD 28 (0.99 ± 0.02) showed a significant decrease ($p<0.05$) when compared to their various control groups (0.51 ± 0.01), ((0.92 ± 0.03), (1.02 ± 0.01), (1.09 ± 0.02).There was no significant difference at GD 20, PD1 and PD4 and in group C at PD 1 pups (0.18±0.01) respectively

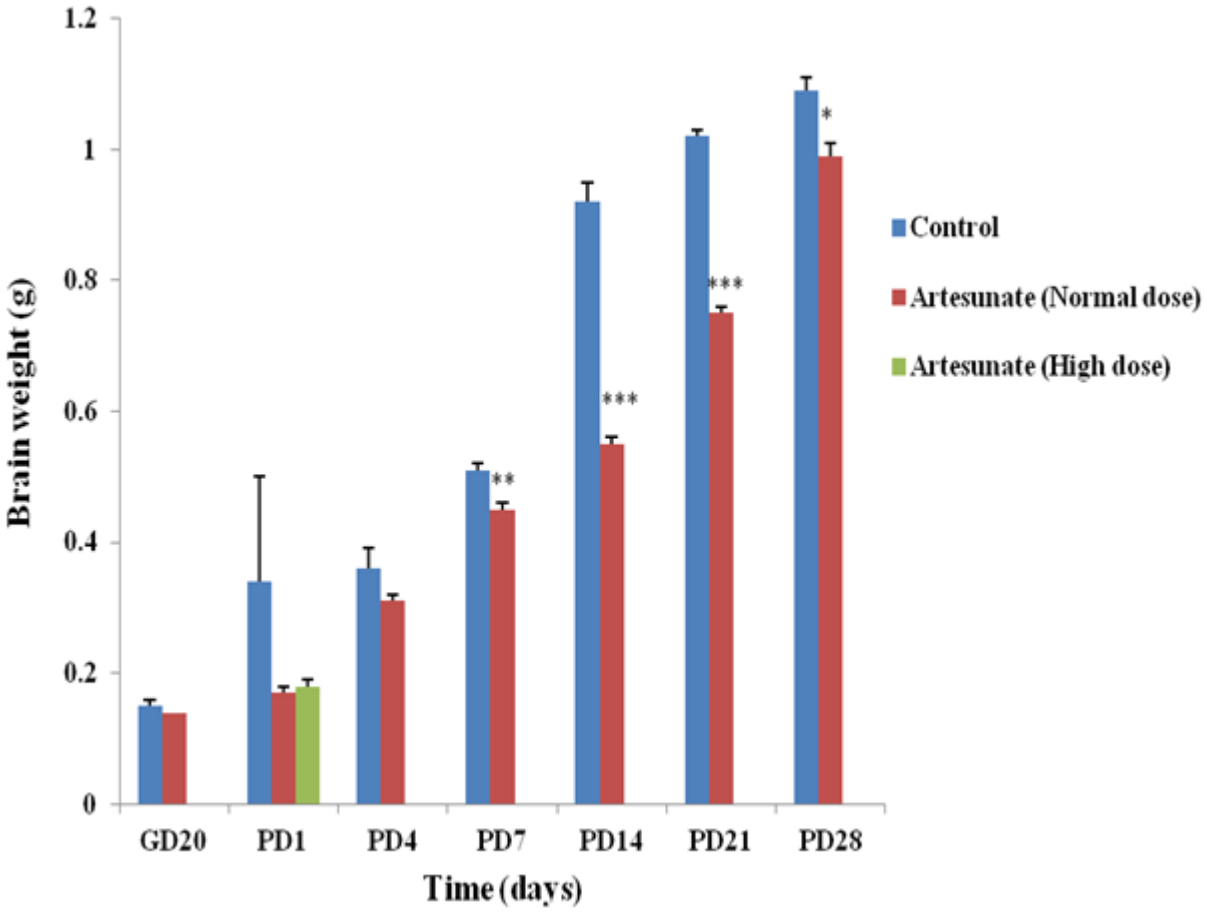


Figure 4.2: Effect of Artesunate on Brain weight of pups * = $p<0.05$, ** = $p<0.01$, ***= $p<0.001$ One-way ANOVA followed by Dunnett post hoc test, n= 5, GD = gestation day, PD = postnatal day

4.2.1.3 Brain Length

There was a significant decrease in group B at postnatal PD 14 (1.71 ± 0.01) and PD 21 (1.93 ± 0.03) when compared to the control (1.90 ±0.00 and 2.10 ±0.35) groups. The brain length in group B, GD 20 (0.93 ±0.02) PD 1 (0.77 ±0.17) PD 4 (1.14 ± 0.02) PD 7 (1.53 ± 0.07) and PD 28 (2.11 ± 0.06) group C, PD 1 (0.5±0.40) showed no significant changes observed in the brain length (cm) respectively.

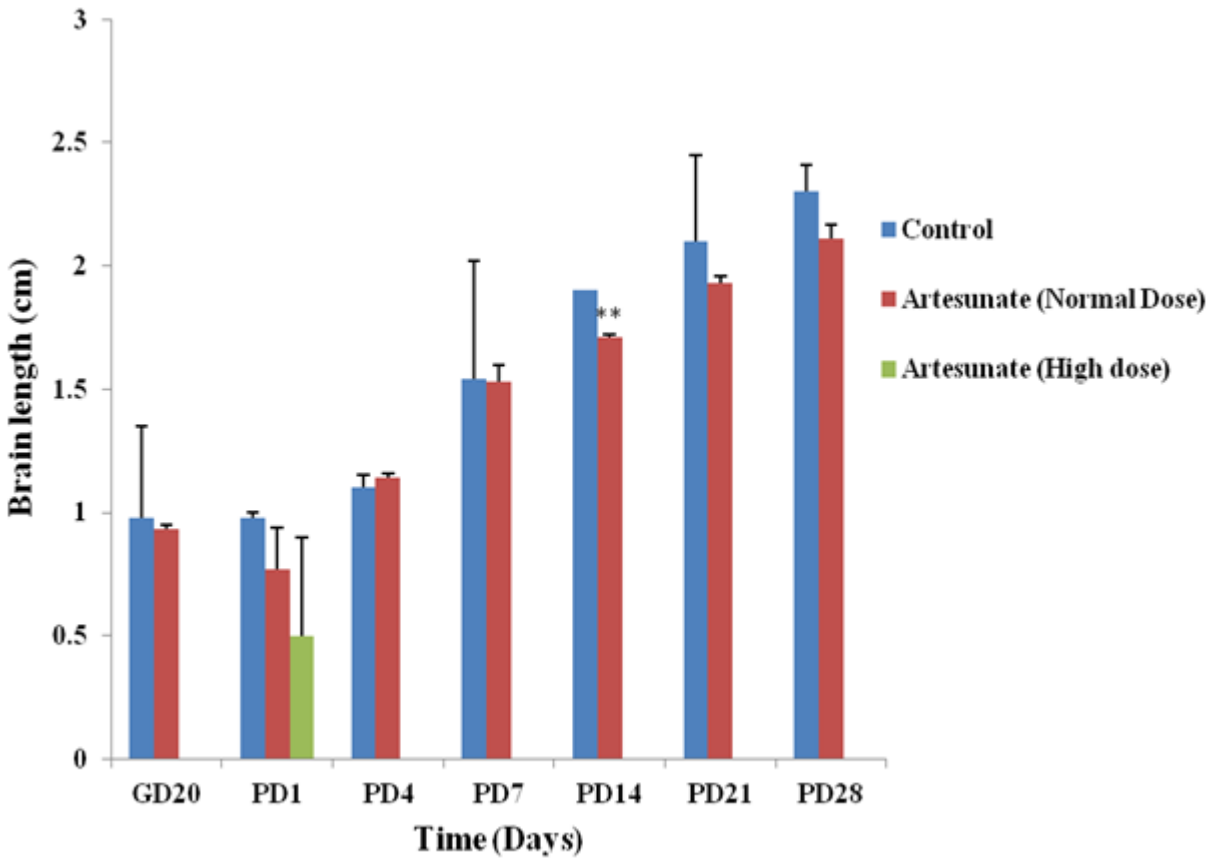


Figure 4.3: Effect of Artesunate on the Brain length of pups ** = p<0.01, ***= p<0.001 – One-way ANOVA followed by Dunnett post hoc test, n= 5, GD = gestation day, PD = postnatal day.

4.2.1.4 Brain Width

The Brain width (cm) in group B pups at postnatal days PD 4 (0.83 ± 0.01) PD 7 (0.93 ± 0.01) PD 14 (1.00± 0.00), and PD 21 (1.08 ± 0.02) showed a significant decrease while pups in group B animals that were treated with 4mg/kg for 3 days and 2 mg/kg for 4 days of artesunate showed no significant difference at their respective groups when compared to the control groups of PD 4 (0.89 ± 0.01), PD 7 (1.00 ± 0.02), PD 14 (1.15 ± 0.02) and PD 21 (1.22 ± 0.01) respectively, while (0.58 ± 0.02) in GD 20, (0.60 ± 0.16) in PD 1 (1.21 ± 0.01) in PD 28, and in group C, PD1 (0.55±0.00).

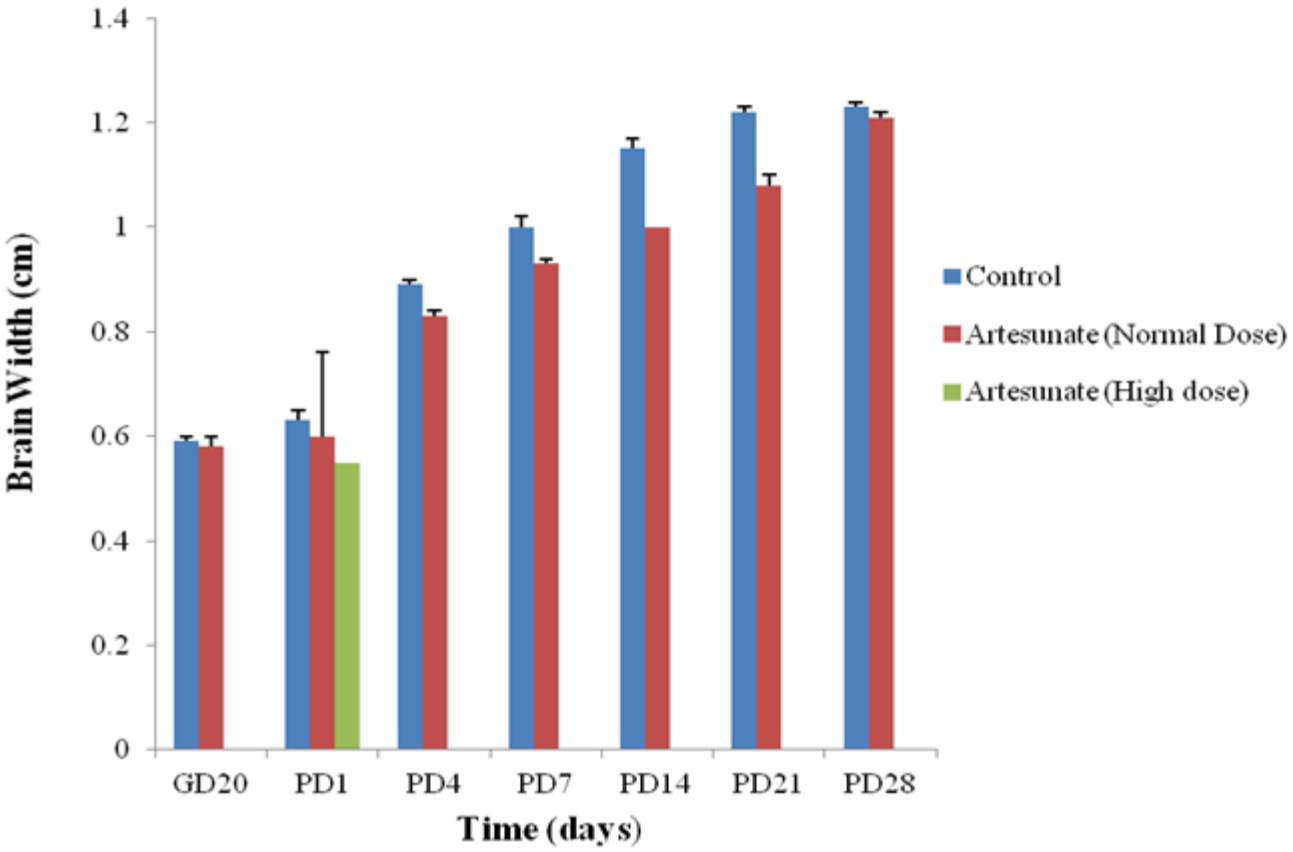


Figure 4.4: Effect of Artesunate on Brain width of pups * = $p<0.05$, ** = $p<0.01$, ***= $p<0.001$ – One-way ANOVA followed by Dunnett post hoc tests, n= 5, GD = gestation day, PD =postnatal day.

4.2.1.5 Forelimb Length

The forelimb length (cm) in group B pups at postnatal days PD1, 7, 14 and 21 (1.16 ± 0.01, 2.18 ±0.25, 2.52 ± 0.04, 2.69 ± 0.01) and also in group C at PD 1 (0.95±0.05) were statically significant decrease when compared to the control (1.31 ± 0.19, 2.36 ± 0.04, 3.10 ± 0.21, 3.82 ± 0.06) groups respectively. But in prenatal day GD20 (1.18± 0.02), postnatal days PD4 (1.78 ± 0.02), PD28 (3.22 ± 0 .05) showed no significance when compared to the control groups.

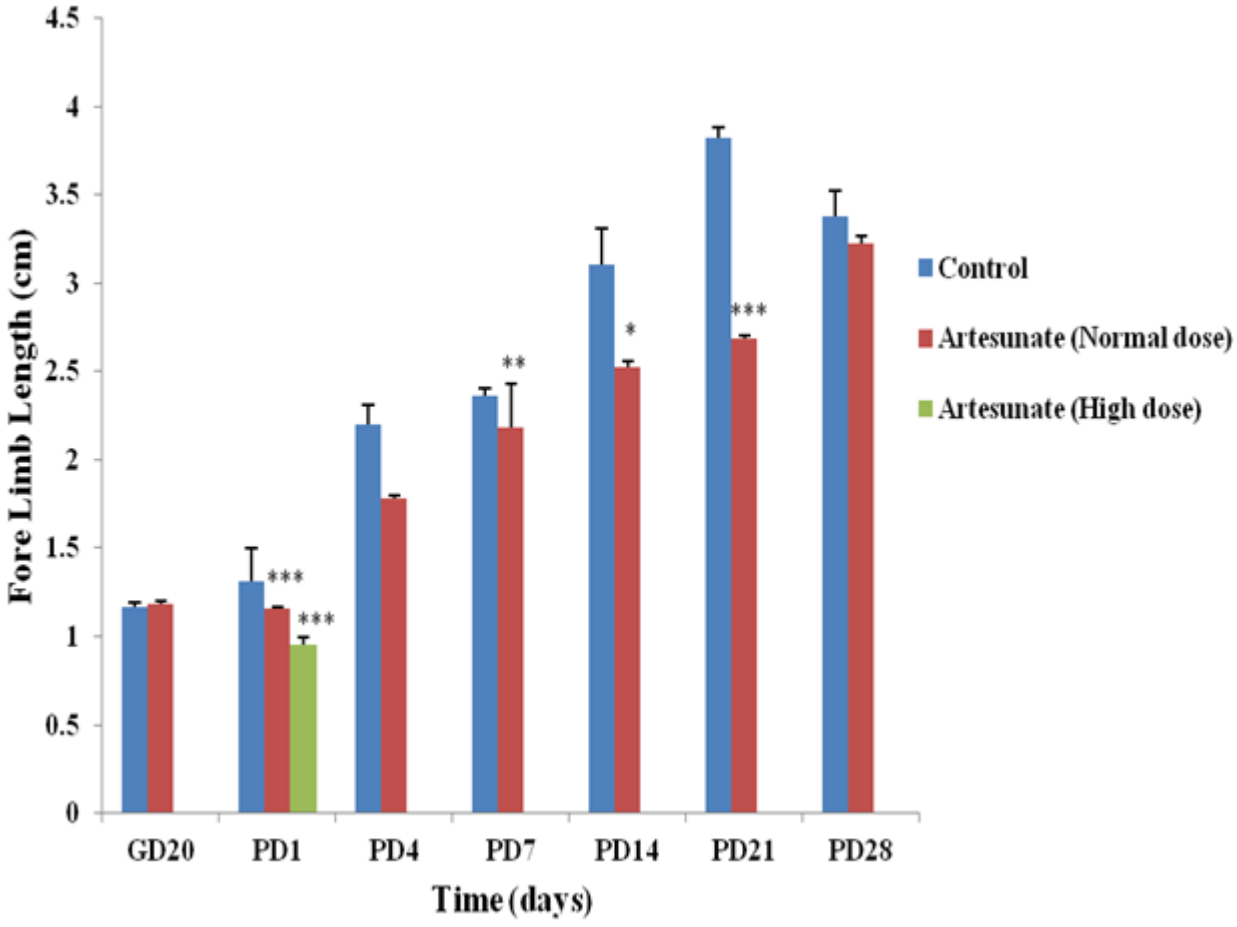


Figure 4.5: Effect of Artesunate on Fore limb length of pups * = $p<0.05$, ** = $p<0.01$, ***= $p<0.001$ – One-way ANOVA followed by Dunnett post hoc test, n= 5, GD = gestation day, PD = postnatal day.

4.2.1.6 Hind Limb Length

Hind limb length (cm) were significantly lower in group B pups at postnatal days PD1 (1.16 ± 0.01), PD7 (2.18 ±0.25), PD14 (2.52 ± 0.04), and PD21 (2.69 ± 0.01) when compared to the control (1.31 ± 0.19, 2.36 ± 0.04, 3.10 ± 0.21, 3.82 ± 0.06) groups respectively. While at prenatal day GD20 (1.18± 0.02), postnatal days PD4 (1.78 ± 0.02), PD28 (3.22 ± 0 .05) and in group C, PD 1 (1.40±0.00) showed no significance when compared to the controls.

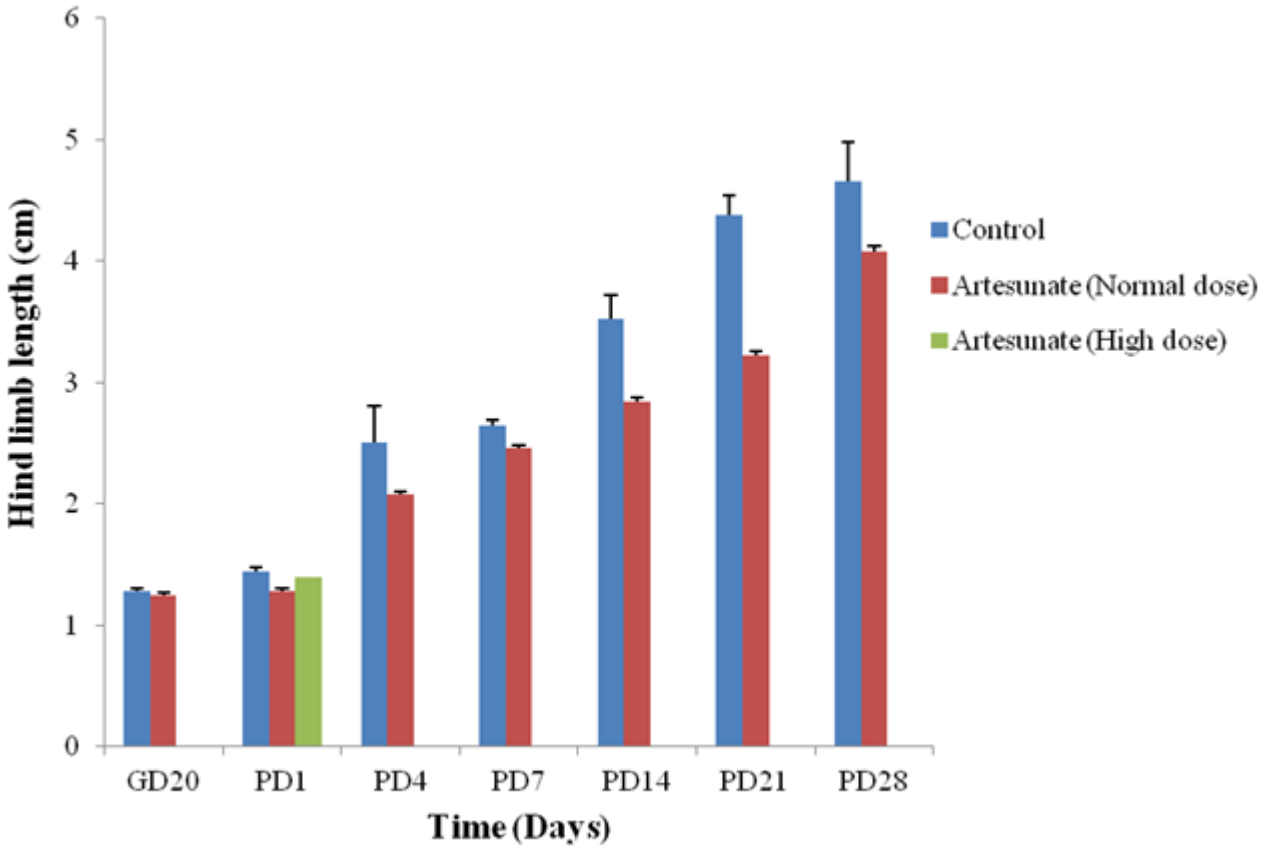


Figure 4.6: Effect of Artesunate on Hind limb length of pups * = $p<0.05$, ** = $p<0.01$, ***= $p<0.001$ – One-way ANOVA followed by Dunnett post hoc test, n= 5, GD = gestation day, PD = postnatal day.

4.2.1.7 Tail Length

The tail length (cm) in group B at PD1 (1.16 ± 0.01), PD 4 (2.18 ±0.25), PD 14 (2.52 ± 0.04), PD 21 (2.69 ± 0.01) PD28 (3.22 ± 0 .05) and in group C, PD1 (1.40±0.00) were significantly reduced than the corresponding values in the control groups (1.62±0.04, 2.64 ±0.13, 5.33±0.36, 6.92±0.15, 7.96 ± 0.37) respectively. There was no significant change in GD20 (1.36±0.02) and PD4 (2.85 ± 0.03) when compared to the control groups.

