EFFECT OF ETHANOL ON CEREBRAL AND CEREBELLAR CORTICES, NEUROBEHAVIOUR AND TRACE ELEMENTS OF NEONATAL WISTAR RATS AFTER INTRAUTERINE EXPOSURE

By

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Declaration

I hereby declare that the research work reported in this thesis entitled "**Effect of Ethanol on Cerebral and Cerebellar Cortices, Neurobehaviour and Trace Elements of Neonatal Wistar Rats After Intrauterine Exposure**" was carried by me in the Department of Human Anatomy, Faculty of Medicine, A.B.U, Zaria, under the supervision of Prof.S.S. Adebisi and Dr. W.O. Hamman. The information derived from the literatures has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree in any institution.

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Certification

This thesis entitled "**Effect of Ethanol on Cerebral and Cerebellar Cortices, Neurobehaviour and Trace Elements of Neonatal Wistar Rats After Intrauterine Exposure**" by Shehu Ibrahim El-ladan meet the regulation governing the award of Master of Science (M.Sc.) degree in Department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria, under the supervision of **Prof S.S. Adebisi** and **Dr W.O. Hamman**. It is therefore 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 it out successfully the research reported in this thesis and in loving memory of my friends and brothers Nuru Aliyu Nuhu and Muhammad Usman El-ladan (Kane).

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Abstract

Alcohol is the most widely used and abused psychoactive drug globally. Ethanol is the main ingredient in the three classes of alcoholic beverages. Children of alcoholic pregnant women are prone to wide spectrum of disorder referred to as Foetal Alcoholic Spectrum Disorder (FASD). The present study aimed at evaluating the teratologic effects of ethanol on the histology, neurobehaviour and trace elements of cerebellar and cerebral cortices of the neonatal Wistar rats. Twenty eight (28) female Wistar rats were mated with matured males in ratio 2:1. Pregnant dams were then grouped into 7. Group A served as the control group that received distilled water, Groups B, C and D were administered 0.5ml 20% ethanol for 7, 14 and 20 days during pregnancy respectively. Groups E, F and G were given 0.5ml 30% ethanol in the above stated manner. Following parturition, morphometric indices of the litters were taken at birth. Neuro-behavioural assessments were done on postnatal days 5, 6 and 7. Cerebral and Cerebellar tissues were obtained and processed for paraffin embedding for histological evaluation using Haematoxylin and Eosin and histochemical study using Cresyl Fast Violet Stains. Atomic Absorption Spectrophotometry was used to quantify Iron, Zinc, Copper and Manganese in the cerebrum and cerebellum. The result showed effects on the weight and crown rump length of the pups by exhibiting lower weight and crown rump length values in the ethanol treated neonates. Intrauterine ethanol exposure was shown to affect the development of vestibular and postural reflexes, sensory and motor coordination with significance at $p<0.05$.Histological evaluation of the cerebral cortex revealed histopathological presentations such as pyknosis, karyorhexis, clumping of cells and neural degeneration. Histology of the cerebellar cortex showed degeneration and disorientation of purkinje cells. Histochemical evaluation of the cerebral cortex showed degradation of nissl substance. Moreover, the histochemistry of the cerebellar

cortex demonstrated chromatolysis of the purkinje cells.The concentration of Iron in cerebrum was depleted when compared with the control, there was no statistical significance at $P<0.05$, while the amount of Iron in the cerebellum was significantly elevatedin the treated groups ($P<0.01$, $P<0.001$). The amount of Copper in the cerebrum was also depleted while significantly elevated in the cerebellum ($P<0.05$). The concentration of Manganese in the cerebrum and the cerebellum was elevated in some of the treated groups and depleted in some of the treated groups as well, there was no statistical significance at $P<0.05$. The amount of Zinc in the cerebrum was depleted with high statistical significance ($P<0.001$) in all the ethanol-treated groups when compared with the control group. On the other hand, the concentration of Zinc in the cerebellum was also depleted but there was no statistical significance ($P<0.05$). The findings from this study revealed that, intrauterine ethanol exposure has potency of inducing teratogenicity on the morphometry, neuro-behaviour, histology, and alters the trace element concentrations in the brain of the neonates irrespective of the trimester equivalence.