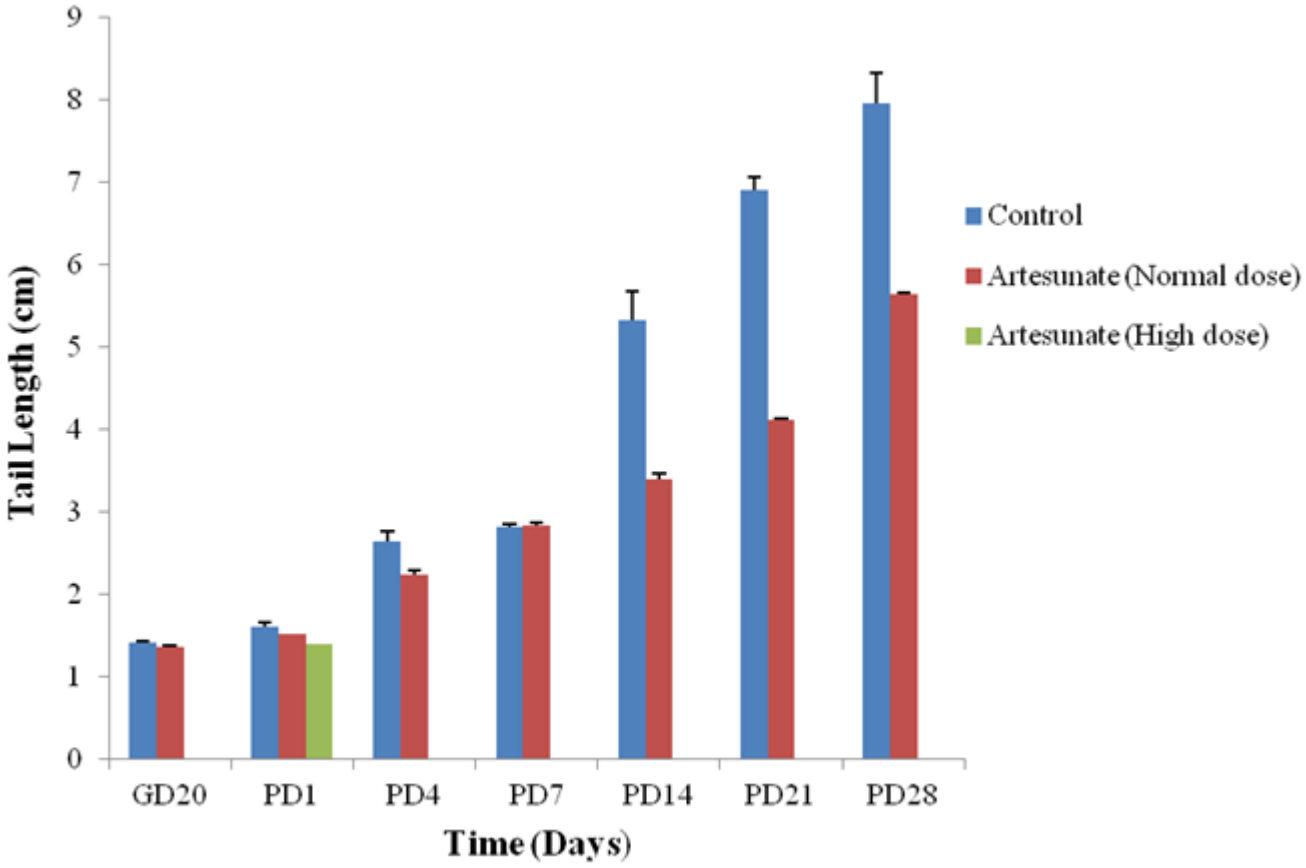

Tail Length (cm)
0
1
2
3
4
5
6
7
8
9
GD20
PD1
PD4
PD7
PD14
PD21
PD28
Time (Days)
Control
Artesunate (Normal dose)
Artesunate (High dose)

Figure 4.7: Effect of Artesunate on Tail length of pups * = $p<0.05$, ** = $p<0.01$, ***= $p<0.001$ – One-way ANOVA followed by Dunnett post hoc test, n= 5, GD = gestation day, PD = postnatal day

4.3 Microscopic Examination of Tissues

4.3.1 Histological Findings

The results of the histological changes using H&E showed differences in layer stratification in the cerebral cortex. The pups in control (group A) consisting of gestation day (GD20), and pups at postnatal day (PD 1) consist of three layers of the developing cerebrum (Plate X) Likewise in postnatal days (PD) 4 and PD 7 is characterize by four layers (Plate XII) with immature pyramidal cells. Pups at PD 14, PD 21 and PD 28 showed complete developed layer stratification (six layers) of the cerebral cortex. They have normal histology of the developing cerebral cortex (Plate XIV) while pups in (group B) that received the therapeutic dose of artesunate showed decrease in cell proliferation with marked difference in the layer stratification. There was a form of neurodegeneration such cytoplasmic vacuations loss of stratification of the developing layers of the cerebral cortex compared to control (Plates XI, XIII and XV).

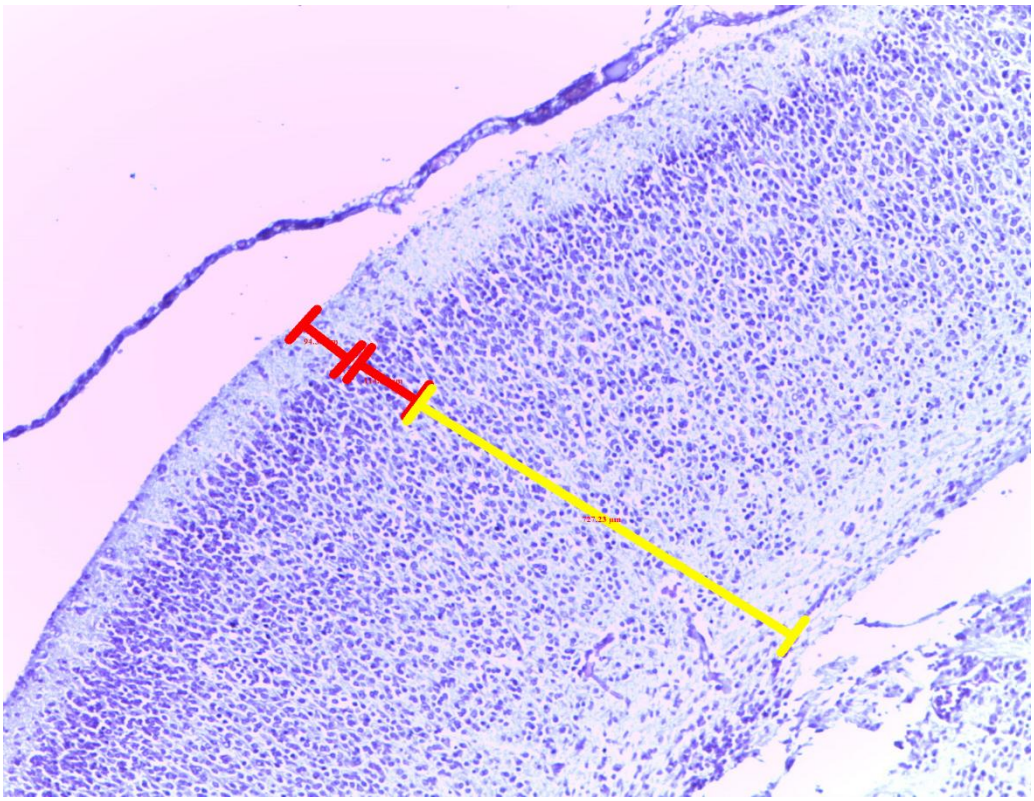


PLATE X: A transverse section of the developing cerebral cortex of pup at GD20 and PD1 Showing three layers (3) of the cerebral cortex (H and E ×100)

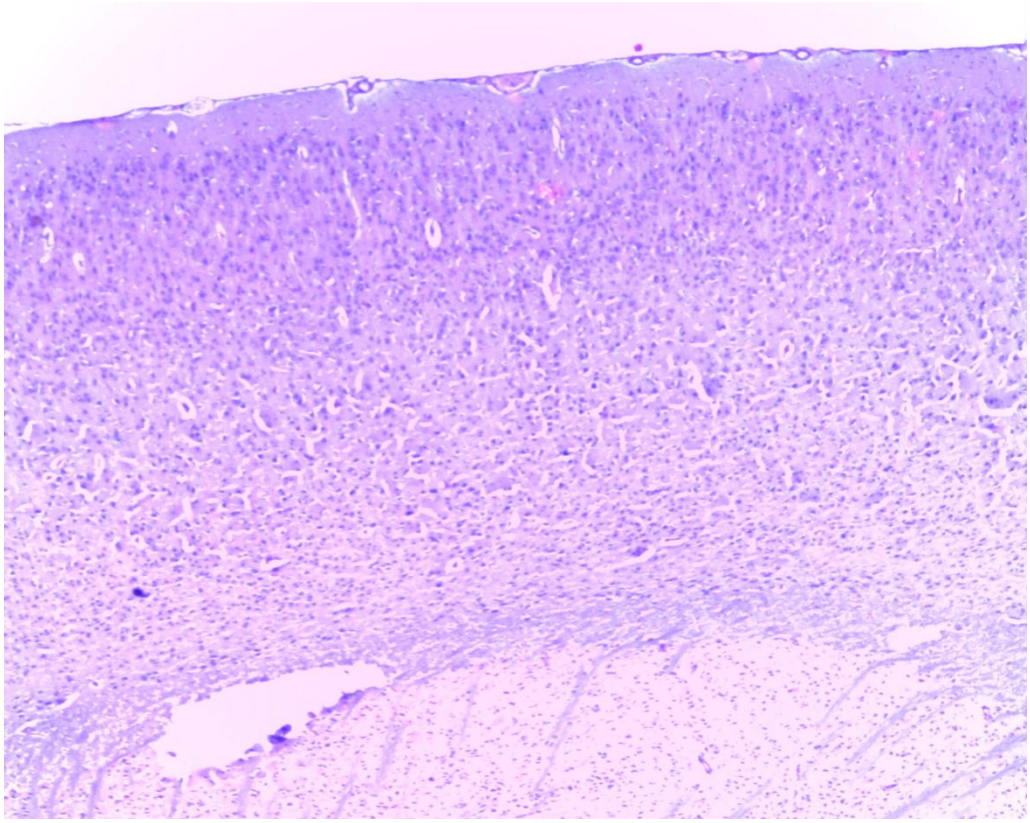


PLATE XI: A transverse section of the developing cerebral cortex of pups at GD20 and PD1 Showing loss of layer stratification and some vacuolation due to exposure to artesunate (H and E ×100)

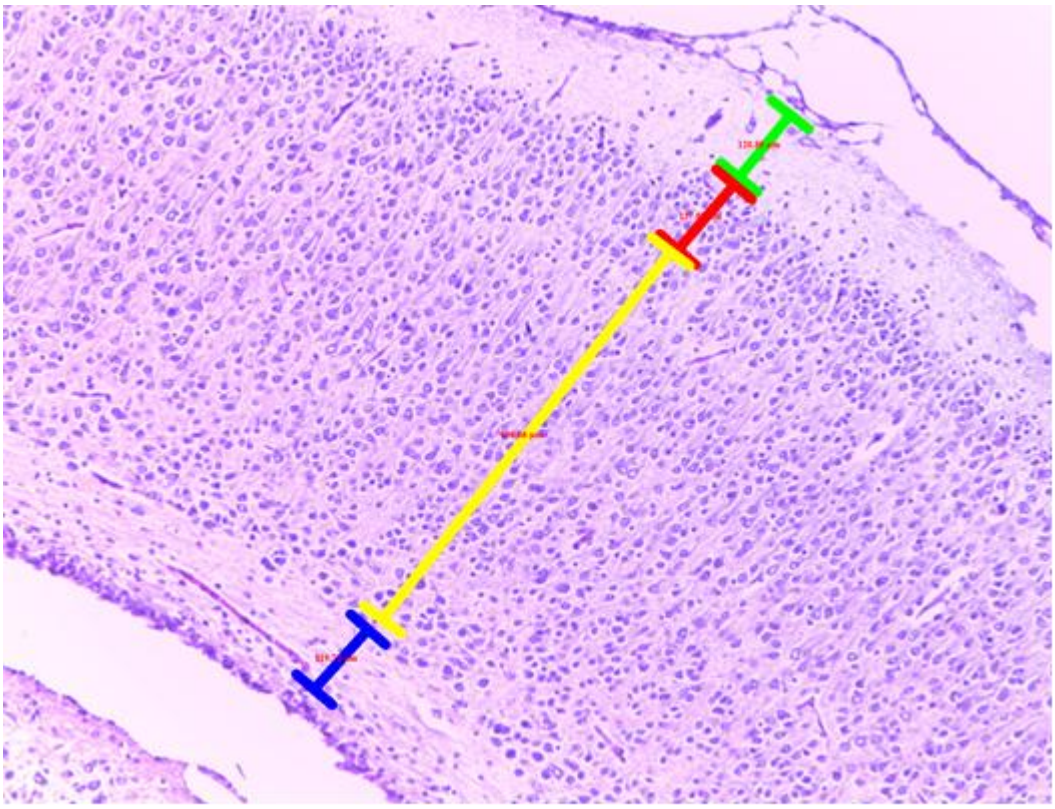


PLATE XII: A transverse section of the developing cerebral cortex of pups at PD4 and PD7 Showing four (4) layers of the cerebral cortex, green- molecular layer red- external granular Layer, yellow-external pyramidal layer, blue-internal granular Layer (H and E ×100).

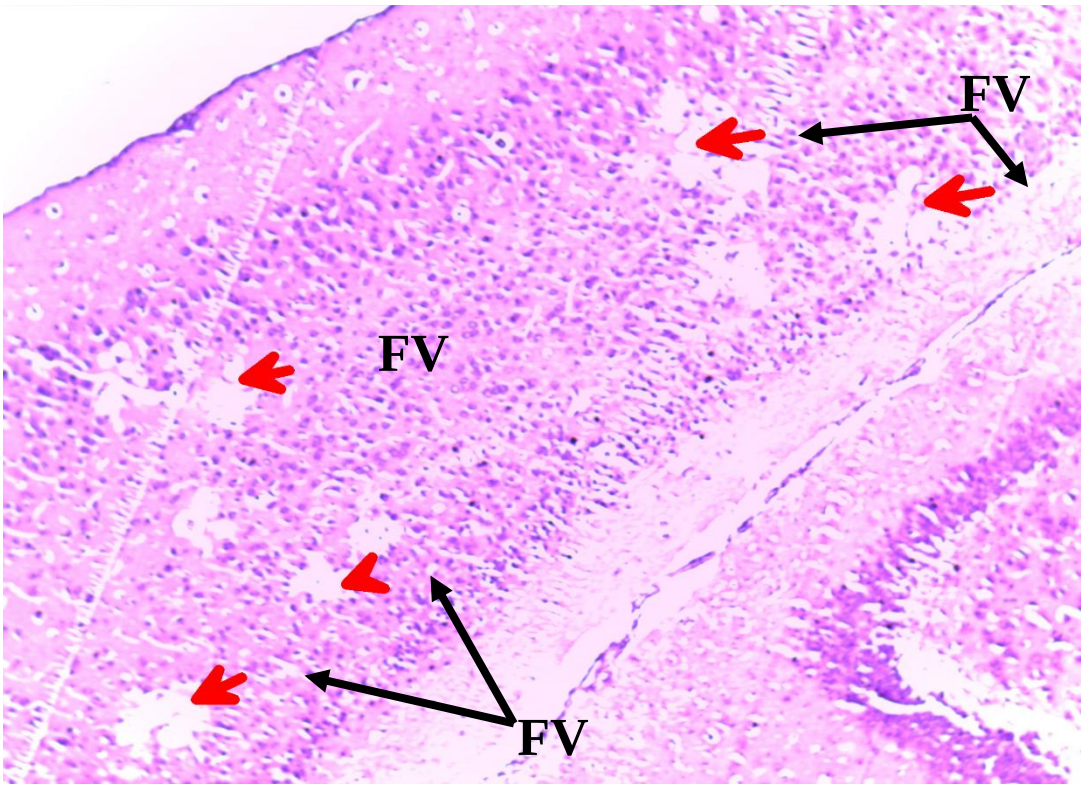


PLATE XIII: A transverse section of the developing cerebral cortex of pups at PD4 and PD7 showed an enlargement of the molecular layer with a remarkable Fats vaculoations (FV) mostly on the external pyramidal layer of the cortex (H and E ×100).

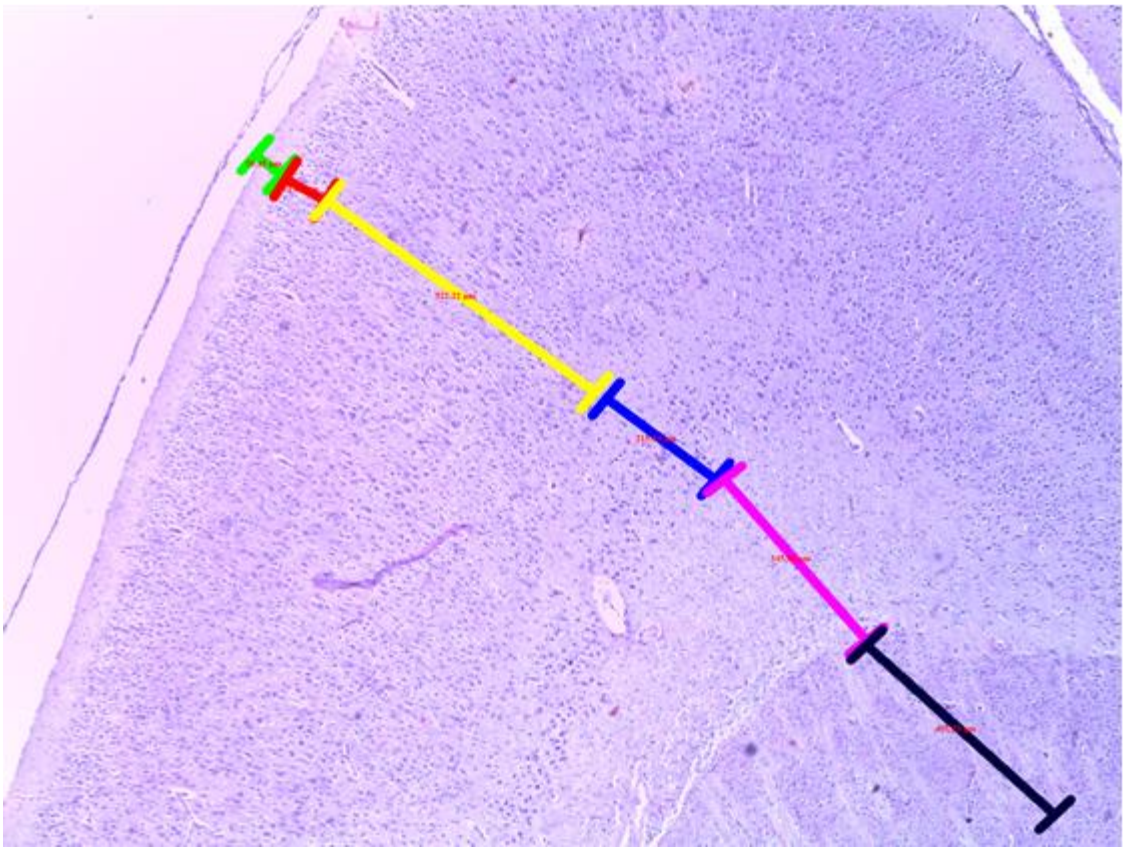


PLATE XIV: A transverse section of the developing cerebral cortex of pups at PD14, PD21and PD28 Showing a well developed six cortical layers of the cerebral cortex Green-

molecular layer, red- external granular Layer, yellow-external pyramidal layer, blue-internal granular Layer, pink- internal pyramidal layer and black- polymorpic or multiform layer (H and E ×100).

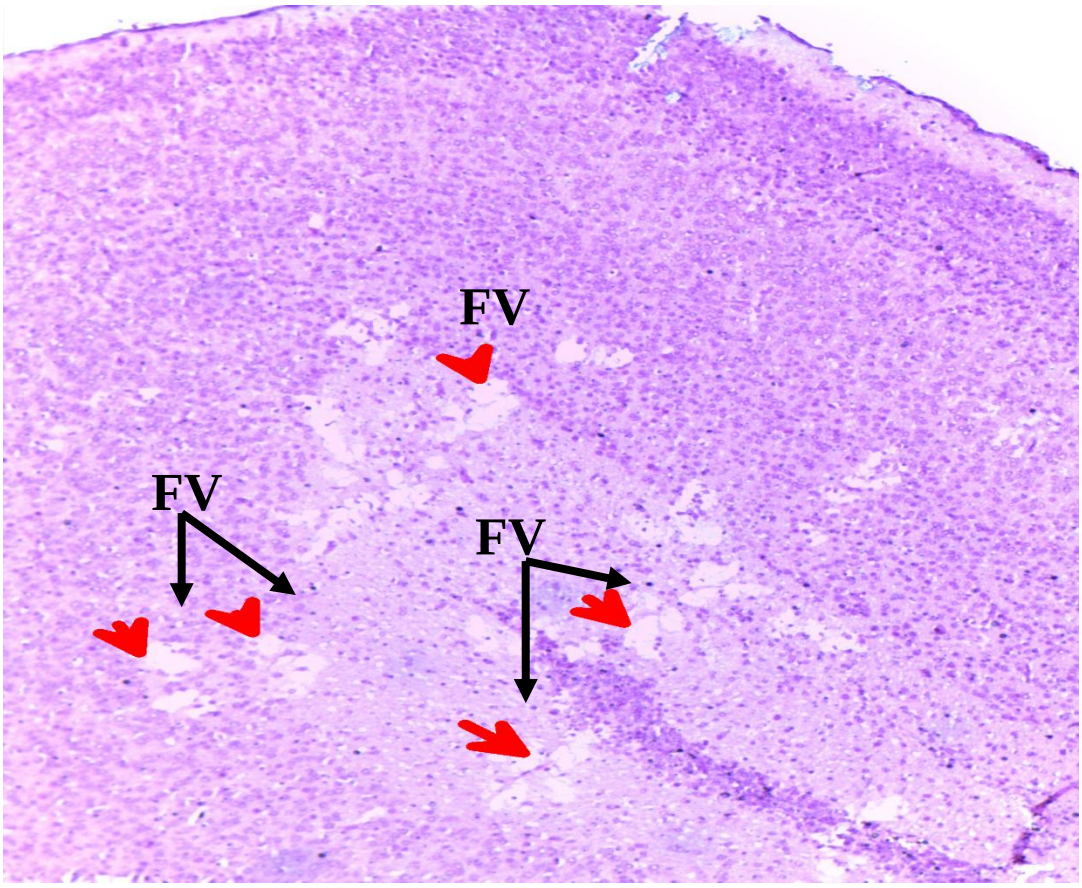


PLATE XV: A transverse section of the developing cerebral cortex of pup at PD14, PD21 and PD28 showed thinning of the molecular layer with remarkable (FV) Fats vaculoations mostly on the internal granular and pyramidal layers of the Cerebral cortex (H and E ×100).

4.3.2 Cerebrum

The results from the histological changes using H and E stain showed animals in the control (group A) to have a normal architecture of the developing pyramidal cells which are immature with absence of arborisation at post-natal days PD 0-4 of the cerebral cortex as shown in (Plate XVI) while pups in (group B) at PD 0-4 respectively showed some area of neuronal degeneration such as degenerated cortical areas with some normal pyramidal cells (Plate XVII). The animals in group C of the corresponding days that received high dose of artesunate showed a more severe form of neurodegeneration characterize by cellular degeneration, cytoplasmic vacuolation, clumping of cells with neuronal aggregation in some areas (Plate XVIII). At postnatal days PD7 and PD14 in control group A showed more prominent pyramidal cells with arborisation as development progressed (plate XIX). Group B of the corresponding post-natal days showed some normal pyramidal cell with neuronal degeneration such as degenerated and pyknotic pyramidal cells (plate XX). while pups in control group A at PD21 and PD28 showed a matured pyramidal cells with normal architecture of the pyramidal cells as development progressed (plate XXI) but group B of the same days showed numerous areas of cellular degeneration, vacuolations, degeneration and loss of neurons in this section as shown in (Plate XXII).

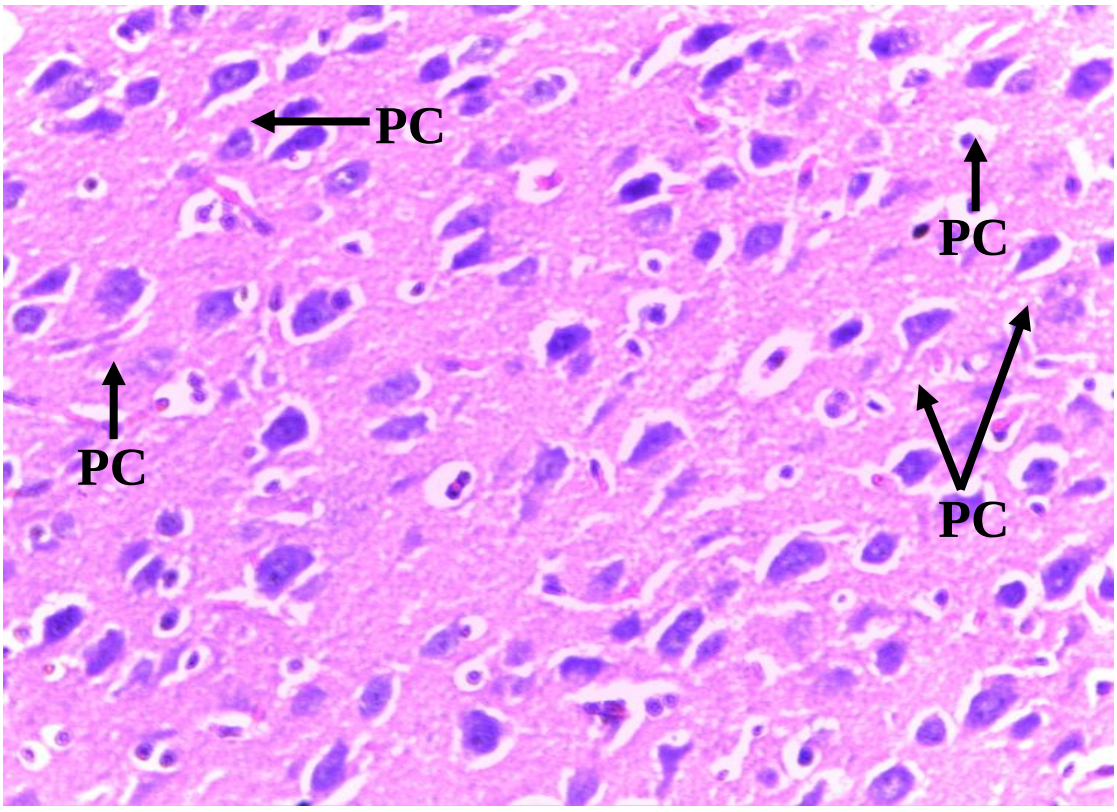


PLATE XVI: A transverse section of the developing cerebral cortex of group A pup at GD20- PD4 showing numerous normal pyramidal cell (PC) (H and E ×400).

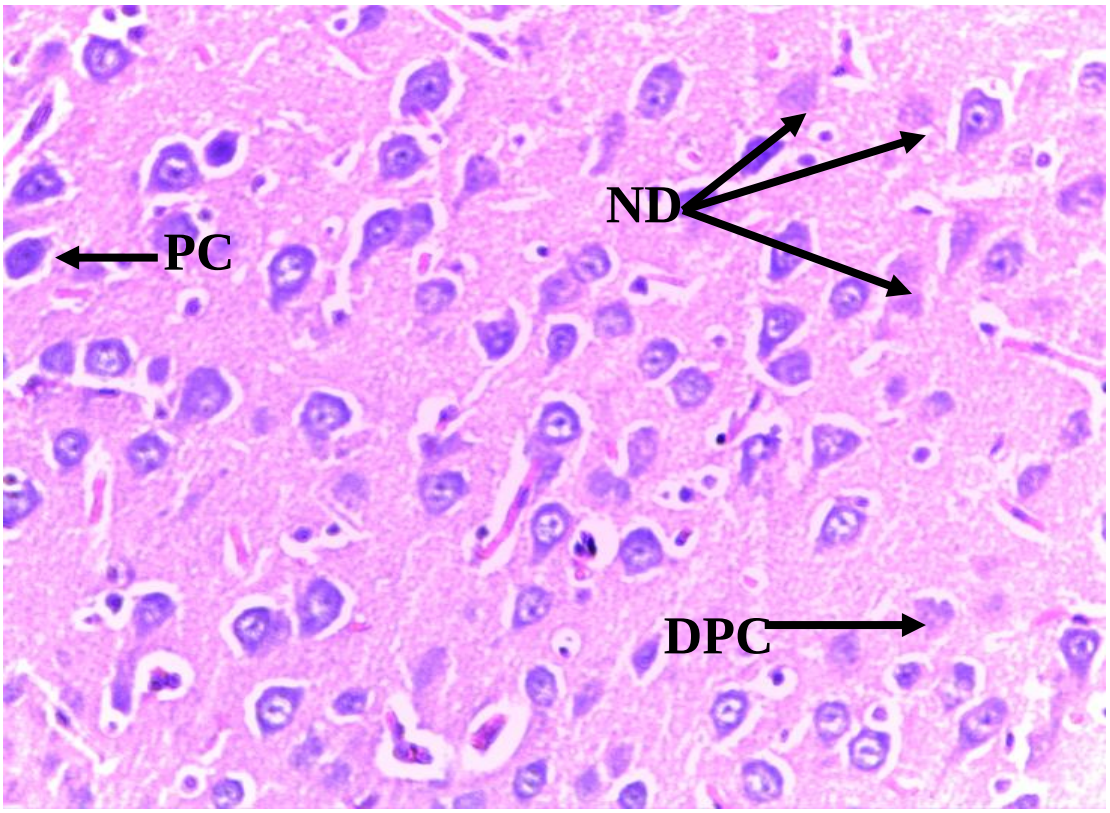


PLATE XVII A transverse section of the developing cerebral cortex of Group B at GD20-PD4 showing some normal pyramidal cell (PC) neuronal degeneration (pyknosis) (ND) with degenerated pyramidal cells (DPC) (H and E ×400).

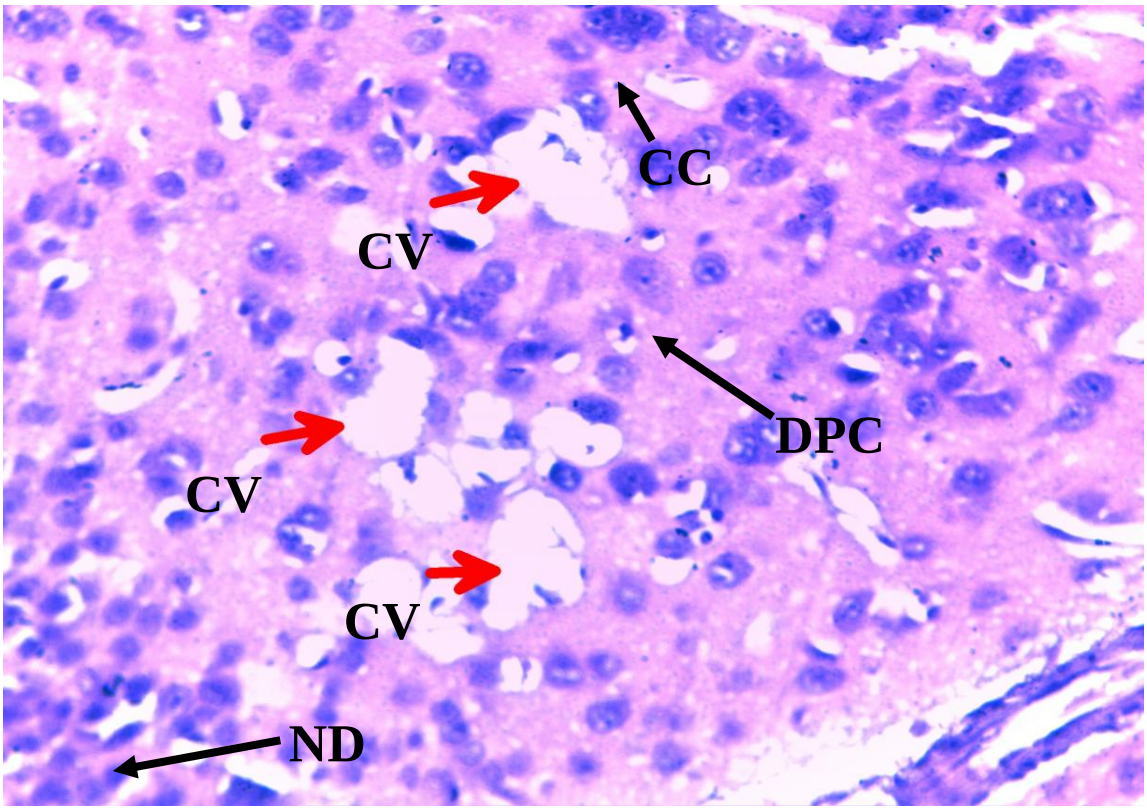


PLATE XVIII A transverse section of the developing cerebral cortex of group C pups at GD20- PD4 showing neuronal degeneration (ND) degenerated pyramidal cells (DPC) cytoplasmic vacuolation (CV) and clumping of cells (CC) (H and E ×400).

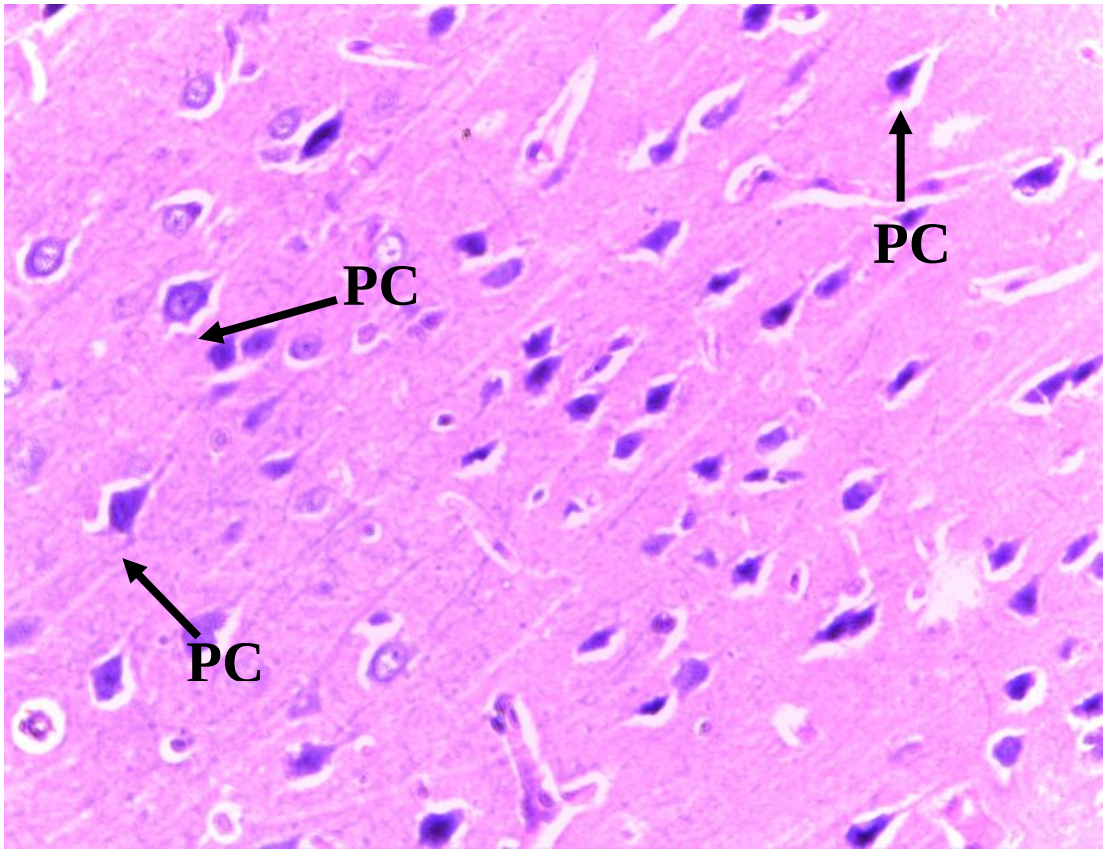


PLATE XIX: A transverse section of the developing cerebral cortex of group A Pups at PD7 & PD14 showing numerous pyramidal cells (PC) without vivid degeneration

present (H and E ×400).

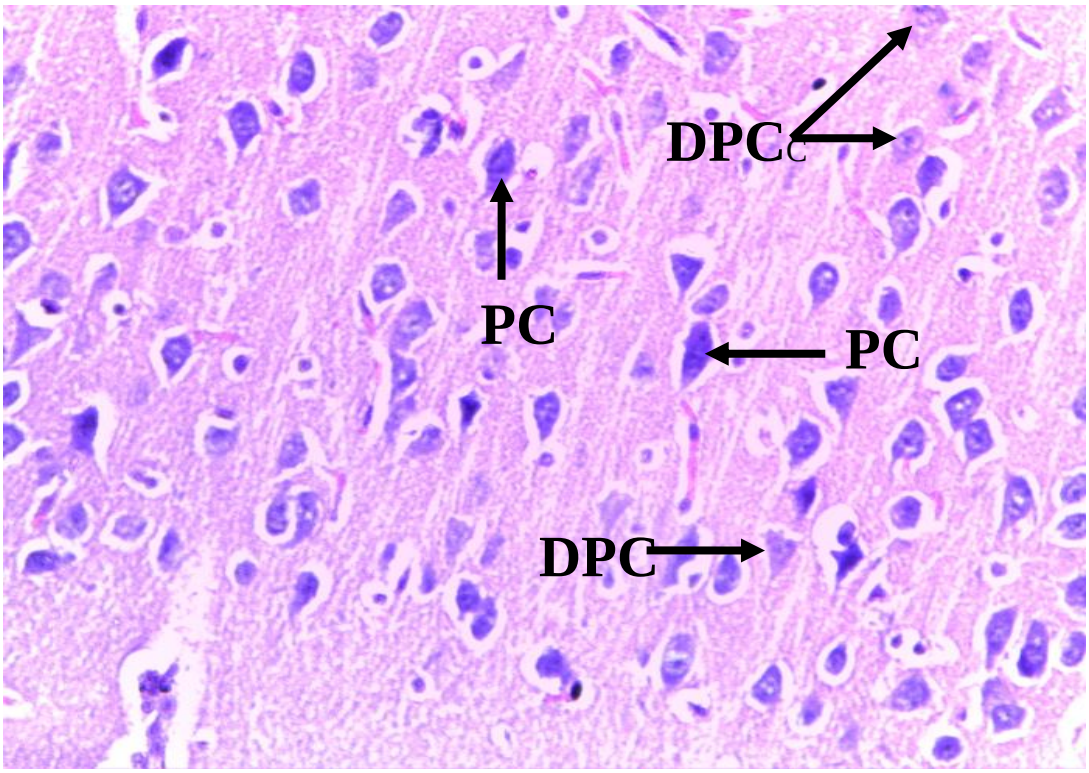


PLATE XX: A transverse section of the developing cerebral cortex of group A Pup at PD7 & PD14 showing normal pyramidal cells (PC), area of neurodegeneration (pyknosis) and degenerated pyramidal cells (DPC) (H and E ×400).

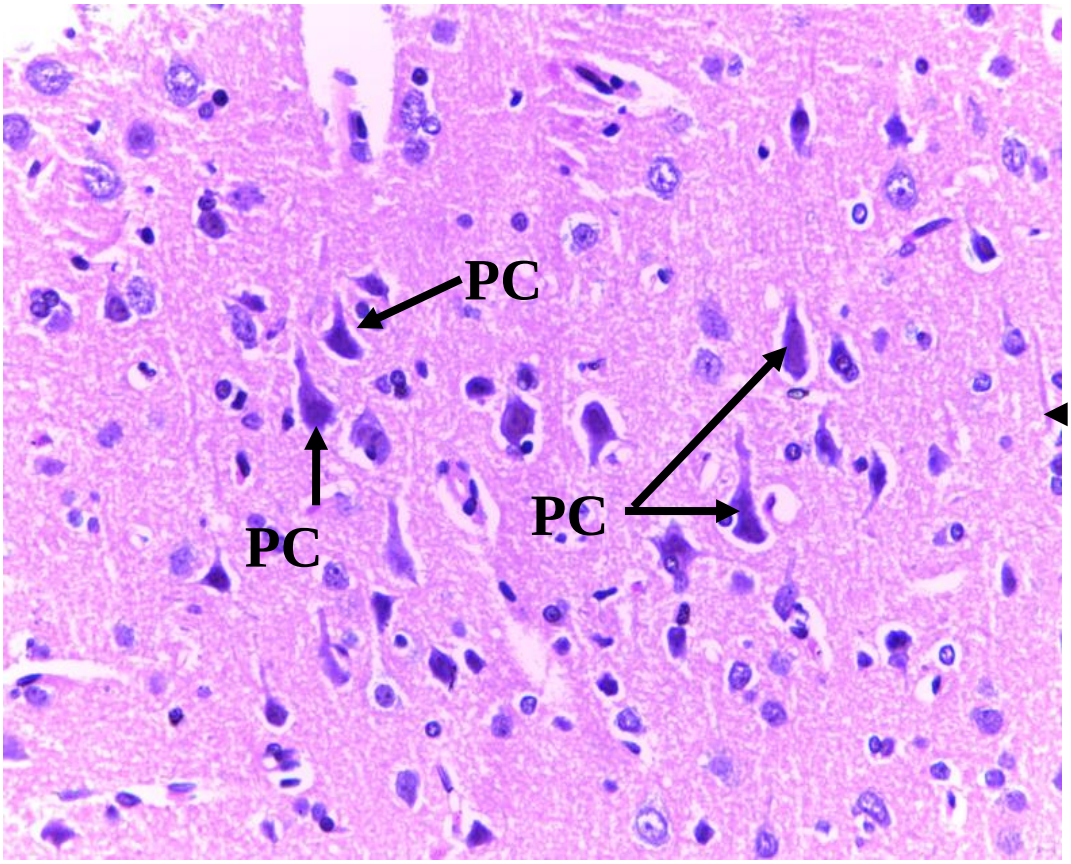

PC
PC
PC

PLATE XXI: A transverse section of the developing cerebral cortex of group Pups at PD21 & PD28 showing normal matured pyramidal cells (PC) without vivid degeneration (H and E ×400).

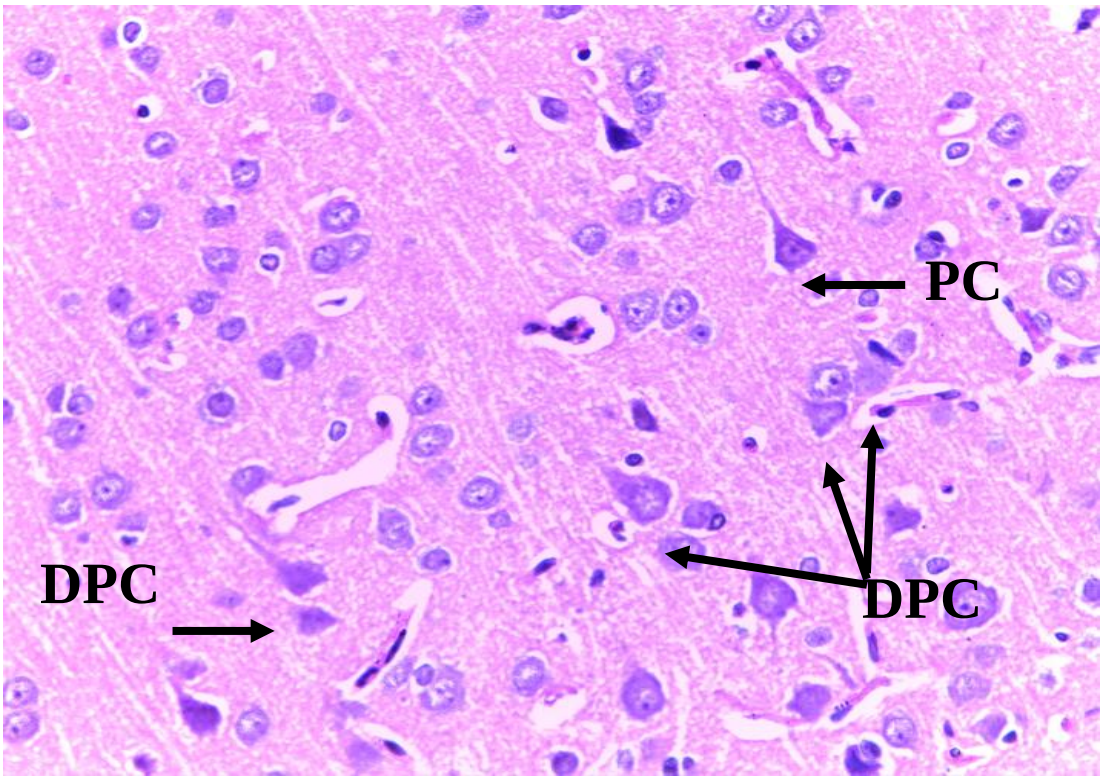


PLATE XXII: A transverse section of the developing cerebral cortex of group B Pups at PD21 & PD28 showing some normal pyramidal cell (PC) neurodegeneration (ND) such as loss of arborisation, degenerating pyramidal cells (DPC) (H and E ×400).

4.3.3 Hippocampus

The results from the histological studies using H and E stain showed animals in the control (group A) to have a normal architecture of the developing pyramidal cells which are immature with absence of arborisation at post-natal days PD 0-4 of the hippocampus as shown in (plate XXIII) while group B of the corresponding days showed a form of neurodegeneration such as degenerated pyramidal cells, cytoplasmic vacuolation and clumping of the cells (plate XIV), While group C pups that received high dose of artesunate showed areas of neuronal degeneration characterize by cytoplasmic vacuolations, degeneration and hypertrophy of the pyramidal cells as shown in (plate XXV). The Pups at PD7 and PD 14 in the control group A have normal cytoarchitecture of the hippocampus with normal pyramidal cell layer with pyramidal cells as development progressed as shown in (Plate XXVI) while group B animals of the same group showed areas of neuronal degeneration such as degeneration and clumping of the pyramidal cells, vacuolated cells (Plate XXVII). Animals control group A at PD21 and PD28 showed normal, mature architecture of the pyramidal cells with well-organized pyramidal cell layer

as development progressed as shown in plate (plate XVIII) while group B pups in the respective showed disorientation of the pyramidal cell layer with loss of pyramidal cells, degeneration and clumping of pyramidal cells as shown in (Plate XXIX).

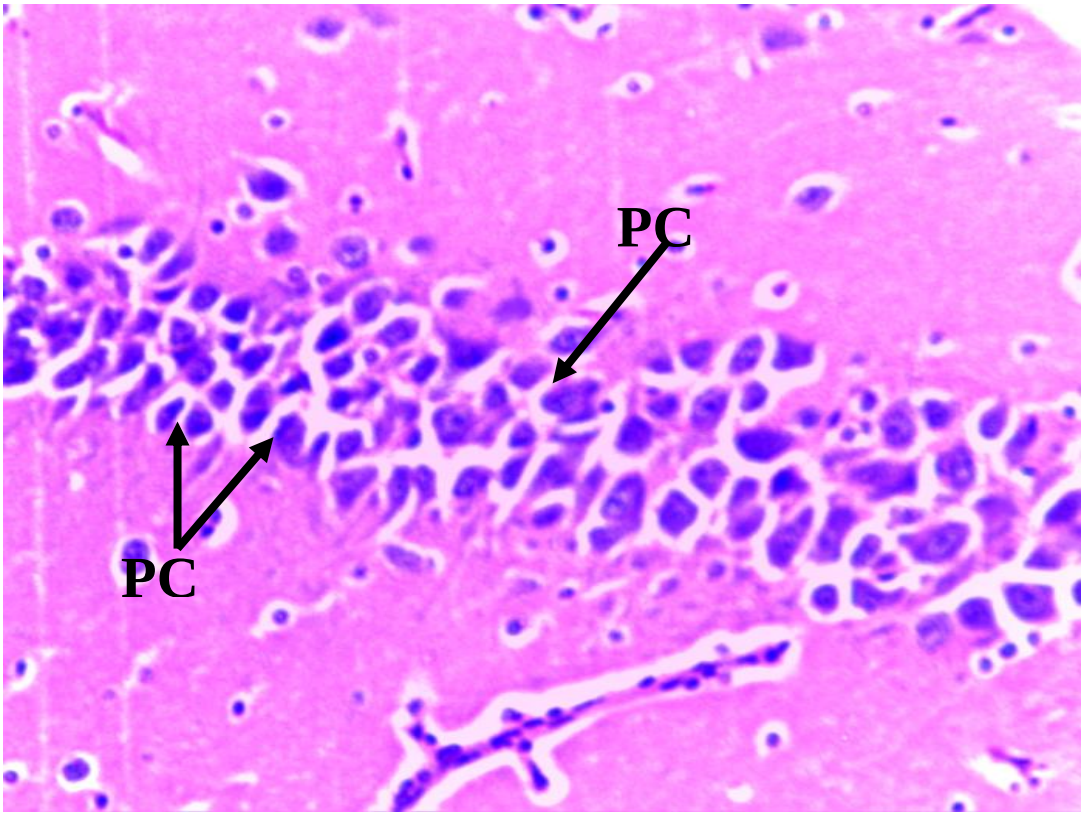


PLATE XXIII: A transverse section of the developing hippocampus of group A pup at GD20- PD4 showing a normal architecture of numerous immature pyramidal cell (PC) (H and E ×400).