CHAPTER ONE

1.0 Introduction

1.1 Background

Alcohol is the most widely used and abused psychoactive drug in many regions of Nigeria (WHO, 2004). Common slangs used include booze, bubbly, firewater, joy juice, sauce, liquid courage, and many others (NIAAA, 2000). Legal for those aged 18years and above in Nigeria (WHO, 2014) and the National legal Blood Alcohol Concentration (BAC) while driving is 0.05% (WHO, 2014). While there are many types of alcohol which is (an entire class of chemicals), the type that is found in drinks and medicines is known as 'ethyl alcohol' or 'ethanol.' A yeast enzyme changes the simple sugars that are found in grapes, potatoes, or corn into ethanol – the alcohol found in beer, malt liquor, wine, liquors such as vodka and whiskey, wine coolers, and liqueurs like Irish cream (NIAAA, 2000). One of the ways ethanol is thought to effect its toxicity is by the inhibition of folic acid uptake by the intestinal bacteria, and its metabolism in the liver. Folic acid is a well-known essential co-factor in the synthesis of purine and pyrimidine components of DNA and RNA, which are important in the formation of protein for normal development, growth, and repair of tissues (Adebisi, 2003a). Ethanol is the main ingredient in the three classes of alcoholic beverages: distilled spirit, wine and beer. Other forms such as methanol have immediate toxic effects that make them unsuitable for drinking (Adebisi, 2004).

The ethanol concentration for common types of alcoholic drinks is as follows:

Beer: 4-6%, Malt liquor: 5-8%, Wine: 7-15%, Wine coolers: 5-10%, Champagne: 8-14%, Hard liquor (Distilled spirits - vodka, rum, whiskey, etc.): 40-95%, and Grain Alcohol: 95-97.5%. (NIAAA, 2000). A standard drink (wine and champagne) contains

18 grams of pure ethanol – approximately the amount found in; Beer 12 Oz. (1 Can or Bottle), Wine 5 Oz. (1 Glass), Hard Liquor 1.5 Oz. (1 Shot) (NIAAA, 2014).

Alcohol is widely recognized as a neuroteratogen (Allam and Abdulhamid, 2013; Moutard *et al.*, 2012; Ohrtman, 2006; Maier and West, 2003; Sandra and Michael, 2003; and Thomas *et al*., 1998). Some women who drink heavily during pregnancy may have their children affected with alcohol-related deficits, such as neuroanatomical malformations (Alex and Feldmann, 2012; Clarren *et al*., 1978), cognitive dysfunction (Coles *et al*., 1991), or other behavioral disorders (Thanabhorn, 2006; Coles *et al*., 1985; Ernhart *et al*., 1985). Other reported observations include absence of skull vault, under-ossification and asymmetry of the constituent skull bones resulting in severe reduction of the cranial volume in rats (Adebisi, 2002a; Adebisi 2002b).

Foetal alcohol syndrome (FAS) results from maternal consumption of alcohol during pregnancy and represents the extreme end of a continuum of foetal alcohol spectrum disorders (FASD). A diagnosis of FAS is made on the basis of three defining characteristics: central nervous system (CNS) damage or dysfunction, pre- and/or post-natal growth retardation with height or weight at/or below the 10th percentile, and distinct dysmorphic facial anomalies including a smooth philtrum, thin upper lip and small palpebral fissures (Jones and Smith, 1973). Growth impairment either antenatal, postnatal or both is a feature of FAS and a common finding in the mild effect called foetal alcoholic effects (FAE). Severe growth retarded children do not show significant accelerated growth following rehabilitation (Adebisi, 2002a; Adebisi 2002b).

The most severe end of the spectrum is foetal alcohol syndrome (FAS) (Warren *et al.*, 2001; Warren and Bast, 1988; Abel, 1984; Streissguth *et al.*, 1980), for which facial abnormalities, growth deficits and central nervous system (CNS) abnormalities are the defining diagnostic features. Despite efforts to educate the public about FASD, the prevalence of ethanol consumption in women of child-bearing age has remained essentially the same (Caetano *et al.*, 2006; CDC, 2004; NIAAA, 2000). Moreover, diminution in cranial and limb bone dimensions, body emaciation, low skeletal weights and decrease in size of ethanol-treated animals had been observed (Adebisi, 2004; Adebisi, 2003a; Adebisi, 2003b; Adebisi, 2000; Adebisi, 1995).