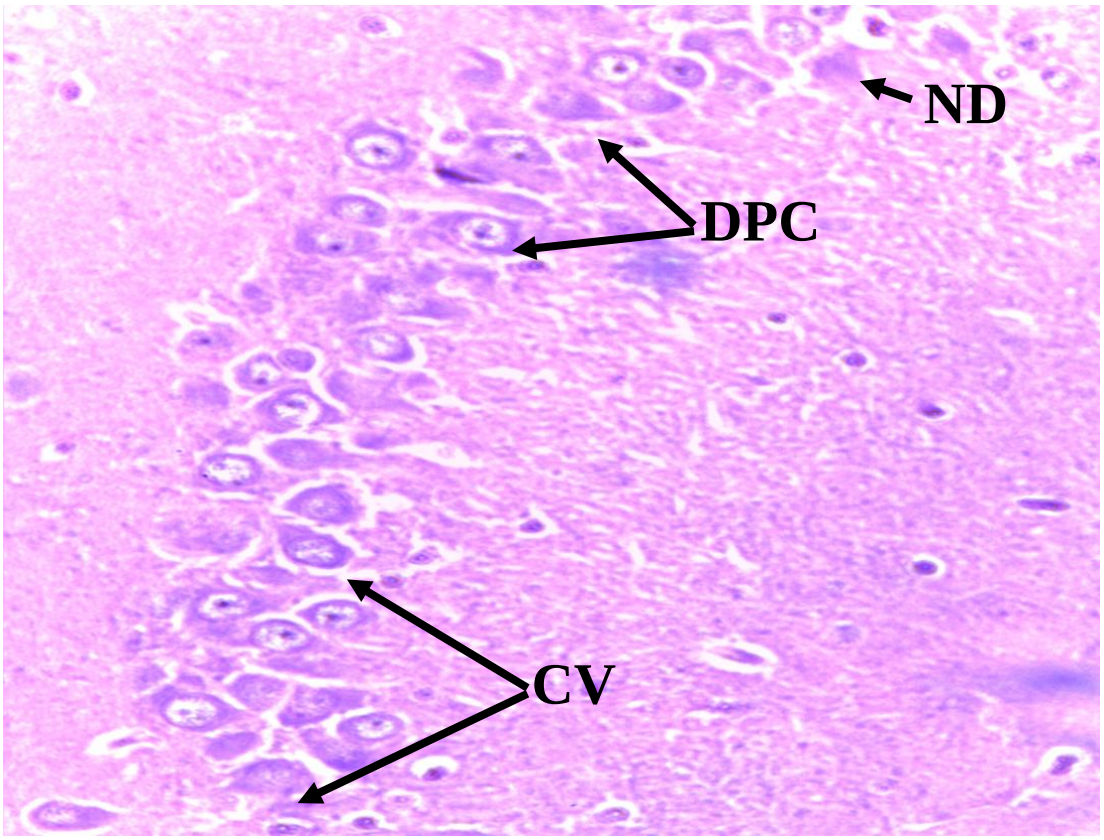


PLATE XXIV: A transverse section of the developing hippocampus of group B pups at GD20- PD4 showing an area of neurodegeneration such as degenerated pyramidal cells (DPC), Pyknotic cells, and cytoplasmic vacuolation (CV) (H and E ×400).

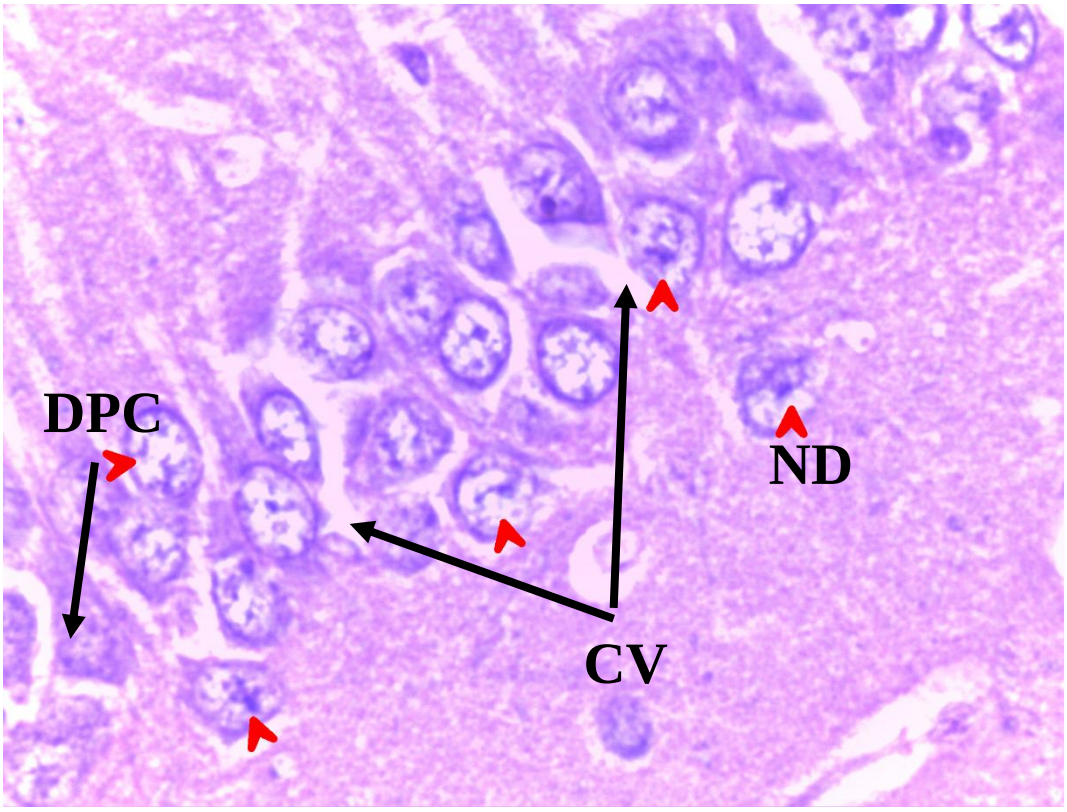


PLATE XXV: A transverse section of the developing hippocampus of group C pups at GD20- PD4 showing severe form of neurodegeneration such as degenerated pyramidal cells (DPC), hypertrophy and Pyknotic cells, cytoplasmic vacuolation (CV) (H and E ×400).

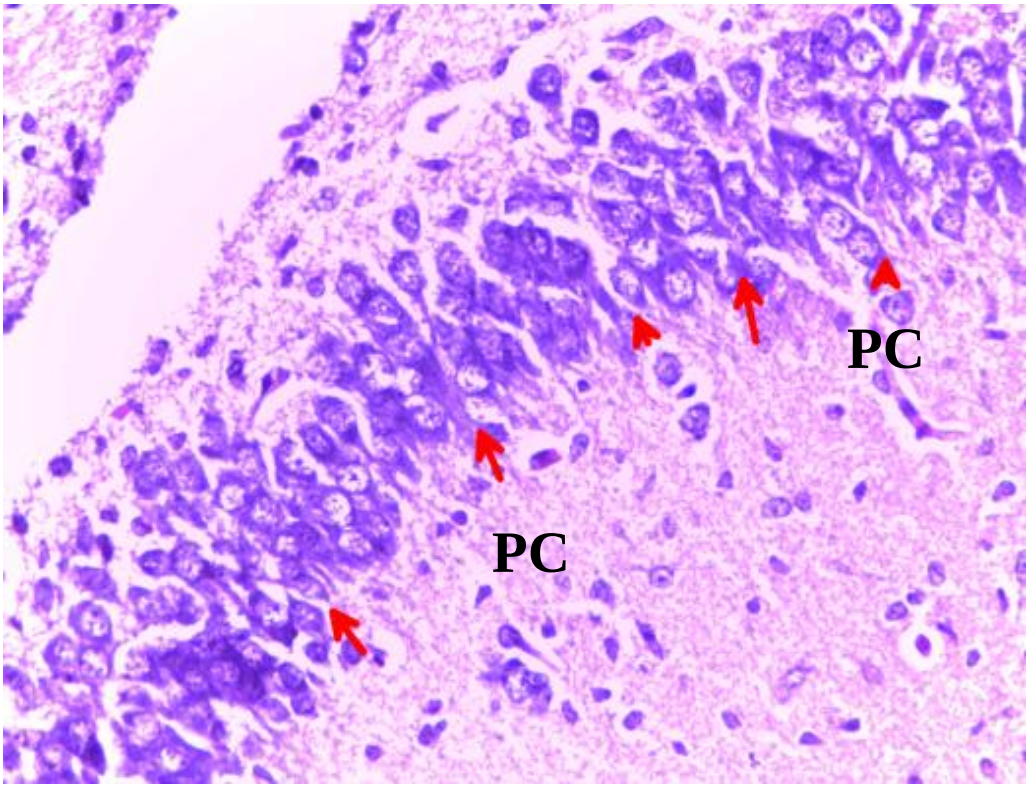


PLATE XXVI: A transverse section of the developing hippocampus of group A pups at PD7 & PD14 showing normal immature pyramidal cells (PC) witha well-organized pyramidal cell layer (H and E×400).

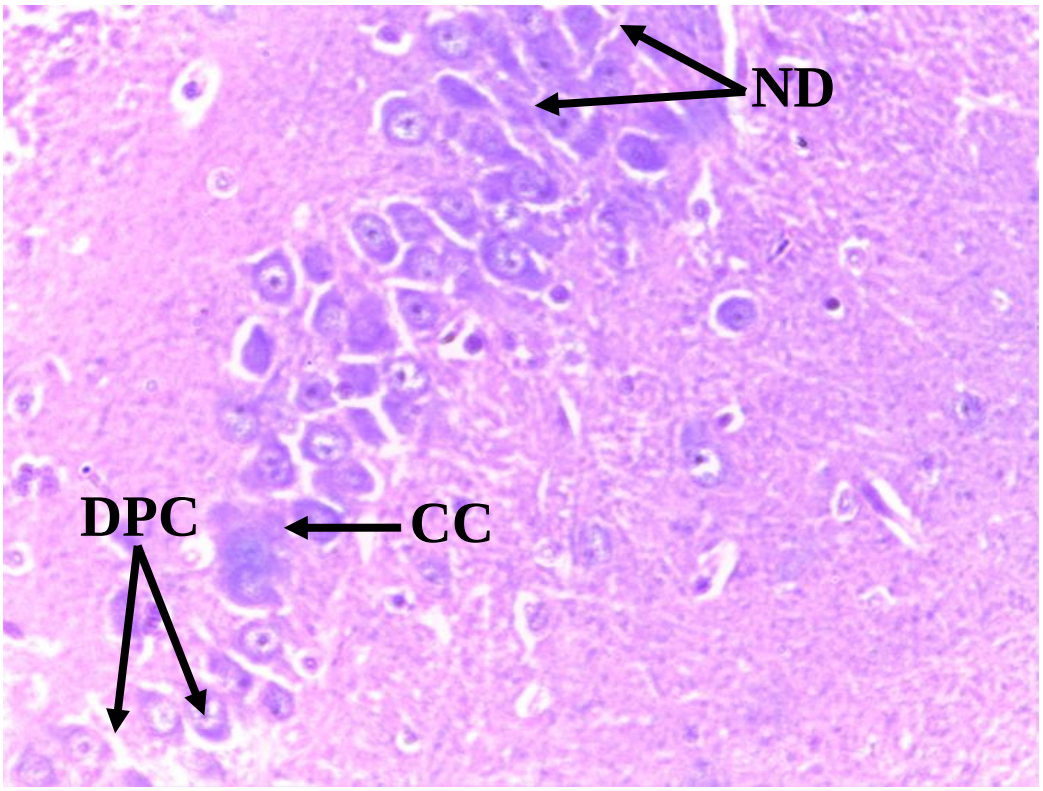


PLATE XXVII: A transverse section of the developing hippocampus of group B pups at PD7 & PD14 showing neuronal degeneration (ND) such as clumping of cells (CC)and degenerated pyramidal cell (DPC) (H and E×400).

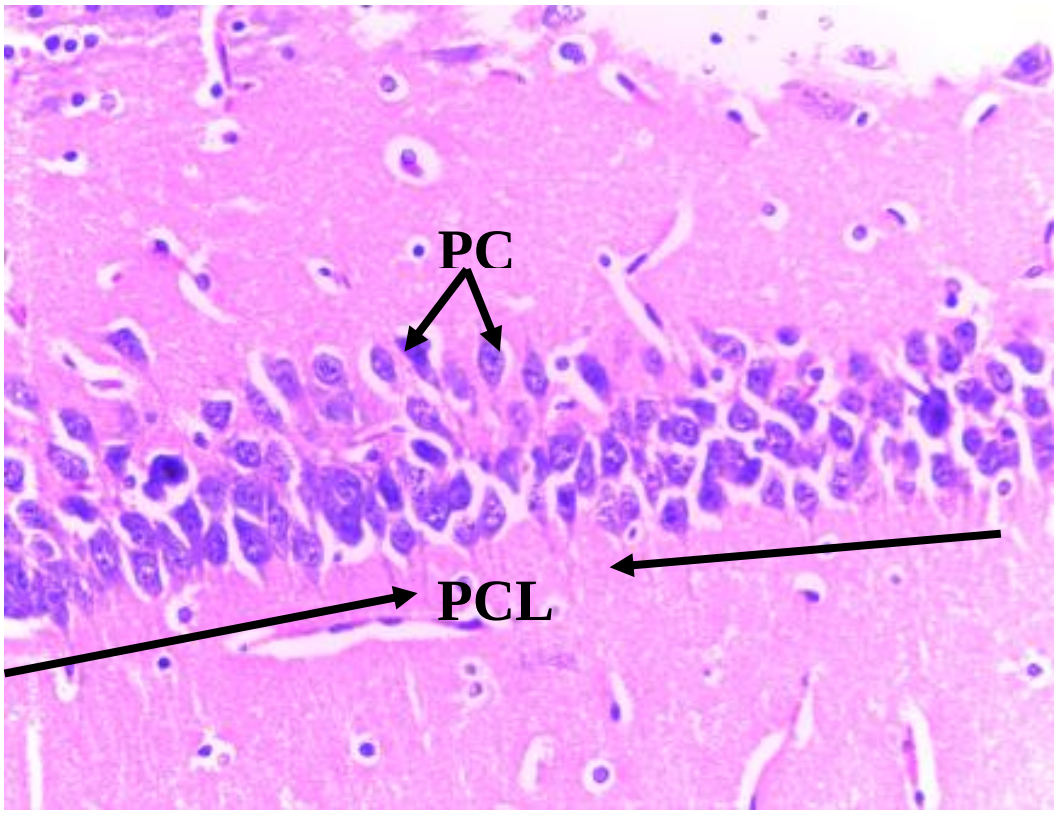


PLATE XXVIII: A transverse section of the developing hippocampus of group Apups at PD 21 & 28 showing a matured pyramidal cells (PC) with well organized pyramidal cell layer (PCL) (H and E ×400).

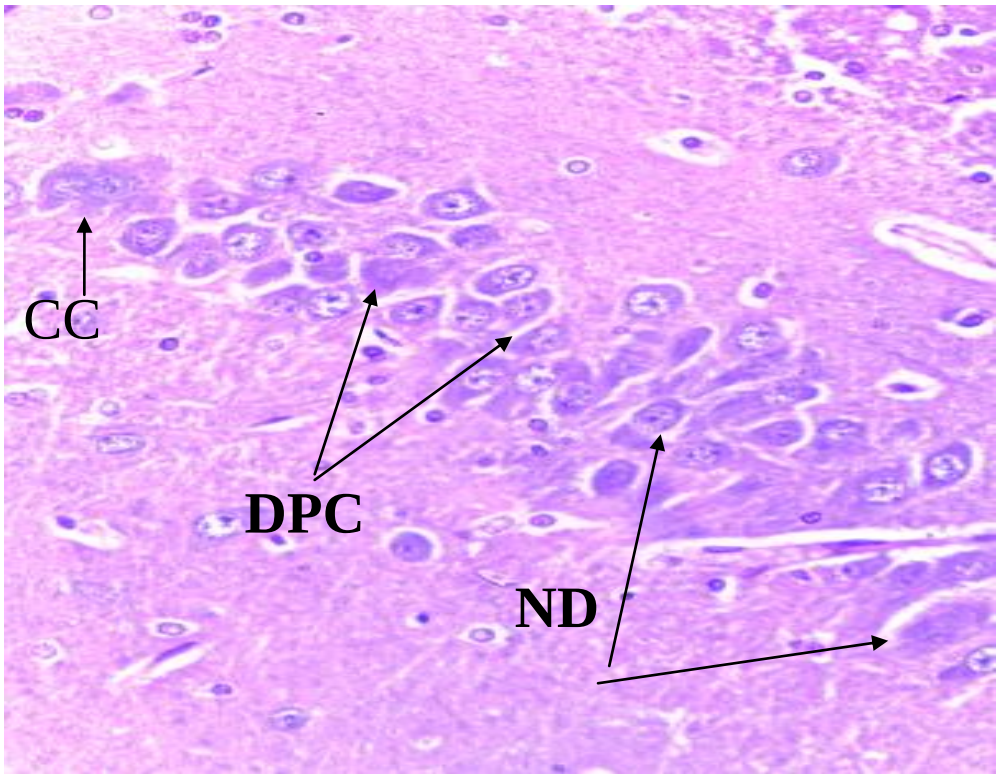


PLATE XXIX: A transverse section of the developing hippocampus of group B at PD 21 & PD 28 showing degenerated pyramidal cell (DPC) neuronal Degeneration (ND) and clumped cells (CC) (H and E ×400).

4.4 Toluidine Blue Stain

4.4.1 Cerebrum

The results from the histochemical changes using toluidine blue stain in the developing cerebral cortex showed pups in control (group A), consisting of gestation day (GD20), and

postnatal days PD1-PD4 revealed a distinct and intensely stained pyramidal cells (Nissl substances),with normal architecture of the immature developing neurons in the cortical layers as shown in (plate XXX). while group B of the corresponding days showed a form of neurodegeneration such as numerous degenerated pyramidal cells, loss of arborisation, reduced staining intensity and migration of the nuclear material towards the periphery of the perikaryon (central chromatolysis) as shown in (plate XXXI). While group C pups that received high dose of artesunate showed areas of severe form of neuronal degeneration characterize by rounding and fading of cells, cytoplasmic vacuolations, degenerated pyramidal cells, loss of arborisation and staining intensity and finally central chromatolysis as shown in (plate XXXII). At postnatal days PD7 and PD14 in control group A showed more prominent pyramidal cells that are distinct and intensely stained as development progressed (plates XXXIII) while group B pups of the corresponding post-natal days showed areas of neuronal degeneration such as degenerated and vacuolated pyramidal cells, reduced staining intensity with central chromatolysis as seen in (plate XXXIV). Pups in control group A at PD21 and PD28 showed a more matured normal architecture of the pyramidal cells that are distinct and intensely stained (XXXV) but group B of the same days showed area of neurodegeneration such as degeneration of the pyramidal cells, faintly stained and vacuolated cells with central chromatolysis as shown in (plate XXXVI).

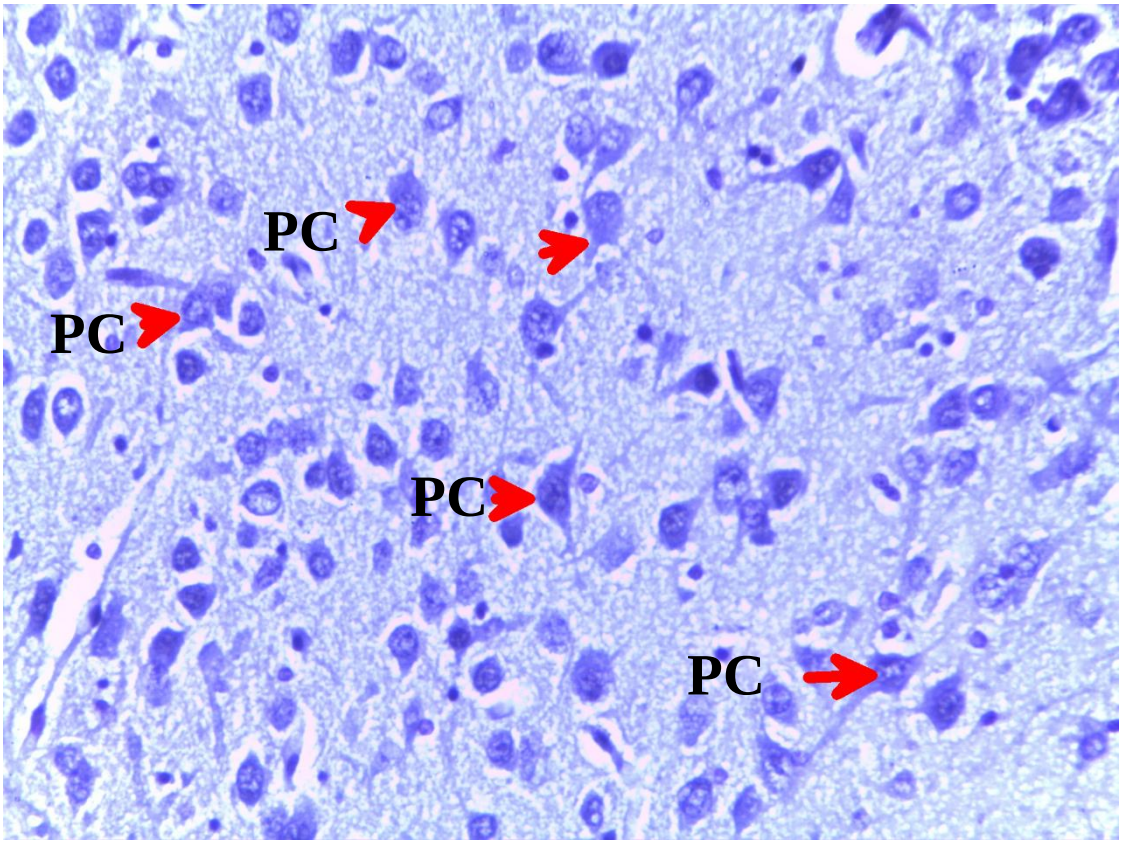
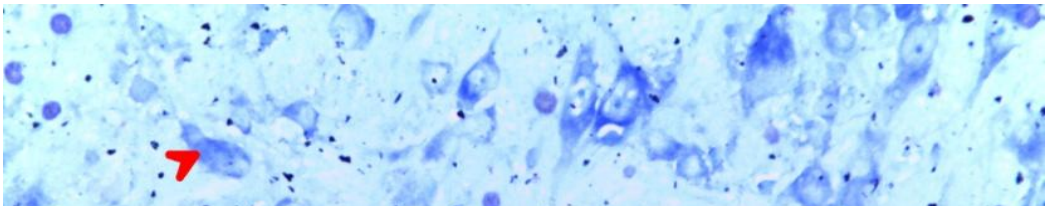


PLATE XXX: A transverse section of the developing cerebral cortex of group A Pups at GD20 & PD1 showing an immature distinct and intensely stained pyramidal cells(Toluidine Blue ×400).



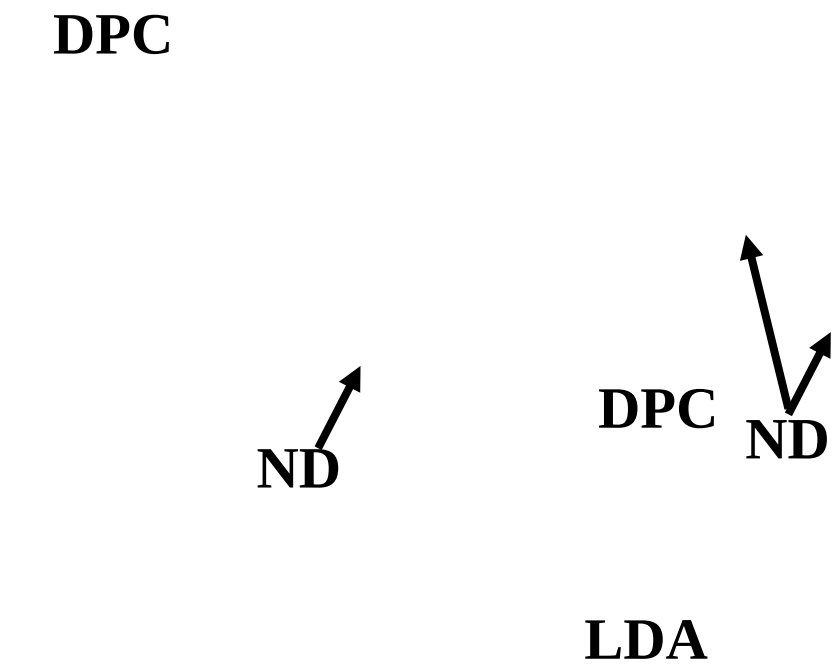
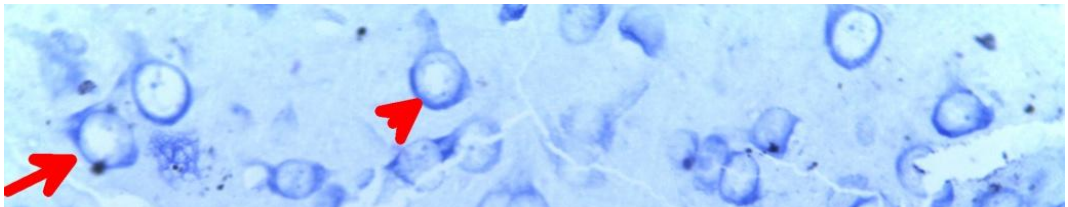


PLATE XXXI: A transverse section of the developing cerebral cortex of group B Pups at GD20 & PD1- 4 showed a form of neuronal degeneration characterize byReduced staining intensity, degenerated pyramidal cells (DPC) with loss of dendriticarborisation (LDA) and central chromatolysis (Toluidine Blue ×400).



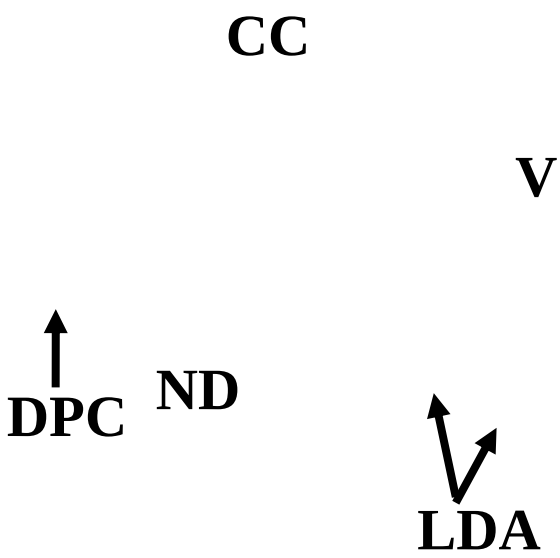


PLATE XXXII: A transverse section of the developing cerebral cortex of group C Pups at GD20 & PD1-4 showed a form of neuronal degeneration (ND) characterize by loss of dendritic arborisation (LDA) rounding and fading of cells, vacoulated Cell (VC) reduced staining intensity of cells and chromatolysis (Toluidine Blue ×400).

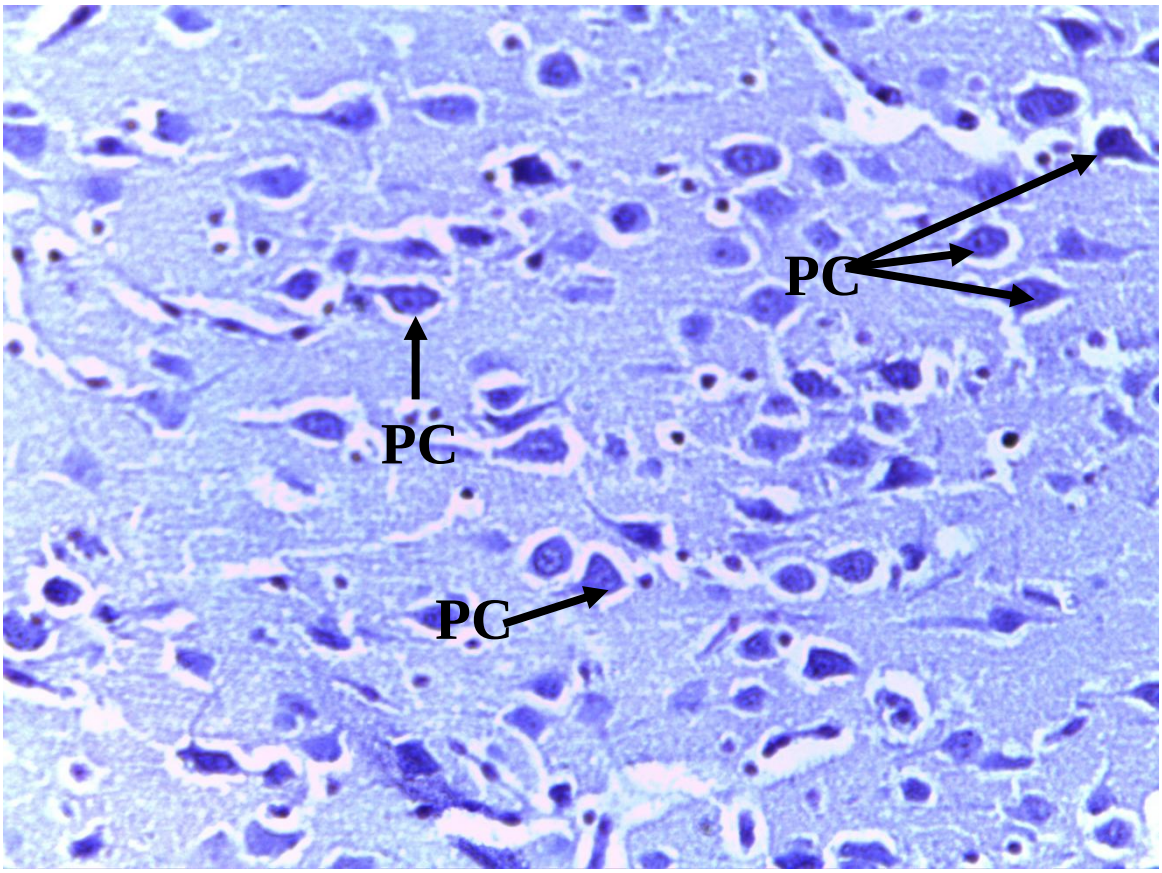


PLATE XXXIII: A transverse section of the developing cerebral cortex of groupA Pups at PD7 & PD14 showing numerous and normal distinct and intensely stained pyramidal cells (Toluidine Blue ×400).

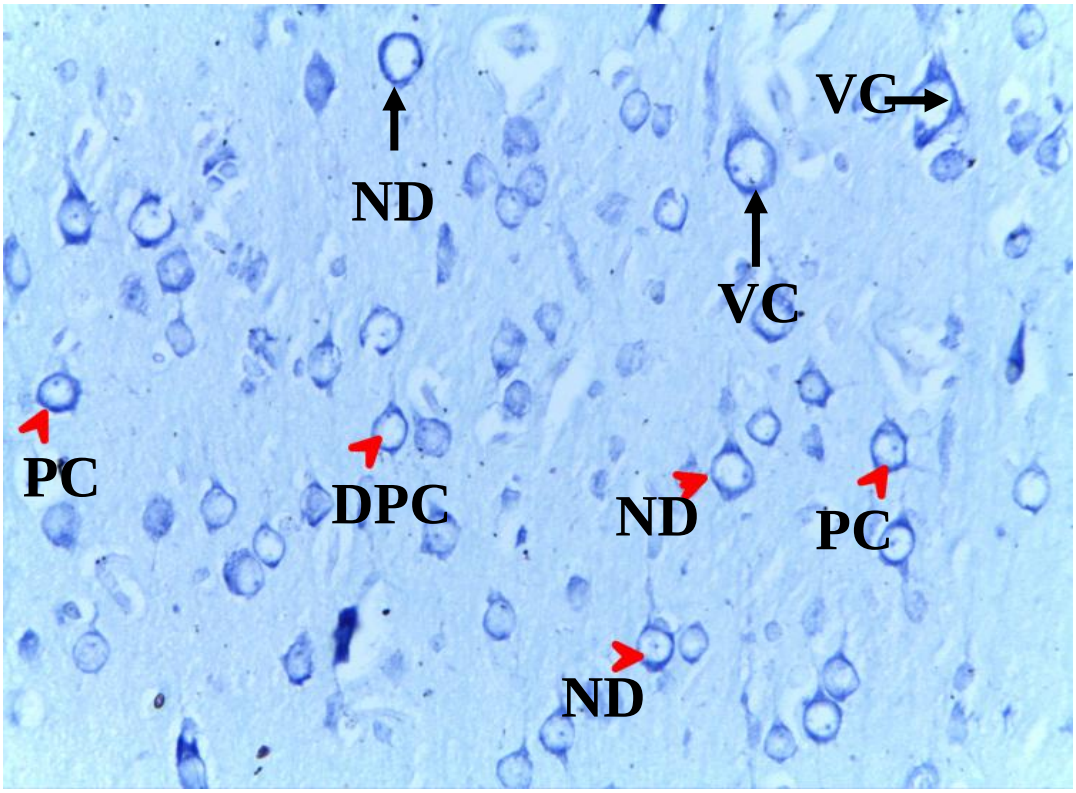


PLATE XXXIV: A transverse section of the developing cerebral cortex of group B Pups at PD7 & PD14 showed a form of neuronal degeneration (ND) such as degenerated pyramidal (DPC), vacuolated cells with less staining intensity and central chromatolysis (Toluidine Blue ×400).

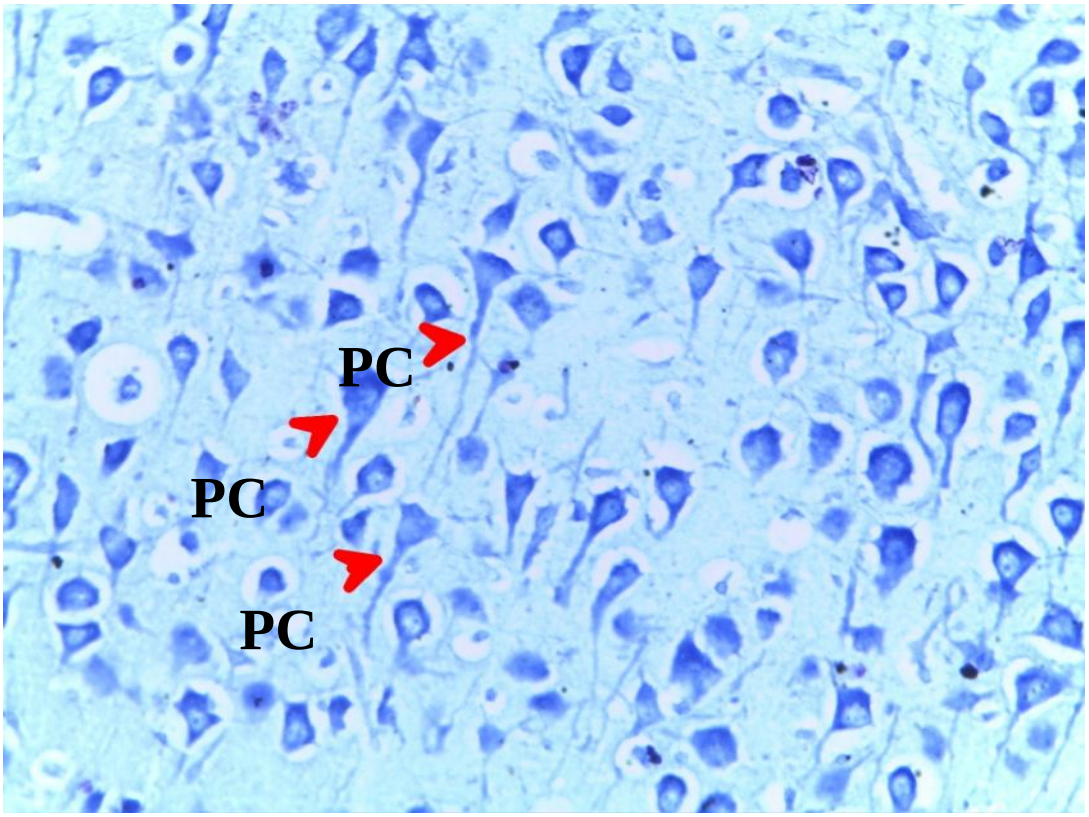


PLATE XXXV: A transverse section of the developing cerebral cortex of group A Pups at PD21 & PD28 showing normal and numerous pyramidal cells (PC) that are distinct and intensely stained (Toluidine Blue ×400).

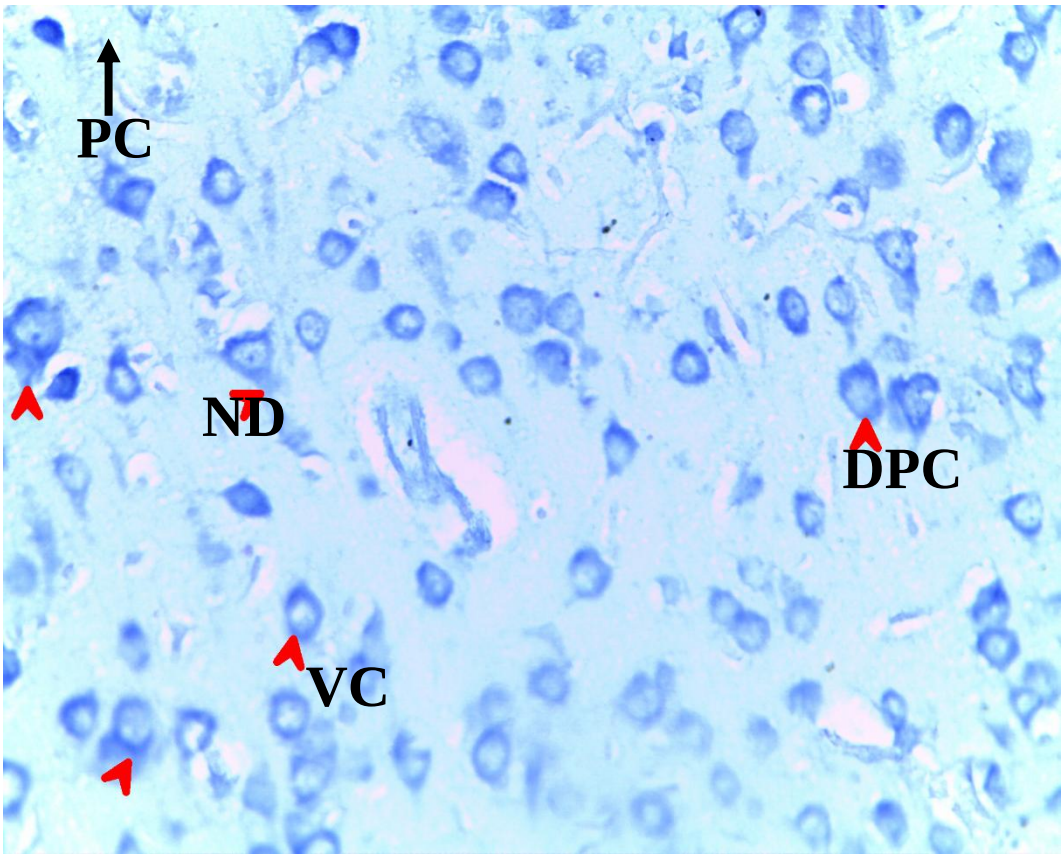


PLATE XXVI: A transverse section of the developing cerebral cortex of group B Pups at PD21 & PD28 showed neuronal degeneration such as faintly stained cells degenerated Pyramidal cells (DPC) and vacuolation cells(VC), with some normal pyramidal cells (Toluidine Blue ×400).

4.4.2 Hippocampus

The results from the histochemical changes using Toluidine blue stain in the developing hippocampus showed pups in control (group A), consisting of gestation day (GD20), and postnatal days PD1-PD4 revealed a distinct and intensely stained pyramidal cells (Nissl

substances), with normal architecture of the immature developing neurons in the hippocampus as shown in (plate XXXVII). Group B of the corresponding days showed a form of neurodegeneration such as numerous degenerated pyramidal cells, loss of arborisation, reduced staining intensity, vacuolated cells and cytoplasmic vacuolation as shown in (plate XXXVIII). While group C pups that received high dose of artesunate showed areas of severe form of neuronal degeneration characterize by rounding and fading of cells, cytoplasmic vacuolations, degenerated pyramidal cells, loss of arborisation and staining intensity, central chromatolysis and finally distortion of the pyramidal cell layer as shown in (plate XXXIX). At postnatal days PD7 and PD14 in control group A showed more prominent pyramidal cells that are distinct and intensely stained as development progressed with an organized pyramidal cell layer (plates XL) while group B pups of the corresponding post natal days showed areas of neuronal degeneration such as degenerated and clumping of pyramidal cells, reduced staining intensity with central chromatolysis with mild distortion of the cell layer as seen in (plate XLI). Pups in control group A at PD21 and PD28 showed a more matured normal architecture of the pyramidal cells that are distinct and intensely stained with a prominent axon and well organized pyramidal cell layer (XLII) but group B of the same days showed area of neurodegeneration such as degeneration of the pyramidal cells, faintly stained and clumped cells, disorientation of the pyramidal cell layer with central chromatolysis as shown in (plate XLIII).

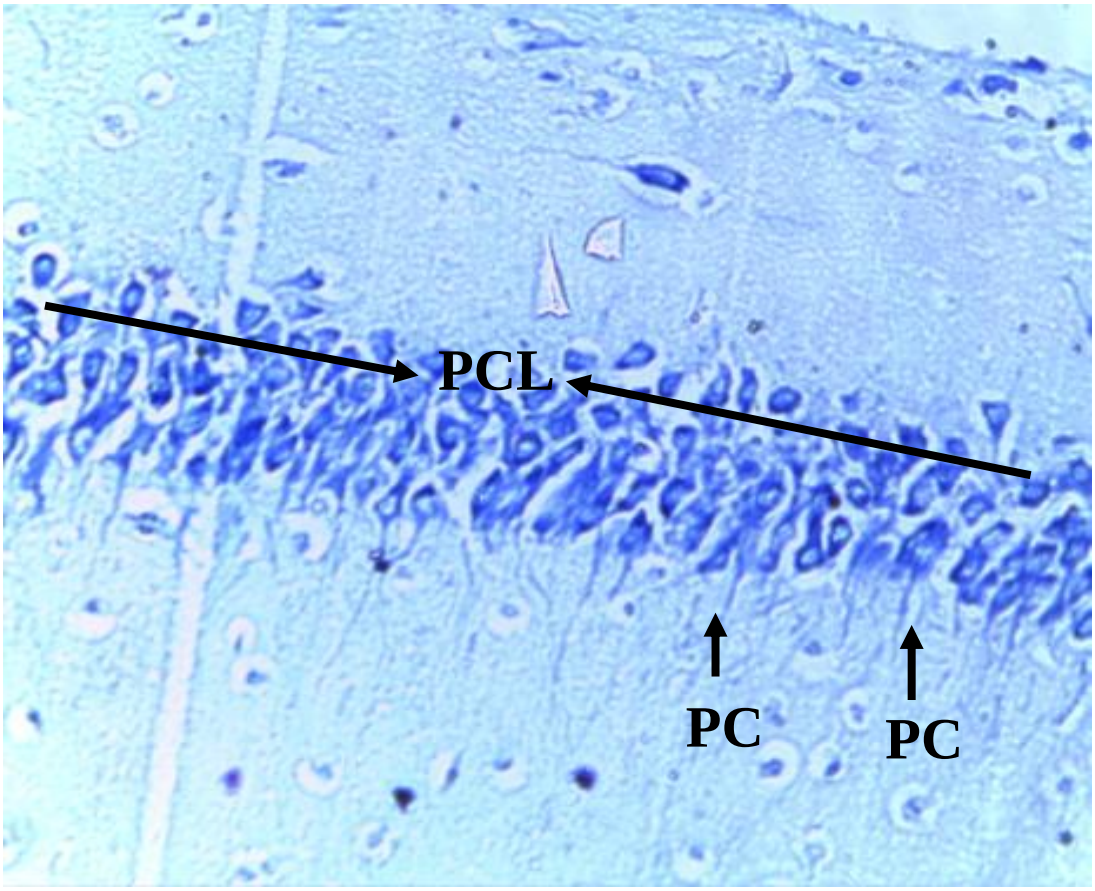


PLATE XXXVII: A transverse section of the developing hippocampus of group A Pups at GD20 & PD1-4 showed an immature and normal pyramidal cells (PC), that are distinct and intensely stained with organized cell layer (PCL), (Toluidine Blue ×400).

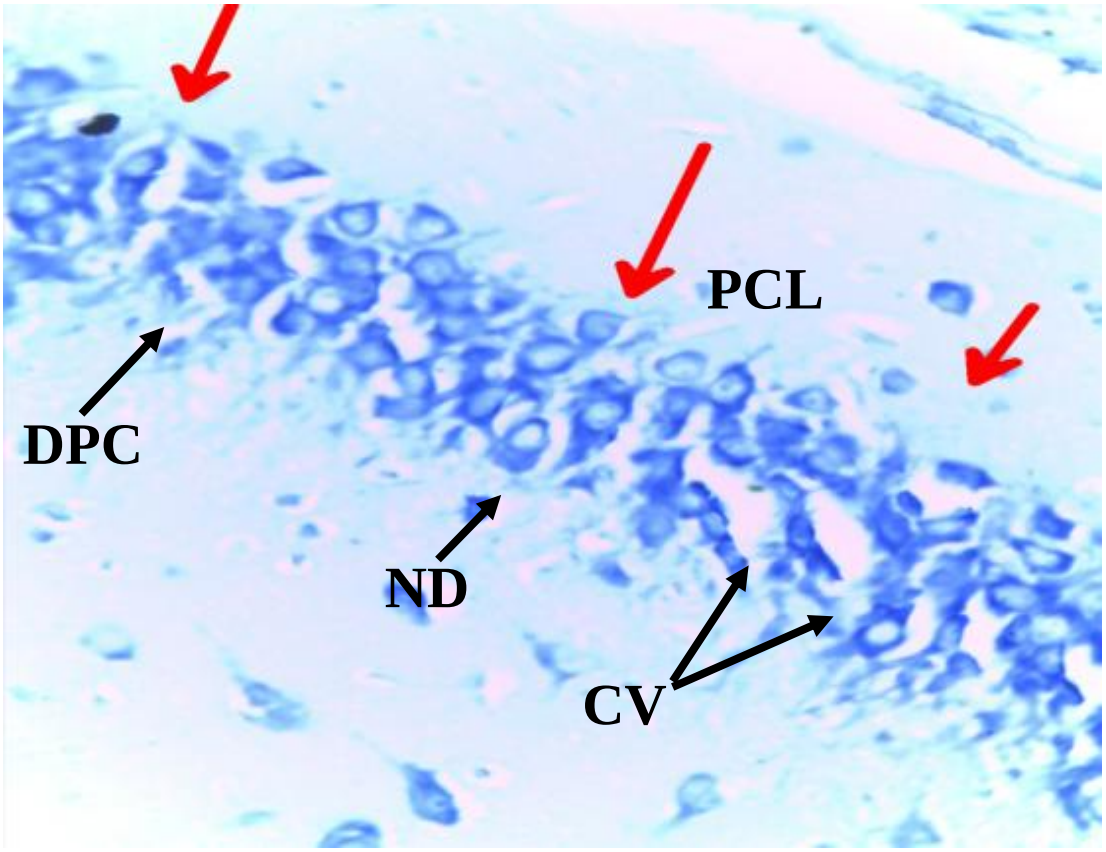


PLATE XXXVIII: A transverse section of the developing hippocampus of group B Pups at GD20 & PD1-4 showed a form of neuronal degeneration (ND) cytoplasmic vacuolation (CV) degenerated pyramidal (DPC), with less staining intensity and central chromatolysis (Toluidine Blue ×400).

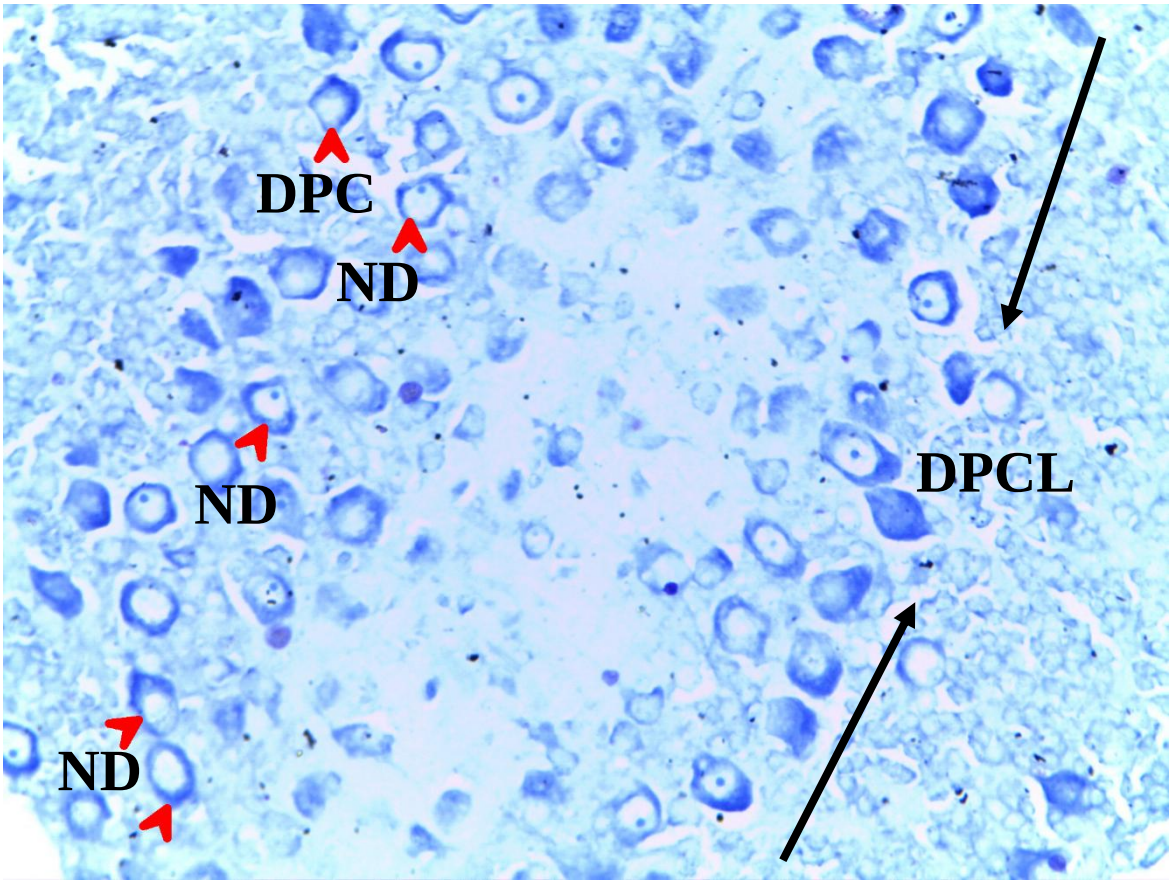


PLATE XXXIX: A transverse section of the developing hippocampus of group C Pups at GD20 & PD1-4 showed a form of neuronal degeneration (ND), degenerated pyramidal (DPC) scattered cells with less staining intensity and central chromatolysis with disorientation of the pyramidal cell layer (DPCL) (Toluidine Blue ×400).