Some of the common traditional alcoholic beverages in Nigeria are: ***Burukutu*** which is a popular alcoholic beverage of a vinegar-like flavour prepared from sorghum grains and fermented guinea corn and consumed in the Northern Guinea savannah region of Nigeria (Haard *et al.,* 1999). It is also typically consumed in the Ibadan region and ranges in alcohol content from 3–6% (Bennet *et al.,* 1998). *Burukutu* is the most popular alcoholic beverage in the rural areas of northern Nigeria and in poor urban neighbourhoods because it is more affordable than commercially brewed beer. It is often consumed as food because it is thick and heavy. The producers of *burukutu* are overwhelmingly women (Obot, 2000). **Palm wine** is to Southern Nigerians what *burukutu* is to the Northerners. Unlike *burukutu,* it is not synthesised but obtained as a natural whitish sap collected in vessels attached to the base of the tree from where some leaves have been removed. Fresh wine from these sources is sweet and contains little alcohol but, with fermentation, the alcohol content increases with time. Unbottled palm wine has a lower alcohol content of around 3% than bottled palm wine which has around 4% (Stanley and Odejide, 2002). In general, palm wine, which has an alcohol

content of 3–6%, is also widely consumed in the Ibadan region of Nigeria (Bennet,1998).

The main alcoholic beverages produced and consumed by the Tiv people of Central Nigeria are ***tashi*** and ***ityo***, also known as palm wine. Both alcoholic beverages contain nutrients rich in vitamins such as B and C found in *ityo* and complex carbohydrates in *tashi*. ***Akpetashi***, a native gin to some Ghanians community, is distilled from *tashi* (Gire and Dimah, 2001). ***Pito*** is the traditional beverage of the Binis in the mid-western part of Nigeria. It is now very popularly consumed throughout Nigeria owing to its low price. Prepared from cereal grains mainly maize, sorghum or a combination of both, pito is a dark brown liquid which varies in taste from sweet to bitter. It contains lactic acid, sugars, amino acids and has an alcohol content of 3% (Haard, 1999). ***Emu*** common among the Yorubas in the Southwest part of Nigeria is produced from sugary palm saps. The most frequently tapped palms are raphia palms and the oil palm. It has an alcoholic content of around 5% (SCTD, 2004). ***Ogogoro*** (also known as *kinkana* and *apetesi*) is a gin-like drink distilled from oil or raffia palm wine. In Nigeria, distillation takes place in small sheds dotted along the coastal areas and in villages across the South. The end product is a clear liquid with alcohol content often higher than 40% (Obot, 2000).

In the rural town of Igbo-Ora, guinea corn is malted and fermented to produce ***oti baba*** or ***oti'ka***, with *baba* and *ka* being local names for the corn. There is also ***agadangidi***, a fermented beverage made from mashed ripe plantain, fresh chili peppers and water (Mamman *et al.*, 2002).

1.2 Statement of Research Problem

Despite the fact that ethanol is known to have numerous toxic effects on humans, the level of alcohol abuse and alcoholism remain a very pressing issue throughout the world (WHO, 2014b). There are evident data on billions of Dollars being spent by developed countries on Foetal Alcohol Spectrum Disorder (FASD). In Canada, the annual cost of FASD amounts to 5.3billion Dollars (Stade *et al.,* 2009). There are reports by numerous researchers on the use and misuse of alcohol by women in different parts of Nigeria (WHO, 2014b). Alcohol is also known to have toxic effects on nervous system and ethanol is known to have potency in inducing teratogenicity (Allam and Abdulhamid, 2013). However, some proportion of pregnant women still take alcohol either in moderate or in binge drinking pattern (Allam and Abdulhamid, 2013).

1.3 Significance of the Study

Children of alcoholic women are exposed to the potential risk of ethanol-induced disorders as a result of ethanol ingestion during pregnancy, as such, this study will create awareness on the risks associated with alcohol intake during pregnancy with emphasis on the nervous system. The result of the study will provide scientific data on the pattern of teratologic effects of ethanol as a function of timing and dosage on nervous tissues.

1.4 Justification

There are evidences that ethanol has toxic effects on the nervous system of adult mice and rats, but little is known of its prenatal effects on the nervous system. The study seeks to provide information on prenatally administered effects of ethanol on the

developing cerebrum and cerebellum. The study outcome could serve as the basis on which further studies could be carried out.