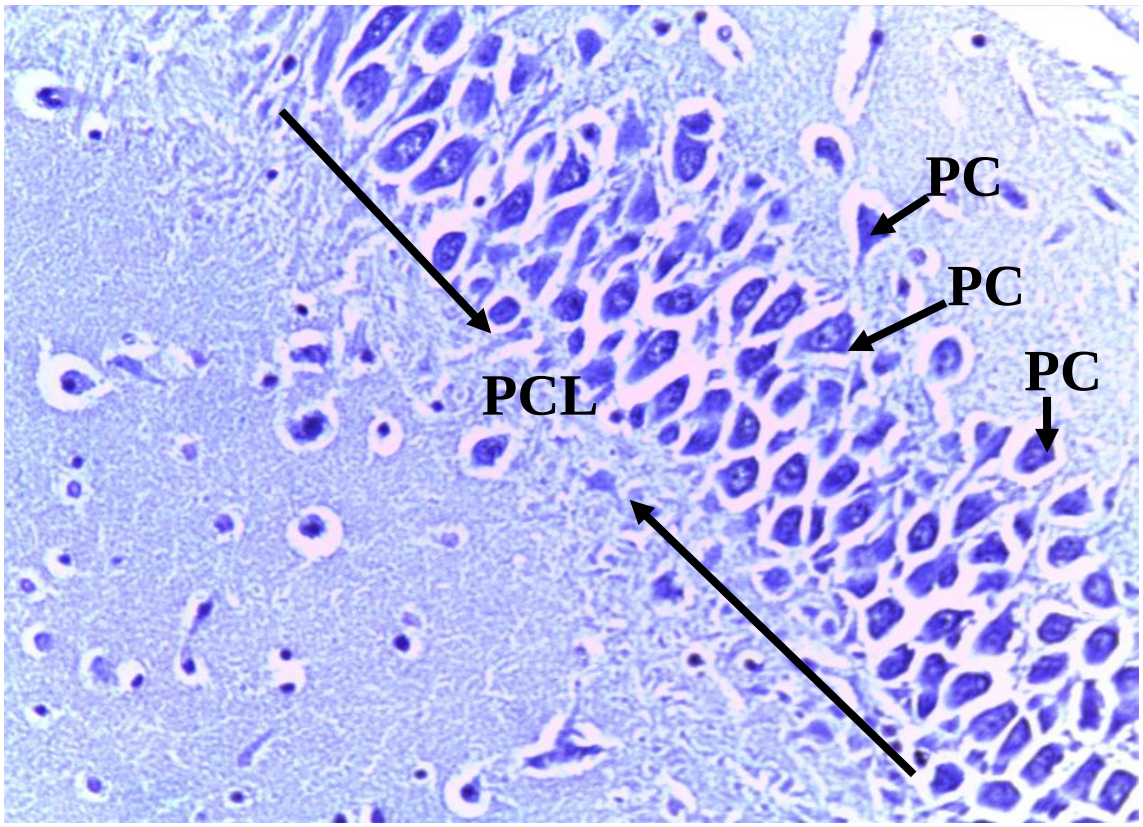


PLATE XL: A transverse section of the developing hippocampus of group A Pups at PD7 & PD14 showed a distinct and well stained normal pyramidal cell (PC) with a well organised pyramidal cell layer (PCL) (Toluidine Blue ×400).

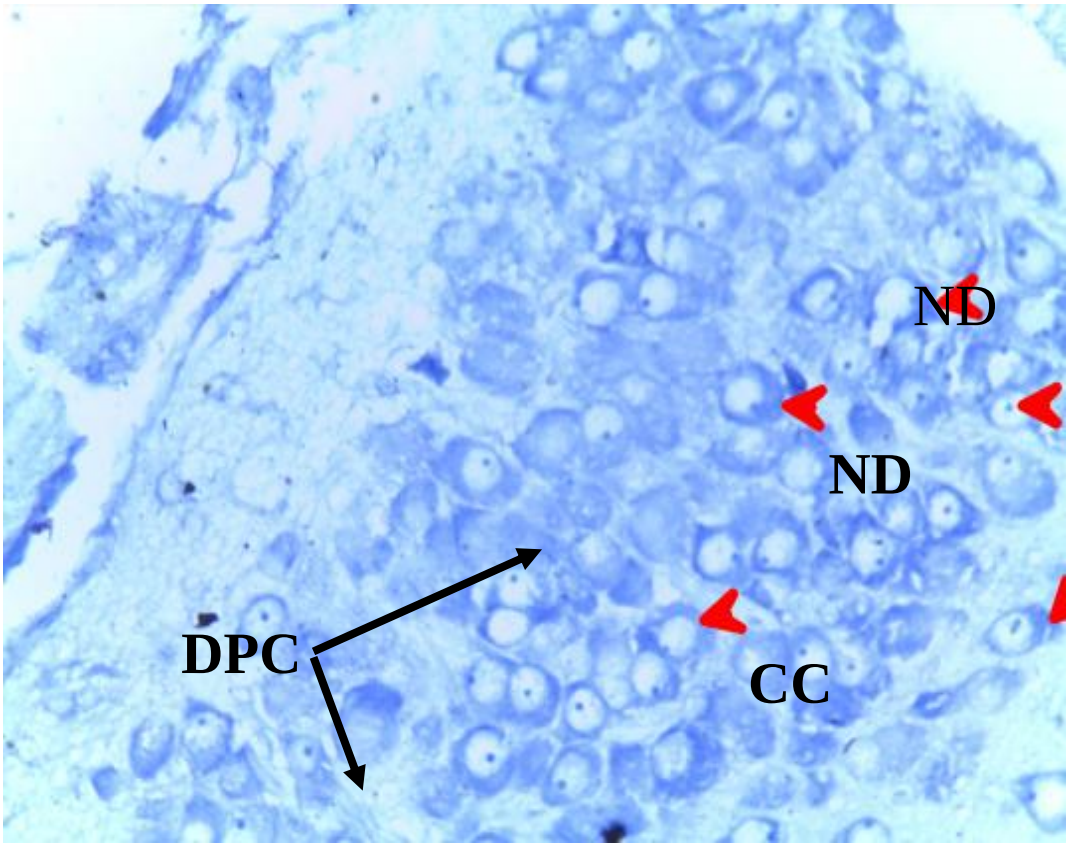


PLATE XLI: A transverse section of the developing hippocampus of group B Pups at PD7 & PD14 showing a form of neuronal degeneration (ND) such as clumping cells (CC) degenerated pyramidal cell (DPC) and disorientation of the pyramidal cell layer (DPCL) (Toluidine Blue ×400).

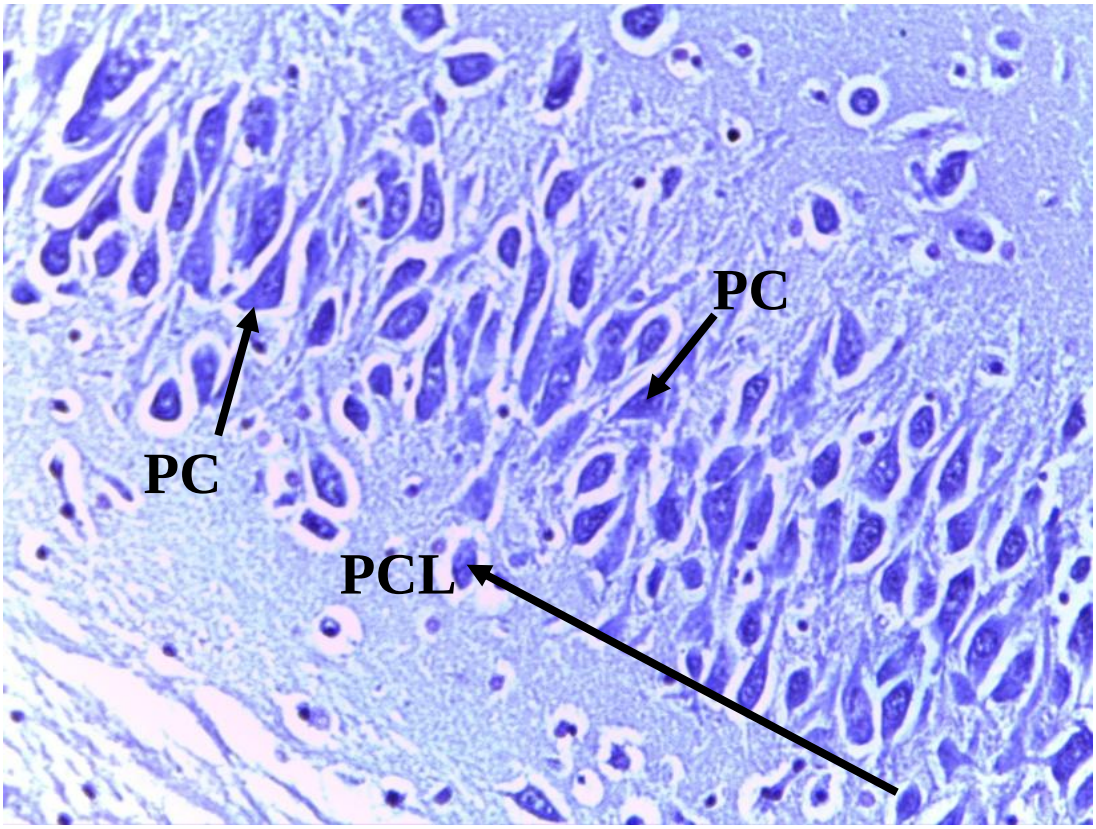
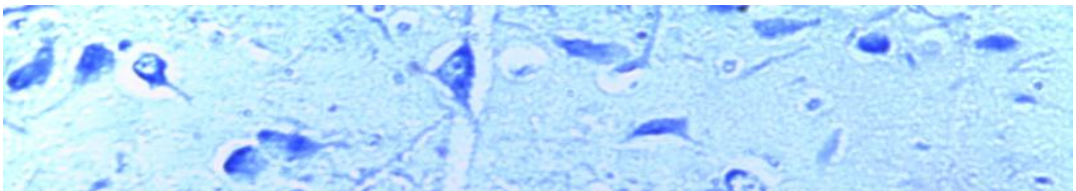


PLATE XLII: A transverse section of the developing hippocampus of group A Pups at PD21 & PD28 showed normal matured pyramidal cell (PC), distinctly and stainedPC, Intact and organized pyramidal cell layer (PCL), (Toluidine Blue ×400).



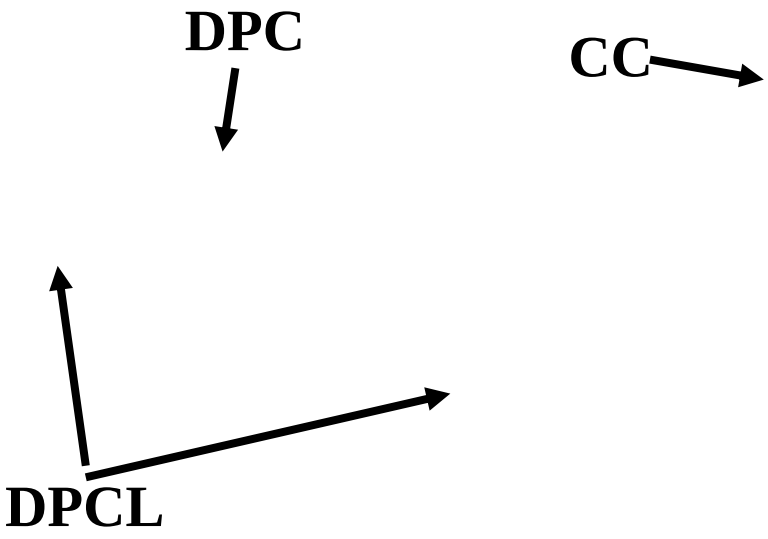


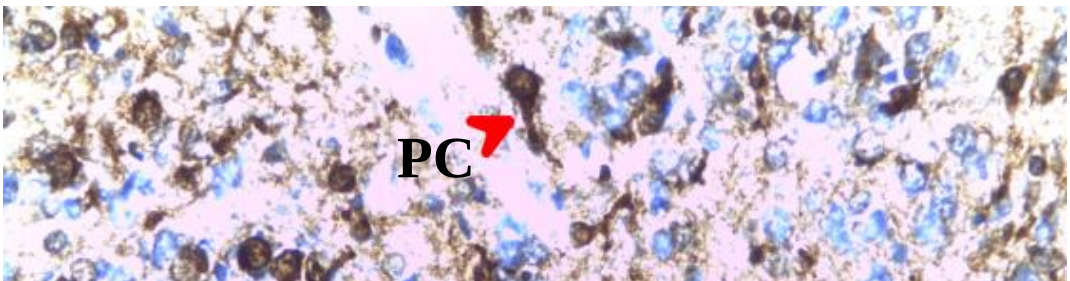
PLATE XLIII: A transverse section of the developing hippocampus of groupB Pups at PD21 & PD28 showing neuronal degeneration such as clumped cellsCC) disorientation of the pyramidal cell layer (DPCL), degenerated pyramidal cells (DPC) and faintly stained cells (Toluidine Blue ×400).

4.5 Immunohistochemical Expression of Calbindin D28K.

4.5.1 Cerebrum

The result from the immunhistochemical localization of calcium binding protein calbindin D28k in the control group A at gestation day GD20 and postnatal days PD 1, PD 4, PD 7, PD 14, PD 21 and PD 28 respectively showed a positive expression of Calbindin D28K in

the pyramidal cells of the cerebral cortex. The pyramidal cells were distinct and deeply stained indicating positive immunolabelling with Calbindin across the control groups as shown in plates (XLIV, XLVII, XLIX). While in group B that received therapeutic dose of artesunate showed a reduced staining intensity with loss of cellular components indicating mild expression of Calbindin. Some pyramidal cells showed an area of neurodegeneration due to aresunate administration across the groups as shown in plates (XLV, XLVIII, L). But in group C that received high dose of artesunate showed a negative immunoexpression of Calbindin in the pyramidal cells with a severe form of neurodegeneration and faintly stained neuropil as shown in plate (XLVI)



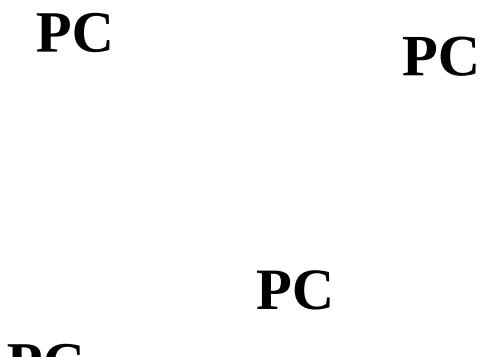


PLATE XLIV: A transverse section of the developing cerebral cortex of group APups at GD20 & PD1-4 showed a positive expression of Calbindin in the Pyramidalcells (Mag ×400).

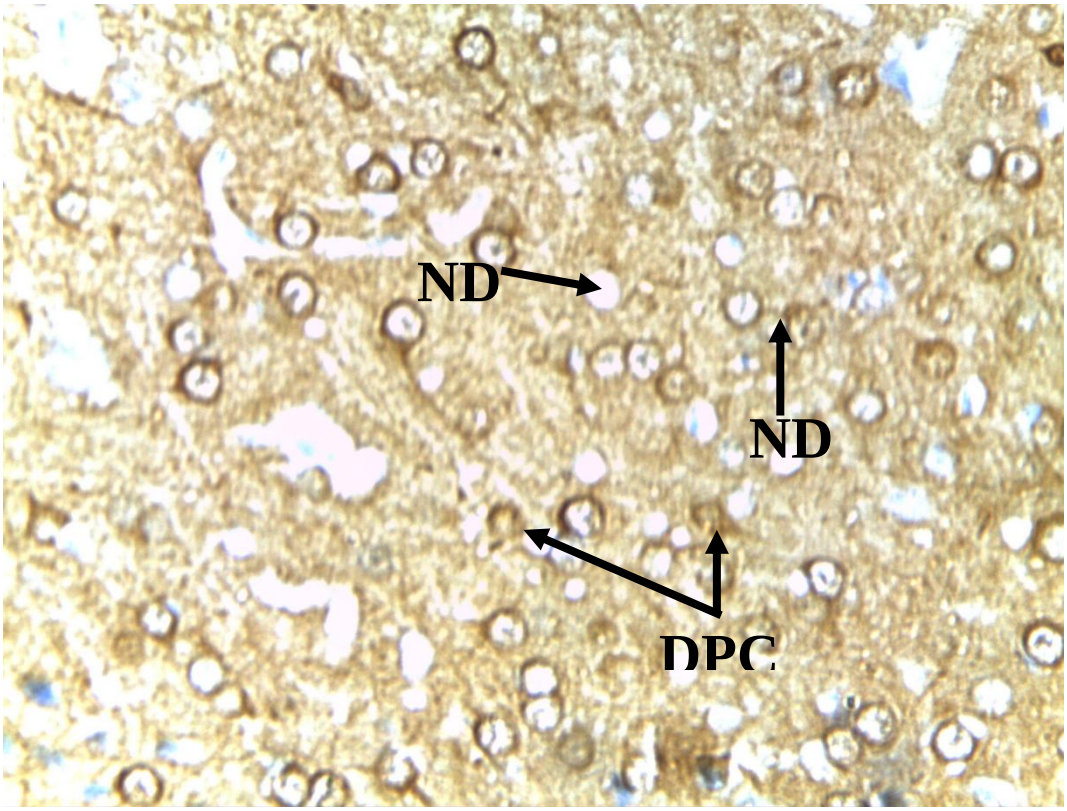


PLATE XLV: A transverse section of the developing cerebral cortex of group B Pups at GD20 & PD1-4 showed a decreased intensity in immunoreaction in the Pyramidal cell with areas of neurodegeneration and degenerating pyramidal cells, vacuolated cells (Mag ×400).

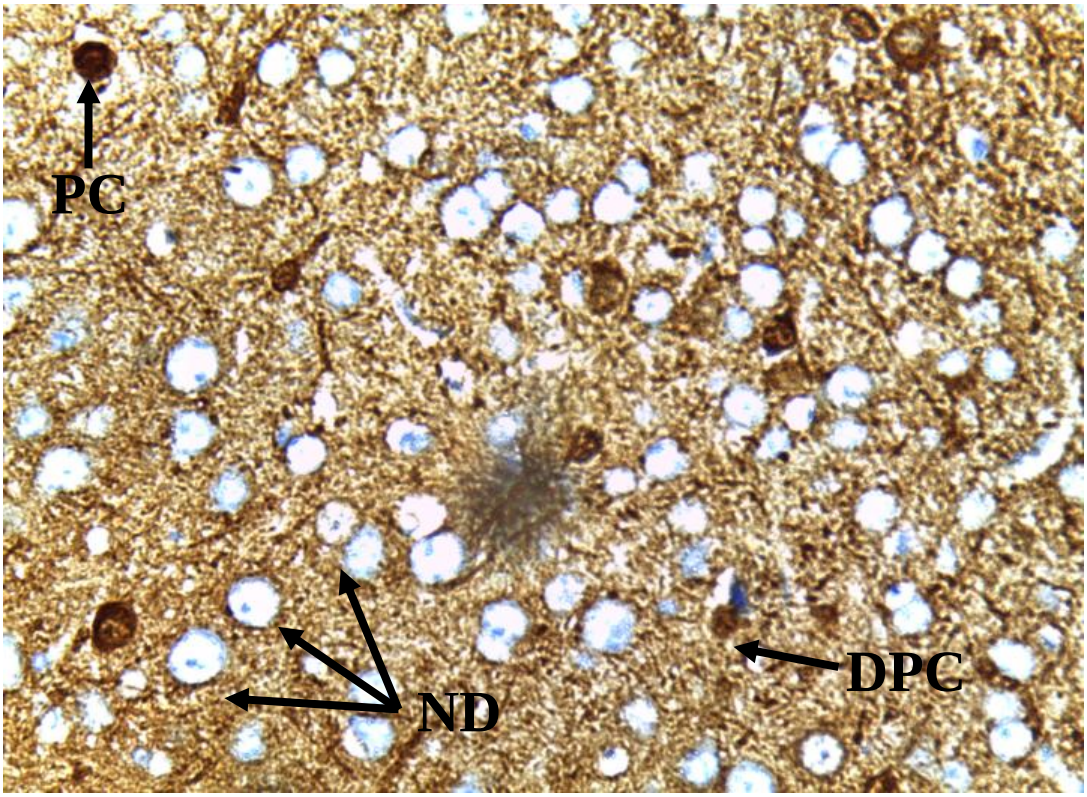


PLATE XLVI: A transverse section of the developing cerebral cortex of group C Pups at GD20 & PD1-4 showed a decreased intensity in the immunoreactions in the neuropil and negative expression of Calbindin in the Pyramidal cells and degenerating pyramidal cells (Mag ×400).

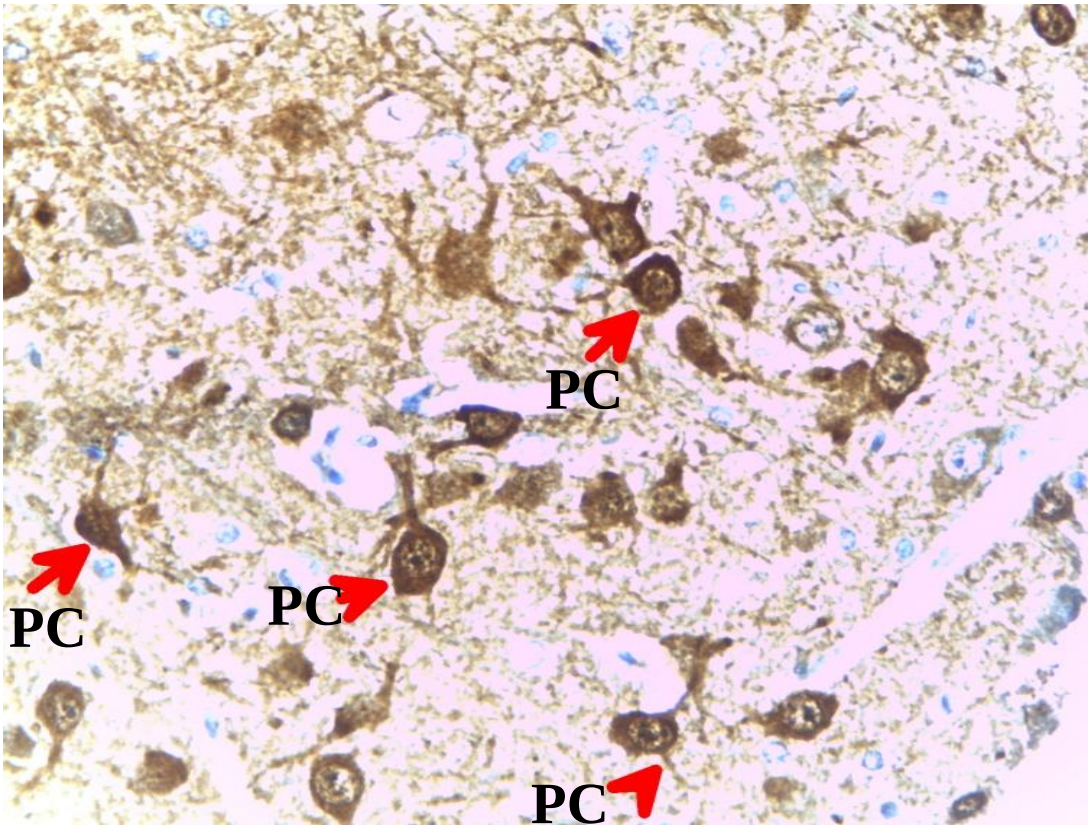


PLATE XLVII: A transverse section of the developing cerebral cortex of group Pups at PD7&PD14 showed intense and positive imunolabelling of Calbindin in the Pyramidal cells with Strong immunoreactivity detected in the processes (Mag ×400)

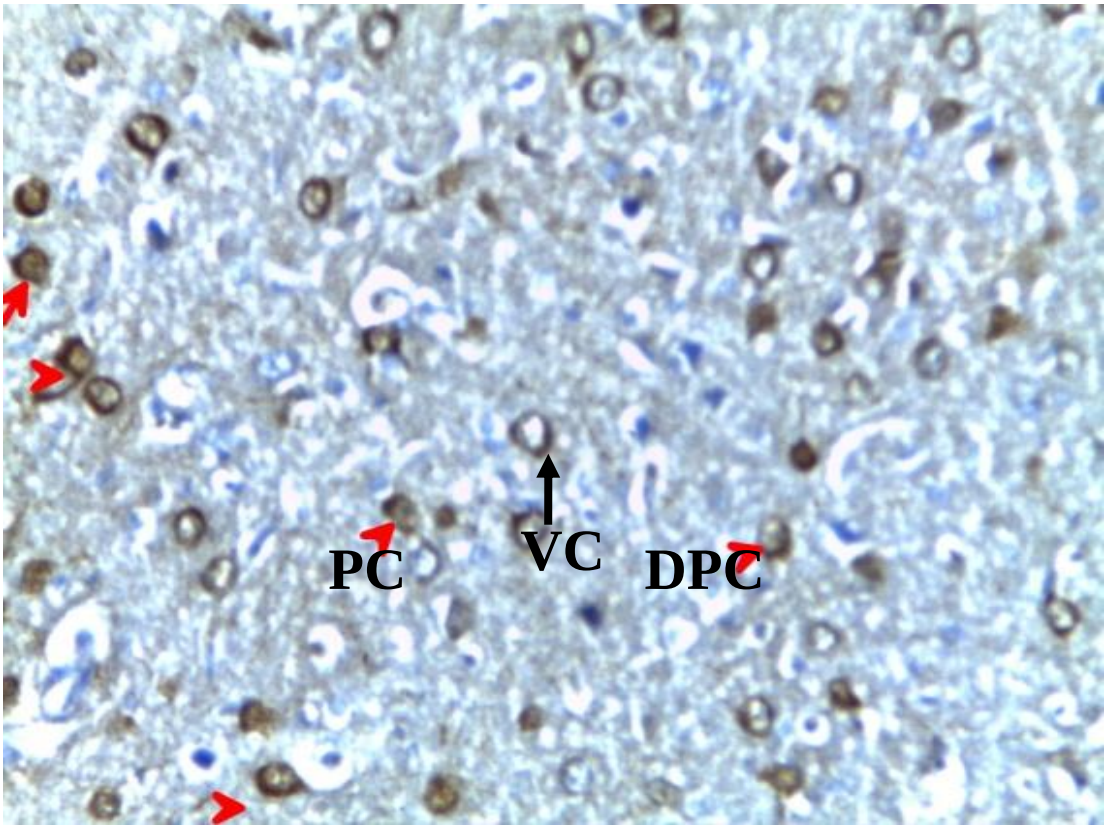
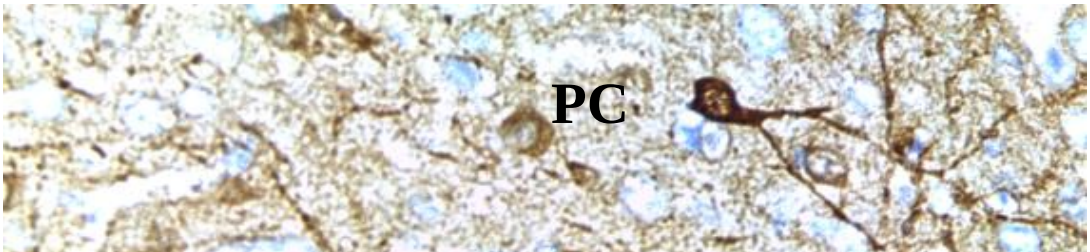


PLATE XLVIII: A transverse section of the developing cerebral cortex of group BPups at PD7 & PD14 showed a reduced immunoreaction of calbindin in the Pyramidalcell with degenerating pyramidal cells, vacuolated cells, (Mag×400).



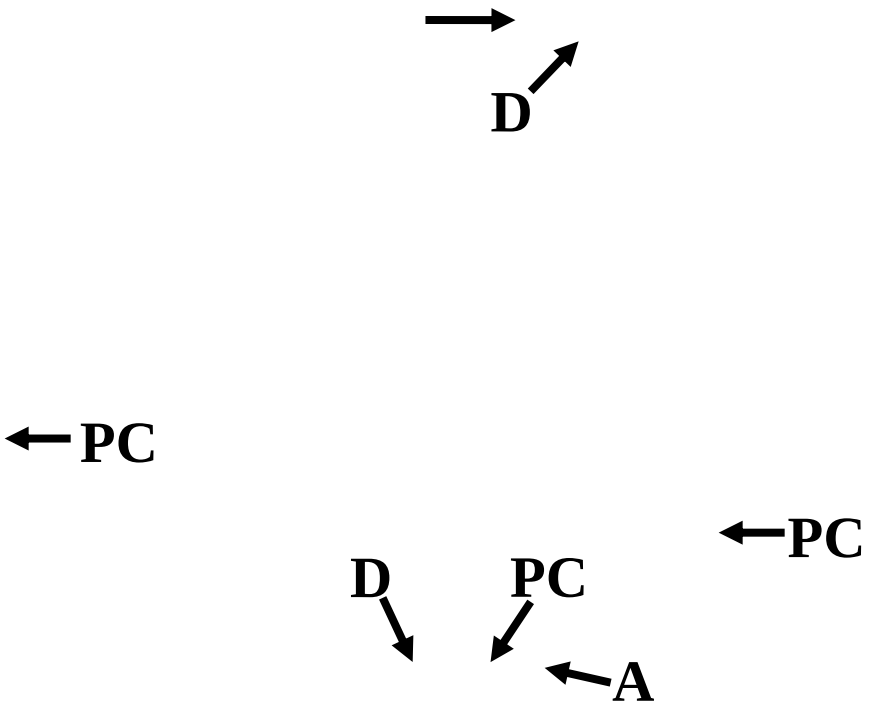


PLATE XLIX: A transverse section of the developing cerebral cortex of group APups at PD 21& PD 28 showed a positive expression of calbindin in the Pyramidal Cells and extends into its dendritic arborisation (×400).

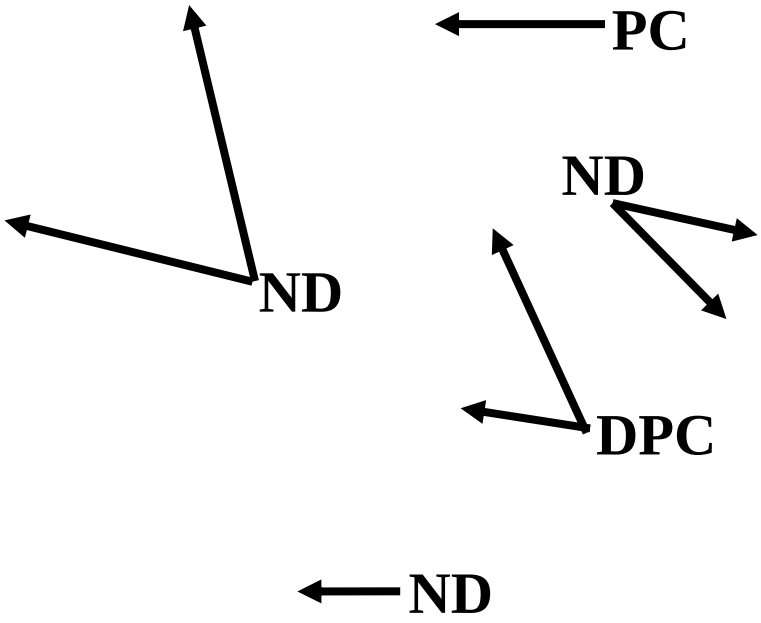


PLATE L: A transverse section of the developing cerebral cortex of group B Pupsat PD21 & PD28 showed a decreased intensity in the immunoreactions in thePyramidal cells, degenerating pyramidal cells with a form of neuronal degeneration(ND) and loss of dendriticarborisation (Mag×400).

4.5.2 Hippocampus

The result from the immunhistochemical localization of calcium binding protein calbindin D28k in the control group A at gestation day GD20 and postnatal days PD 1, PD 4, PD 7, PD 14, PD 21 and PD 28 respectively showed a positive expression of Calbindin D28K in

the pyramidal cells of the hippocampus. The pyramidal cells were distinct and deeply stained indicating positive immunolabelling with Calbindin across the control groups as shown in plates (LI, LIV, LVII). While in group B that received therapeutic dose of artesunate showed a reduced staining intensity with loss of cellular components indicating mild expression of Calbindin. Some pyramidal cells showed an area of neurodegeneration due to artesunate administration across the groups as shown in (LII, LV, and LVII). Group C that received high dose of artesunate showed a negative immunoexpression of Calbindin in the pyramidal cells with a severe form of neurodegeneration and faintly stained neurophil as shown in plate (LIII).



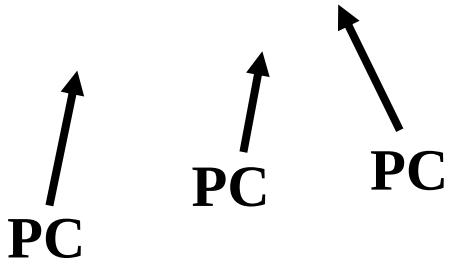


PLATE LI: A transverse section of the developing hippocampus of group A Pups at GD20 & PD1-4 showed a strong immunoreaction with calbindin in thepyramidal cells with immunopositive neuropil (Mag ×400).

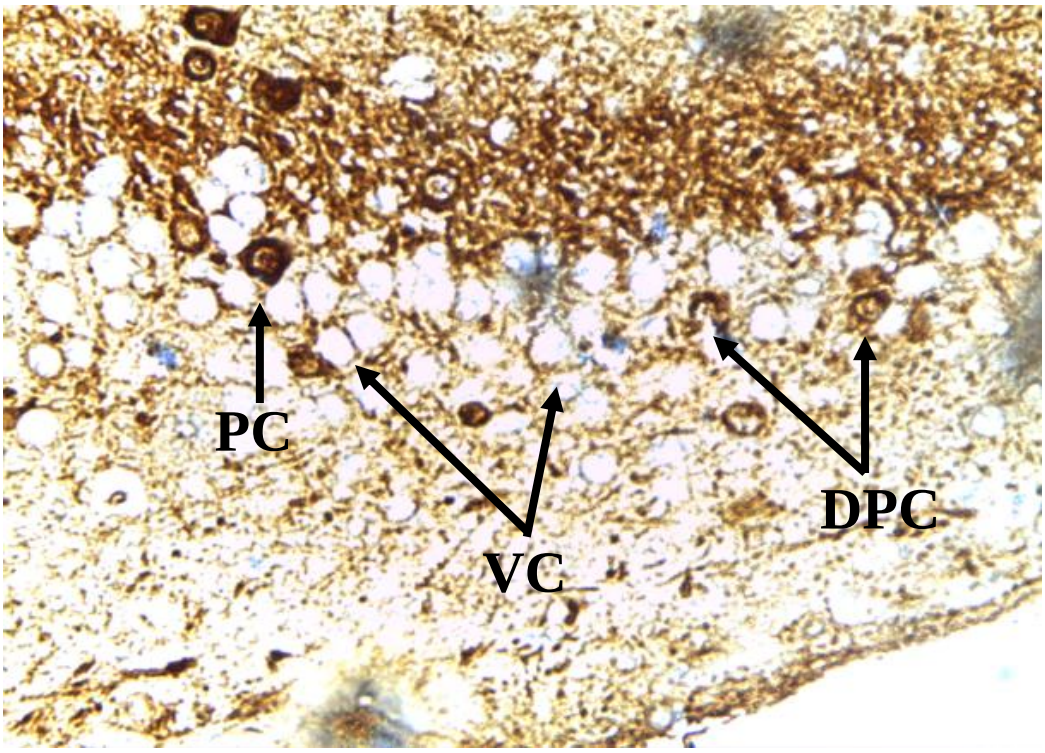


PLATE LII: A transverse section of the developing hippocampus of group B Pups at GD20 & PD1-4 showed intense labelling in the molecular layer with decreased intensity in the Pyramidal cells with few positive pyramidal cells(Mag ×400).



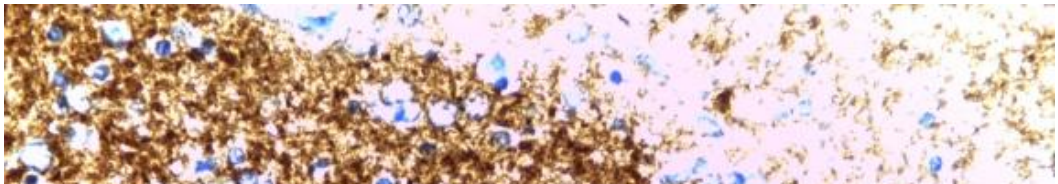
DPC

DPCL

CC

DPC

PLATE LIII: A transverse section of the developing hippocampus of group CPups at GD20 & PD1-4 showing decrease in staining intensity of Calbindin in the pyramidal cells, degenerating pyramidal cells (DPC), clumping(CC), scattered and distortion of the pyramidal cell layer (Mag ×400).



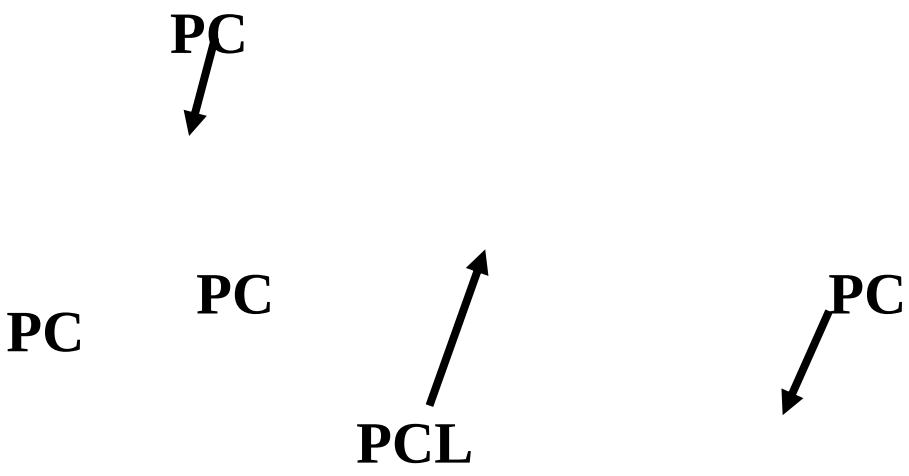
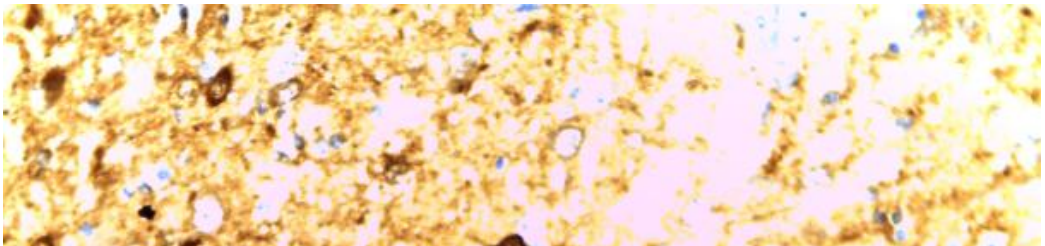


PLATE LIV: A transverse section of the developing hippocampus of group A Pupsat PD7 & PD14 showed an intense labelling of calbindin in the molecular layerPyramidal cell layer with positive expression in the cells (Mag ×400).



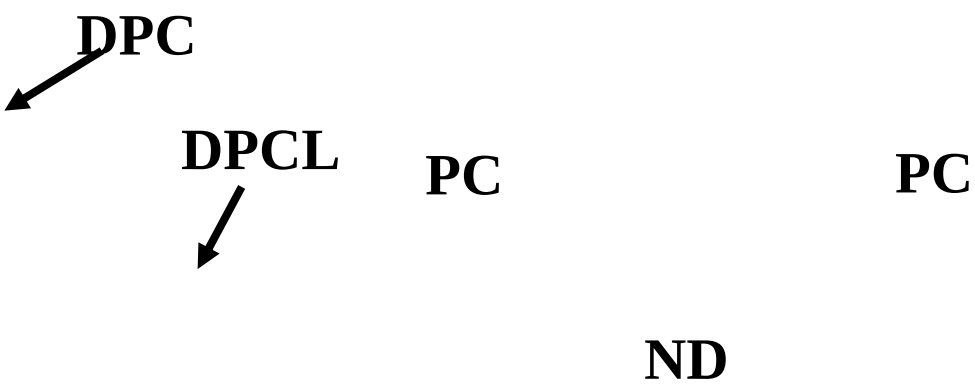


PLATE LV: A transverse section of the developing hippocampus of group B Pupsat PD7 & PD14 showed decrease in staining intensity to Calbindin with few normalpyramidal (PC), degenerating pyramidal cell (DPC) scattered cells and distortion ofpyramidal cell layer (DPCL) (Mag ×400).

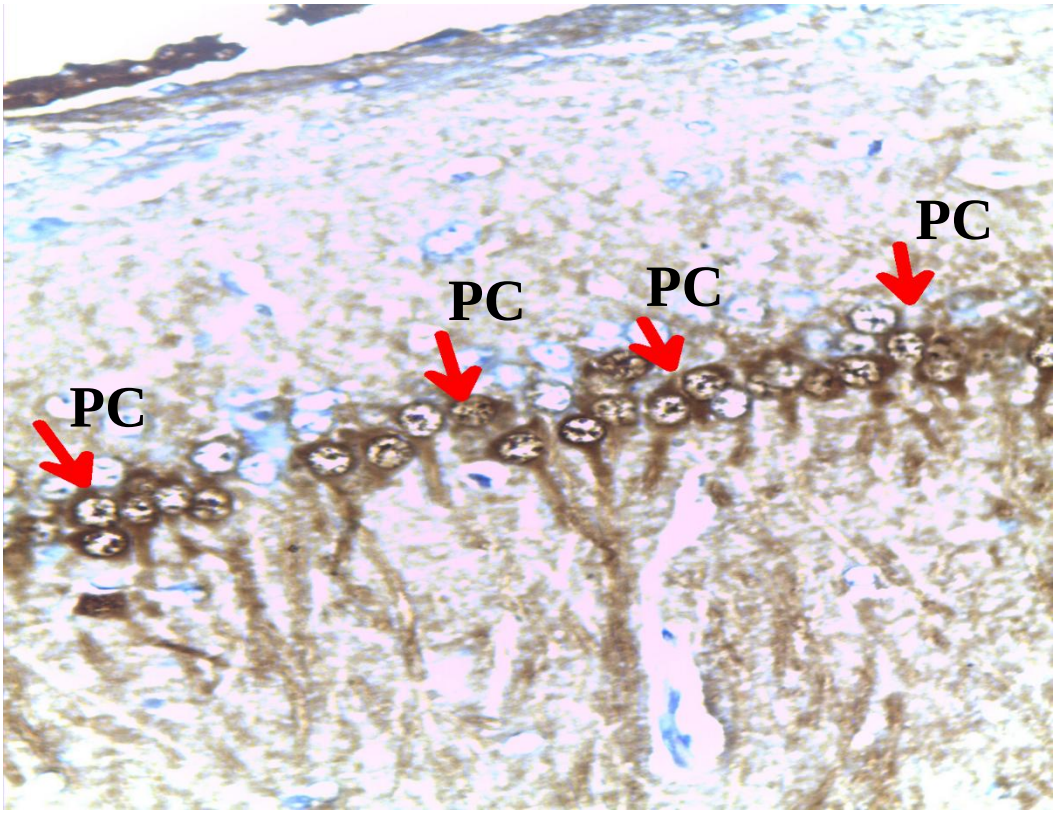
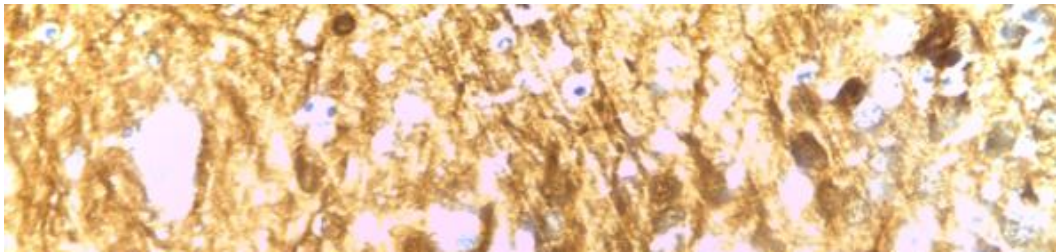


PLATE LVI: A transverse section of the developing hippocampus of group A Pups at PD 21 & PD28 showing intense labeling in the Pyramidal cells. Strong Immunoreactivity is detected in the pyramidal cell layer (Mag ×400).



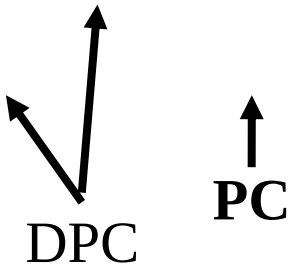




PLATE LVII: A transverse section of the developing hippocampus of group B Pups at PD21 & PD28 showing faintly positive immumoreaction in the pyramidal cellswith some degenerating pyramidal cells and processes (Mag ×400).

CHAPTER FIVE

5.0DISCUSSION

Extensive period of development beginning from the embryonic organogenesis to the postnatal infantile period, the high susceptibility of the undifferentiated neural cells and the lack of self-renewing ability of neurons make the mammalian brain highly vulnerable to the environmental factors (Koestner and Norton, 1991; Inouye, 1995). During early postnatal development of the brain, the proper acquisition of position of the migrating neurons, through an active migration, ultimately affects their morphology, synaptic connectivity and position. Any disturbance to the migrating neurons may cause congenital brain anomalies (Barth, 1987; Hatten, 1990).

The result from the haematoxylin and eosin stain showed degenerative changes and loss of cellular components with reduced population of pyramidal and glial cells in the treatment groups compared with the control section of the cerebral and hippocampal cortex of the developing Wistar rat pups.

Cells death has been reported to result from neuronal degeneration (Waters *et al*., 1994). Cell death may result from necrosis or apoptosis. Apoptosis and necrosis differ morphologically and biochemically. Necrosis is a pathological change which may result from toxic, thermal, traumatic and mechanical factors while apoptosis is an organized form self destruction also known as programme cell death that is mediated by intrinsic and active mechanisms (Wyllie *et al*., 1980). Pathological or accidental cell death is regarded as necrotic and could result from extrinsic insults to the cells such as osmotic, thermal, toxic

and traumatic effect (Faber *et al*., 1981). Cell death in response to neurotoxin might trigger an apoptotic death pathway within brain cells.

Artesunate crosses membrane and affects the cellular integrity of tissue. Artesunate in this study possibly might have acted as a neurotoxin to the hippocampal, and cerebral cortex thereby affecting neuronal integrity and causing disruption in membrane permeability and tissue homeostasis. The effect of artesunate on the cortical layers of the cerebrum and hippocampus observed in this study may be responsible for its degeneration. This present work is in line with the findings of (Eweka *et al.*, 2008 and Ajibade *et al.*, 2012) who revealed that high dose and long term administration of artesunate causes spares cellular populations, degenerative changes, cellular hypertrophy and vacuolation in the medial geniculate body and cerebellum in adult Wistar rat.

Clinical studies have reported adverse effects with artemisinin derivatives when used alone or in combination with other antimalarial in therapeutic doses. Adverse neurological effects after treatment include acute psychosis, depressant syndrome, sleep disturbance (McIntosh and Olliaro, 2000) and post-cerebellar syndrome-ataxia and slurred speech (Sabchareon *et al*., 1998).

It is not impossible that artesunate had irreversible neurotoxic effect on the cortical layers of the cerebrum and hippocampus which resulted in distortion, loss of cellular component and pyramidal cells in the developing cortical layers of the treated hippocampal and cerebral sections. These findings agree with the report of (Ajibade *et al.*, 2006) on cerebellar cortex of wistar ratsfollowing quinine administration.