1.5 Scope of the Study

The study was on the histological and histochemical changes in the developing cerebrum and cerebellum as a result of prenatal ethanol exposure. Haematoxylin and Eosin (H and E) and Cresyl Fast Violet stains were employed in the study of the tissues. Atomic Absorption Spectrophotomery was also conducted to quantify multi-elements in the tissues of the experimental animals.

1.6 Aim and Objectives of the Study

1.6.1 Aim of the study

The aim of the study is to evaluate the histology, histochemistry, neuro-behaviour and quantify the amount of trace elements on the cerebellar and cerebral cortices of noenatal Wistar rats following intrauterine ethanol exposure.

1.6.2 The objectives of the study are to:

i- Determine the morphometric variables of the pups by measuring the crown rump length and weight of the pups.

ii- Assess the neurobehavioral effects of ethanol on the pups prior to weaning by using cliff avoidance, surface righting and negative geotaxis reflexes methods.

iii- Study the teratologic effects of ethanol on the histology of cerebral and cerebellar cortices of the pups using the routine Haematoxylin and Eosin stain.

iv- Investigate the histochemical changes in the cerebral and cerebellar cortices of the pups using Cresyl Fast Violet to demonstrate nissl substance.

v- Study the effects of intra-uterine ethanol exposure on the concentration of trace elements (Zn, Cu, Fe and Mn) present in the noenatal brain tissue using Atomic Absorption Spectrophotometry.

CHAPTER TWO

2.0 Literature Review

2.1 Review of Related Literature

Since 1973, it has become clear that exposure of otherwise normal human fetuses to high levels of alcohol damages a substantial number of the exposed brains in a wide variety of ways nowadays referred to collectively as the foetal alcohol spectrum disorders (FASDs). Often this damage can be seen directly in brain images obtained much later in life (Fred, 2009). Stratton *et al.,* (1996) reported Alcohol Related Neurodevelopmental Disorder (ARND) which is an evidence of CNS abnormality (such as an abnormally neurodevelopmental small head, abnormal brain structures, and disorder (ARND) neurological signs); evidence of a behavioral or cognitive disorder inconsistent with the expected developmental level, with hereditary factors, or with the environment; or both.

One of the most devastating and extreme consequences of developmental alcohol exposure is the loss of neurons, and the data documenting neuronal loss are derived mostly from animal studies. Although selective programmed neuronal death is a normal aspect of CNS development, excessive neuronal death disrupts the development of normal neural networks and may lead to cognitive and behavioral dysfunctions (in both humans and animals). Distinct regions and specific cell types appear to be affected by alcohol-induced neuronal loss. Studies in rats have clearly demonstrated that alcohol causes a reduction of cerebellar Purkinje cells, cells of the olfactory bulb, and pyramidal cells in a part of the hippocampus known as the CA1 region (Livy *et al*., 2003; Chen *et al*., 1999*a*, 1998). In other neuronal populations, however, such as those in the

ventrolateral nucleus of the thalamus (an area important for voluntary movement) and the locus coeruleus (a group of nerve cells involved in arousal and vigilance), alcohol does not cause cell loss (Livy *et al*., 2001; Chen *et al*., 1999*b*).This pattern of alcohol-induced loss of neurons is important for two reasons: (1) it establishes a correlation between the severity of neuronal loss of a specific neuronal population and the degree of behavioural deficits associated with such a cell population, and (2) it provides insight into the specific characteristics of neuronal populations that are most vulnerable or most resistant to alcohol-induced neuronal loss. Proper CNS communication depends on an adequate number of dendritic spines and dendritic branching, because dendrites are the sites of contact for neurons.

Animal studies, both in vivo and in vitro, have demonstrated that developmental alcohol exposure negatively affects the structural integrity of the dendrites and the number of dendritic spines in neurons located in the substantia nigra (a group of nerve cells involved in movement), the cortex, and the hippocampus (Tarelo-Acuna *et al.,* 2000; Yanni and Lindsley, 2000; Shetty *et al.,* 1993; Fabregues *et al.,* 1985). For example, Tarelo-Acuna and colleagues (2000) showed that prenatal and postnatal alcohol exposure alters the proportions of different spine shapes in the long (apical) dendrites of hippocampal CA1 pyramidal cells.