The sites in the cytoplasm of basophilic ribosomes stain intensely with Toluidine blue. These basophilic regions have been described as early as 19th century and were named according to the cells being studied. They were called Nissl bodies in neurons. When appropriate stains are used Rough Endoplasmic Reticulum (R.E.R) and free ribosomes appear under the light microscope as basophilic granular areas called Nissl bodies. The pyramidal cells appeared distinct when Nissl substances were stained which is consistent with the finding of Gharravi *et al.*, 2007 and Doaa *etal.*, 2014. Neuronal degeneration has been reported to cause a reduction in Nissl bodies (Martin *et al*., 1998). The observed neuronal degeneration in association with loss of Nissl bodies with resultant reduced staining intensity of the Nissl substances in the developing cerebral and hippocampal cortex of the artesunate-treated rats in this study is consistent with findings of Ajibade *et al.*, (2009). Injury to axons or neuronal exhaustion resulting from strong or prolong stimuli causes a reduction in the number of Nissl bodies. This alteration which is called chromatolysis which occurs simultaneously with nuclear migration to the periphery of the perikaryon and consequently the RNA level is reduced (Louis *et al*., 1986).

Chemical and toxic substances affect the Nissl substances thereby influencing their metabolic activity (Davis and Robertson, 1991). The findings from this study agreed with degeneration and vacuolar changes which have been observed in many large brain stem neurons in mice that were treated with trimethyl chloride. These neurons acquired a chromatolytic character with eccentric nuclei and loss of Nissl substances which became progressive in ultra structure (Change *et al*., 1983).

Loss of Nissl substance observed in this study is also supported by the earlier findings of Classen *et al*., (1999) who reported that loss of Nissl substance in neurons and shrinkage of nucleus which became prominent in the cerebellar roof, pontine and vestibular nuclei of dog following intramuscular administration of artemether. Neurons have normal morphology with large cell body and clear seen Nissl bodies. Most of neurons shrinking with condensed nuclei and sparse Nissl bodies. Group C Neurons had larger cell bodies than that of group B, most of neurons had Nissl bodies and milder condensed nuclei compared with that of group B.

Developmental studies have indicated that calbindin is expressed early in embryonic central nervous system (CNS), following the cessation of mitosis, when neurons are ready to migrate (Enderlin *et al.,* 1987). It has been shown to be temporarily expressed during brain development in numerous nerve cell systems and animal species (Hogan *et al.*, 1993; and Friauf, 1994).

In the present study calbindin immunoreactivity was evaluated in the hippocampus and cerebrum to examine the effects of artesunate. The present study noted loss of neurons and dendritic arborisation and a decrease in staining intensity of calbndin in the treated groups. There was also severe decrement of stained neurons. This is Similar to findings of (bernochi *et al*., 2015) who reports that neuronal damage and cell loss in the cornu ammonis (CA) area of the female rat hippocampus was noted after exposure at 900MHz. Bas *et al.,* and Odaci *et al*., 2009 reported that prenatal exposure revealed decrement in the number of granule cells in the dentate gyrus by cell death and the inhibition of the

differentiation of neural stem cells into neurons Salford *et al.*, 2003 also reported that different hippocampal regions showed different susceptibility to injury.

Odaci *et al.*, 2008 findings showed a decrease in calbindin on the cerebral cortices which denotes a decrement in the Calcium buffering capability which might ultimately lead to cell death. It also showed areas of degenerated pyramidal cells, distortion of the cell layers. This is similar to the present findings. Dhiral, *et al.,* (2013) reported that lack of calcium might cause severe damage leading to tissue degeneration.

Schwaller *et al.,* (2002) and Bastianelli *et al.,* (2003) reported that the rodent cerebellum calbindin is detected in the cytosol of one or more populations of neurons at relatively high concentrations. The fast buffer calbindin is exclusively present in Purkinje cells, in the soma dendrite branches and axons. Neurons rich in calbindin appeared relatively resistant to degeneration in several acute or chronic disorders (Dhiral, *et al.,* 2013). In the developing cerebellum, Calbindin is low until embryonic day 18 and reaches a peak at postnatal day 20. Thereafter, the calbindin levels remain high (Bastianelli, 2003). The present study showed that all the postnatal stages of the control rats in the developing cerebral cortex and hippocampus have neurons that were intensely immunopositive to calbindin in all compartments, which is in the soma, dendrite and axon. This finding is in agreement with the study of (Schwaller *et al.,* 2002). There is a good evidence of the presence of calbindin in Pyramidal cells and dendritic spines that may change their morphology to partially compensate for changes in calcium homeostasis due to the loss of calcium binding proteins (Bernocchi et al., 2015).

The intensity of the positive immunolabelling was found to have increased at PD14, 21 andPD28 at the dendrite branches, and soma in cerebral layer (CL). This finding is similar to the findings of (Pisu, 2004; Avella *et al.*, 2006).

In the hippocampus, calbindin (CB) is primarily demonstrated in dentate gyrus (DG) granule cell layer; the somata of granule cells to be intensely immunostained while their dendrites are moderately stained (Freund *et al.,* 1996; Keuker *et al.,* 2003,). Calbindin is also expressed in a CA1 pyramidal cell subpopulation and in some interneurons. It is also present in GABAergic neurons mainly innervating the distal dendrite of the principal cells, and controlling the efficacy of afferent inputs (Freund *et al.,* 1996; Keuker *et al.,* 2003,). Hippocampal calbindin positive interneurons appeared resistant to epileptic injury in rat models (Magloczky *et al.,* 1993).

Studies on the ontogeny of cells which express calbindin at early postnatal stages, and showed that calbindin is present in granule cells of dentategyrus and cornu ammonis during the very early postnatal stages as reported by (Ciaroni *et al.*, 2002; Brandt *et al.*, 2003) is in collaboration with the present finding. Calbindin (CB) has been reported to reappear on PD2 in clusters of neurons in the radiatumand the orienslayers and in the granule cells of the mouse dendate gyrus (DG) (Soriano *et al.*, 1994). Progressive expression of Calbindin appeared in an outside-in pattern in the dentate gyrus (DG) granule cell layer is also reported also by (Abraham *et al.,* 2009*)* between PD11 and PD17 of control rat postnatal development.

Interneurons within the granular layer were also detectable as well as strongly Calbindin labelled interneurons (Piccolini *et al.,* 2013). These latter were found at PD17 in the cornu ammonis (CA) layers, where some groups of pyramidal cells were also stained. Calbindin labelled fibers was also distributed in the molecular layer and hilus of DG and beneath the CA3 pyramidal layer.

The present study showed intense immunolabelling to at PD14, PD21 and PD28.this finding is comparable to studied reported by Ciaroni *et al.*, (2002) and Brandt *et al.*, (2003). Changes in the immunolabelling to Calbindin is indicative of changes in the level of the protein, since it has been shown that loss of Calbindin after commissural kindling stimulation is due to a decrease in the protein (Braun *et al.,* 1990). The expression of Calbindin is regulated by the functional state of the hippocampal circuit (Nagerl *et al.,* 2000), since it is needed for normal short-term synaptic plasticity between the dendate gyrus DG granule cells and CA3 neurons. (Blatow *et al.,* 2003), and modulates the excitability of the CA1 pyramidal cells (Arabadzisz *et al.,* 2002).

Calcium binding proteins (CBPs) has been studied to identify different functional cell types in the brain neocortex, hippocampus, cerebellum and thalamus. (Bastianelli *et al*., 2003; Jinno *et al.*,2006). Calcium binding proteins (CBPs) have been extensively studied as neuronal immunohistochemical markers of specific cell populations in the CNSand have been implicated in neuroprotection in different pathological conditions since they function as buffers for calcium excess (Celio *et al.,* 1990, 1996). Regarding the specific involvement, calbindin (CB) sequester the excess calcium during development and synaptic plasticity. Calbindin (CB) alters the duration of action potentials, and promotes neuronal

activity as protecting molecules against the damages derived from excessive calcium influx (Baimbridge *et al.,* 1992).

In the absence of calcium binding proteins (CBPs), there is a high calcium accumulation inside the cytosol causing hyperexcitability that often leads to neurodegeneration (Bastianelli *et al.,* 2002). CBPs immunoreactivity has been detected in similar populations of neurons in mammals, such as humans, rodents and birds (Garcia-Segura *et al.,* 1984, 2001). Therefore, too high or too low level of intracellular calcium causes both neuronal and functional disturbances.

This suggests that artesunate exposure during pregnancy causes alteration in the immunoreaction of calcium-binding proteins (calbindin D28K) in neurons of the hippocampus and cerebral cortex of rat pups. Changes of immunoreactivity of Calbindin may indicate an important role in calcium-dependent artesunate induced developmental and functional alterations.

The results on the morphology of the pups in the control group showed a normal body structure with no visible physical change. Some pups in the normal dose group showed a mild dark spot on the neck region (cervico-thoracic). Pups in the high dose group showed incidence of craniofacial malformations with a prominent dark spot on the face and both limbs which signifies hemorrhage. This present finding is in line with the findings of **(**Chhandamayee *et al.,* 2011) who reported that lamotrigine (LTG) exposed rat foetus had well defined dark brown swellings over parieto-occipital region of head (exencephaly) and hemorrhages of various sizes over the body, especially over the cervicothoracic junction. The present findings contradict the reports of (Longo *et al.*,2006) who observed an

umbilical hernia as the only malformation detected in the fetuses when 14 mg/kg of artesunate was administered on days 6 to 8 of pregnancy.

There was a reduction in the number of litters delivered in the artesunate treated animals when compared with control groups. The number of litters delivered differs significantly in the groups. In the control groups, the litter number was between seven to nine (7-9). While the group that received normal dose of artesunate 4mg/kg for three days and 2mg/kg 4days, the number of litters delivered ranges from five to seven (5-7) while the high dose group reduced to two (2) with a lot of resorption.

This is in line with the work of (Adebisi 2010 and Clark *et al*., 2004) The findings in this study was similar to the studyof Padmanabhan*et al.,* (2003) who reported that lamotrigine (LTG) induces intrauterine growth retardation in mice, whereas multiple doses caused a dose dependant increase in embryonic resorption and craniofacial malformations. White *et al.,* (2004) reported a totally resorped litters and fetuses with bent and or shortened bones.

Artemesinin administered between pregnancy days 6 and 15 showed about 50% loss of embryos was observed at the lowest dose, while at the higher doses total loss was observed again (WHO, 2003). Longo *et al.,* (2006) also observed large embryonic losses at relatively modest doses of Artesunate in rats. In mice, 100% resorptions were observed at a dose of 21.4 mg/kg, while in rats and rabbits 90% resorptions were seen at 10.7 and 2.7 mg/kg, respectively. These present study shows that there were about 98% of resorptions in pregnant dams at the high dose group that received 10mg/kg for 3days and 4mg/kg for the remaining four days.

The mechanisms of teratogenic effect have been reported by (White *et al.*, 2005) that the most sensitive period for teratogenic effect in rats is between days 10 to 11 of pregnancy. Artesunate produces a marked reduction in the numbers of embryonic erythroid cells around 18 - 24 hours after a single dose, with the effect persisting for several days. Laffan *et al.*, (2005) showed that embryonic erythroid cells were more sensitive to the cytotoxic and lytic action of artesunate than adult erythrocytes, it also suggests that an inhibitory action on the development of early erythroid precursor cells leading to anaemia and hypoxia, and resulting finally in damage to growing tissues and embryonic death.

Longo *et al.*, (2006), reported that this toxicity could be attributable to inhibition of angiogenesis in the yolk sac. Artesunate may affect pituitary hormone secretion, leading to complete resorption by a mechanism that does not operate in humans (Nosten *et al.*, 2006), with the view that wide use of artemisinin especially in multi drug-resistant *P. falciparum* malaria in pregnant women.

Several studies have demonstrated a correlation between intrauterine growth retardation and teratogenesis (Mesembe *et al.*, (2004). The parameters measured in the study include fetal body weight, brain weight, brain length, brain width, forelimb, hindlimb, and tail lengths. In the present study the teratogenic potentials of artesunate in most of the above named parameters were significantly reduced in the therapeutic and high dose groups when compared with the control groups. The present study also corroborates with those reports of developmental toxicity of artesunate on crown rump length (CRL), tail length and brain weight (Xu and Zhang, 1996; WHO, 2002a; WHO, 2002b; Lou *et al.*, 2003 and Bhabagrahi

et al., 2010) who reported that the fetal skeletons revealed significantly short crown rump length (CRL) and tail length (TL) in rats treated with lower doses of artesunate and arteether. Four of the 12 fetuses (artesunate 10 mg/kg) had short limb bones, with humerus, femur, tibia and fibula. Adebisi, (2010) reported that artesunate even at relatively low dose appears to be hazardous to the structural integrity of the bones particularly when administered at critical period of development and also noted bone marrow changes-hypoplasia in both myeloid and erythroid population.

The findings in the present study agree with the reports of (Mesembe *et al*., 2004) who observed a significant reduction in the crown rump length and tail length in rats treated with 0.4 and 0.8 mg/kg of artesunate and (Clark *et al.*, 2004) who studied the effects of artesunate as well as of the combination of artesunate with chlorproguanil and dapsone and observed the foetuses consisted of a low incidence of cardiovascular defects (ventricular septal and vessel defects) and of syndrome of skeletal defects, especially with long bone shortening and bending. Iqbal *et al.,* 2001 and Rahmani *et al*., 2006, studied the teratogenic effects of lamotrigine on mouse fetus and noted reduction of body weight and height with increased malformations of vertebral columns and limbs.

Earlier studies of other teratogens have shown an intimate relationship between malformation of the nervous system and the various brain measurements (antero-posterior diameter, medial antero-posterior diameter, and transverse diameter), length and diameter of spinal cord, (Cragg, 1972; Singh and Padmanabhan, 1978; Igiri *et al.*, 1999), brain weight (Rahmani *et al.*, 2006). This confirms earlier reports that a teratogenic insult on the

developing brain during neurogenic period induces anomalies (Singh *et al.,* 1972; Sturrock, 1978; Akpan *et al.,* 1992; Igiri *et al.,* 1999).

Artesunate exert their antimalarial action via an iron catalysed decomposition of the endoperoxide bridge to produce free radicals (Meshnick, 2001) which destroys the parasite possibly by alkylation of the plasmodial proteins (Asawamahaskda *et al.,* 1994; Bhisutthibhan *et al.,* 1998). The generated free radicals may cross the placenta and elaborate a cascade of teratogenic influence (Kosh *etal.,* 1995).

In the present study artesunate was found to have significant embryo fetal toxicity in rats with an incidence of craniofacial malformation. Lou *et al,* (2003) has suggested that artesunate induces its developmental toxicity via lipid-peroxidation and by neutralizing the antioxidant defense mechanism of the organism. It is also conceivable that artesunate exerted its toxic effect by promoting DNA-strand breakage, thereby damaging other genomic structures (Clarkson and Thompson, 2000) leading to a block in protein synthesis and elaboration of enzymes.

CHAPTER SIX

6.0SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

In the present study, artesunate (AS) was found to have an embryotoxic effect on rats with apparent reduced number of foetuses and litters with severe resorption at the high dose group. There was an incidence of craniofacial malformation, dark spot on both limbs (haemorrhage) was also observed. Morphometrical changes on the foetal body and the developing brain were significantly ($p \leq 0.05$) lower in all the parameters at the therapeutic and high dose groups. This could be attributed to the degree of maturation in the brain and as well as normal and high administration of artesunate which leads to intra uterine growth restriction. The main finding in the study was on the histopathology. The histopathological changes ranges from mild to severe degeneration of cerebral and hippocampal pyramidal cells, others include necrotic and clumping of cells, vacuolated, scattered, swollen, faded cells and distortion of the cortical cell layers with loss of branching patterns and eventually cell death.

There was an apparent loss of Nissl's substances with reduced staining intensity. The nuclear materials migrate towards the periphery of the perikaryon called central chromatolysis. Immunolabelling of neurons specific for calcium binding protein Calbindin D28K demonstrated fewer positive cells with less staining intensity of the pyramidal cells in the treated pups compared to the control. These results indicate that artesunate (AS) has a narrow safety margin in rats and could induce teratogenic and neurotoxicological effects in which the mechanism may involve calcium homeostasis in the brain.

6.2 Conclusion

The present study has enabled the various observations to the general conclusions of the consequences of exposure to artesunate as dose and time dependent. These results indicate that artesunate has a narrow safety margin in rats and could induce teratogenic and intra-uterine growth restriction. Histologically, the safe dose showed decrease in cell proliferation with marked difference in the layer stratification of the cerebrum while high dose showed complete retardation of cerebral and hippocampal development in the few pups.

Imunolabelling of neurons specific for the calcium binding protein Calbindin D28k demonstrated fewer primitive cells characterized by less intensity of the stain in the pyramidal cells in all the treatment groups which the mechanism may involve calcium homeostasis in the brain.

6.3 Recommendations

1. It is recommended that further work should be carried out in other parts of the brain prenatally.
2. It is also recommended that neurobehavioral studies should be done to ascertain functional level of the damage caused by Artesunate.
3. Quantification of calbindin in the developing cerebrum and hippocampus of wistar rat pups should be investigated using an immunohistochemical profiler or image J.

6.4 Contribution to Knowledge

The present study was able to highlight morphometric changes in the rat pups due to prenatal administration of Artesunate and these changes were significant at:

Brain weight in Group B at PD 7 (0.45 ± 0.01) PD 14 (0.55 ± 0.01) PD 21 (0.75 ± 0.01) and PD 28 (0.99 ± 0.02). Tail length in Group B at PD1 (1.16 ± 0.01), PD 4 (2.18 ±0.25), PD 14 (2.52 ± 0.04), PD 21 (2.69 ± 0.01) PD28 (3.22 ± 0 .05) and in Group C, PD1 (1.40±0.00) and also imunolabelling of neurons specific for the calcium binding protein Calbindin D28k demonstrated fewer primitive cells characterized by less intensity of the stain in the pyramidal cells in all the treatment groups as a result of artesunate exposure.

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APPENDICES

APPENDIX I

Drug used for the research = Artesunate (AS)

LD_{50} of Oral Artesunate = 900mg/kg body weight

Concentration for artesunate ======= 160mg/80ml

Dose ===== 4mg/kg for 3 days

2mg/kg for 4 days

80ml ==== 160mg

Stock Solution is = 2mg/ml

Artesunateadministration base on body weight of animals used

4mg----------1000g

1000g--------- 4mg of AS

Therefore, 200g animal will receive ======= 4/1000 ×200/1 = 0.8mg

2mg =1ml

0.8mg =1/2 ×0.8/1 =0.4ml

Total volume = 0.4×3+0.2×4 =====1.2+0.8========== 2ml

APPENDIX II

Table 1: Effect of Artesunate on the Foetal weight of pubs (g)

Time (Days)	Treatment (mg/kg)		
	Control	**Artesunate (Normal dose)**	**Artesunate (High dose)**
GD20	3.73 ± 0.08	3.41 ± 0.13	
PD1	5.46 ± 0.15	4.78 ± 0.16*	4.54 ± 0.17*
PD4	7.53 ± 0.44	7.03 ± 0.21	
PD7	9.97 ± 0.24	11.10 ± 0.20*	
PD14	15.25 ± 1.80	11.39 ± 0.27	
PD21	21.78 ± 0.73	14.33 ± 0.16***	
PD28	28.30 ± 2.14	22.48 ± 0.20	

Values are presented as mean ± S.E.M., * = $p<0.05$, ***= $p<0.001$ – One-way ANOVA followed by Dunnett test, n= 5, GD =gestation day, PD =postnatal day

Table 2: Effect of Artesunate on the Brain Weight of Pups

Time (days)	Treatment (mg/kg)		
	Control	**Artesunate(Normal dose)**	**Artesunate(High dose)**
GD20	0.15 ± 0.01	0.14 ± 0.00	
PD1	0.34 ± 0.16	0.17 ± 0.01	0.18 ± 0.01
PD4	0.36 ± 0.03	0.31 ± 0.01	
PD7	0.51 ± 0.01	0.45 ± 0.01**	
PD14	0.92 ± 0.03	0.55 ± 0.01***	
PD21	1.02 ± 0.01	0.75 ± 0.01***	
PD28	1.09 ± 0.02	0.99 ± 0.02*	

Values are presented as mean ± S.E.M., * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ – One-way ANOVA followed by Dunnett test, n= 5, GD =gestation day, PD =postnatal

Table 3: Effect of Artesunate on the Brain Length of Pups

Time (Days)	Treatment (mg/kg)		
	Control	**Artesunate (Normal dose)**	**Artesunate (High dose)**
GD20	0.98 ± 0.37	0.93 ± 0.02	
PD1	0.98 ± 0.02	0.77 ± 0.17	0.5 ± 0.40
PD4	1.10 ± 0.05	1.14 ± 0.02	
PD7	1.54 ± 0.48	1.53 ± 0.07	
PD14	1.90 ± 0.00	1.71 ± 0.01***	
PD21	2.10 ± 0.35	1.93 ± 0.03**	
PD28	2.30 ± 0.11	2.11 ± 0.06	

Values are presented as mean ± S.E.M., **= $p<0.01$, ***= $p<0.001$ – One-way ANOVA followed by Dunnett test, n= 5, GD =gestation day, PD =postnatal

Table 4: Effect of Artesunate on the Brain Width of Pups

Time (Days)	Treatment (mg/kg)		
	Control	**Artesunate (Normal dose)**	**Artesunate (High dose)**
GD20	0.59 ± 0.01	0.58 ± 0.02	
PD1	0.63 ± 0.02	0.60 ± 0.16	0.55 ± 0.00
PD4	0.89 ± 0.01	0.83 ± 0.01**	
PD7	1.00 ± 0.02	0.93 ± 0.01*	
PD14	1.15 ± 0.02	1.00 ± 0.00***	
PD21	1.22 ± 0.01	1.08 ± 0.02***	
PD28	1.23 ± 0.01	1.21 ± 0.01	

Values are presented as mean ± S.E.Mw., * = $p<0.05$, ***= $p<0.001$ - One-way ANOVA followed by Dunnett test, n= 5, GD =gestation day, PD =postnatal

Table 5: Effect of Artesunate on the Fore Limb Length of Pups

Time (Days)	Treatment (mg/kg)		
	Control	**Artesunate (Normal dose)**	**Artesunate(High dose)**
GD20	1.17 ± 0.02	1.18 ± 0.02	
PD1	1.31 ± 0.19	1.16 ± 0.01***	0.95 ± 0.05***
PD4	2.20 ± 0.11	1.78 ± 0.02	
PD7	2.36 ± 0.04	2.18 ± 0.25**	
PD14	3.10 ± 0.21	2.52 ± 0.04*	
PD21	3.82 ± 0.06	2.69 ± 0.01***	
PD28	3.38 ± 0.14	3.22 ± 0.05	

Values are presented as mean ± S.E.M., * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ – One way ANOVA followed by Dunnett test, n= 5, GD =gestation day, PD =postnatal

Table 6: Effect of Artesunate on the Hind Limb Length of pups

Time (Days)	Treatment (mg/kg)		
	Control	**Artesunate(Normal dose)**	**Artesunate (High dose)**
GD20	1.28 ± 0.02	1.25 ± 0.02	
PD1	1.44 ± 0.03	1.28 ± 0.02**	1.40 ± 0.00
PD4	2.50 ± 0.30	2.08 ± 0.02	
PD7	2.64 ± 0.05	2.46 ± 0.02*	
PD14	3.52 ± 0.20	2.84 ± 0.04*	
PD21	4.38 ± 0.16	3.22 ± 0.04***	
PD28	4.66 ± 0.32	4.08 ± 0.04	

Values are presented as mean ± S.E.M., * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ – One-way ANOVA followed by Dunnett test, n= 5, GD =gestation day, PD =postnatal

Table 7: Effect of Artesunate on the Tail Length of pups

Time (Days)	Treatment (mg/kg)		
	Control	**Artesunate (Normal dose)**	**Artesunate (High dose)**
GD20	1.42 ± 0.02	1.36 ± 0.02	
PD1	1.62 ± 0.04	1.52 ± 0.01*	1.40 ± 0.00**
PD4	2.64 ± 0.13	2.24 ± 0.05*	
PD7	2.82 ± 0.04	2.85 ± 0.03	
PD14	5.33 ± 0.36	3.40 ± 0.07**	
PD21	6.92 ± 0.15	4.12 ± 0.02***	
PD28	7.96 ± 0.37	5.64 ± 0.02**	

Values are presented as mean ± S.E.M., * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ – One-way ANOVA followed by Dunnett test, n= 5, GD =gestation day, PD =postnatal