Allam and Abdulhamid reported that postnatal day 21, the outer molecular layer of ethanol treated pups was defined. The apical dendrites were perpendicular to the pial surface in both normal and treated groups. At postnatal day 21, the normal and treated pups showed no obvious lamination in the cortical plate except the outer molecular layer which was sharply defined. The normal cells of the cerebral cortex had spherical

or pyramidal perikaryons whose nuclei were large, also the neurons arranged in a regular pattern. Generally the cerebral neurons appeared more developed toward the white matter.Allam and Abdulhamid reported alteration in the intensity of nissl granules in ethanol treated pups. The nissl substance in the treated groups was either faintly or moderately stained when compared with the control.

2.2 Foetal Alcohol Spectrum Disorders (FASD)

2.2.1 Clinical background on FASD

The maternal consumption of alcohol during pregnancy may lead to neurodevelopmental abnormalities in exposed children (Becker and Randall 1989; Guerri *et al.,* 2009). Children exposed to ethanol prenatally may acquire a continuum of permanent birth defects known as Foetal Alcohol Spectrum Disorders (FASD) (Bertrand *et al.*, 2005; Abel and Sokol, 1987).

FASD affects approximately one in 100 live births (Stade *et al.,* 2009). FASD-related abnormalities may include physical, behavioural, and cognitive dysfunctions of varying degrees (Guerri *et al.,* 2009; Kodituwakku, 2007; Kalberg *et al.*, 2006; Chudley *et al.*, 2005; Steinhausen *et al.,* 1993). Growth deficiency, low birth weight, spatial learning delays, neuromuscular coordination deficits, central nervous system damage, and craniofacial malformations are key features of FASD (Engle and Kerns, 2011; Hellemans *et al.*, 2010; Herman *et al.,* 2008; Kodituwakku, 2007; Colorado *et al.,* 2006; Bhatara *et al.,* 2006; Guerri 1998). Children and adults who have been exposed to alcohol *in utero* may experience problems in attention span, judgment, communication, executive functioning, memory, and social adaptation (Green *et al.,* 2009; Jirikowic *et al.,* 2008; Mattson*et al.,* 1998). Once affected by alcohol, a child is affected for life,

and there is no cure. Even with behavioural interventions, individuals with FASD may live with lifelong learning disabilities and poor social skills (Kodituwakku, 2007).

The spectrum of disabilities resulting from prenatal alcohol exposure ranges from mild defects, defined as Foetal Alcohol Effects (FAE), to more severe and diagnosable cases of Foetal Alcohol Syndrome (FAS). FAE are characterized by the occurrence of minimal abnormalities; meanwhile, FAS is the most severe form on the FASD spectrum (Manning *et al.,* 2007; Steinhausen *et al.,* 2003). FAS is characterized by all, or a combination of the following: severe mental illness, craniofacial abnormalities, intrauterine growth retardation, depression or psychosis, and antisocial behaviour (Steinhausen *et al.,* 2003; Ikonomidou, 2000; Steinhausen *et al.,* 1993). Unlike any other forms of FASD, FAS is an official International Classification of Diseases (ICD) diagnosis (Kvigne *et al.,* 2004).

Other conditions resulting from *in utero* alcohol exposure include Alcohol-Related Neurodevelopmental Disorder (ARND) and Alcohol-Related Birth Defects (ARBD) (Chudley *et al.,* 2005). Hyperactivity, anxiety, emotional problems, and learning disabilities are common across the FASD spectrum (Hellemans *et al.,* 2010; Chudley *et al.,* 2005; Steinhausen *et al.,* 2003). Due to the behavioural heterogeneity in individuals with FASD, it is difficult to diagnose children with different types of FASD. To overcome this limitation, a quantitative behaviour checklist is used to categorize abnormalities observed in children who have been exposed to alcohol prenatally (Steinhausen *et al.,* 2003). In this way, cases can be distinguished based on child-specific behavioural manifestations of FASD disabilities. Specific classifications across the FASD spectrum allow for more thorough insight into how an individual was

developmentally influenced by alcohol, both physically and mentally (Katarzyna, 2013).

2.2.2 Foetal Alcoholic Spectrum Disorder (FASD) is a wide-spread and costly disorder

The prevalence of FASD in Canada and the United States is approximately 9.1 in 1000 live births, as reported by the Public Health Agency of Canada (Stade *et al.,* 2009). In the late 1980's, this number was 1.9 per 1000 live births (Abel and Sokol, 1987). The number of individuals affected with FASD has been steadily increasing. One possibility for the increasing rate of FASD is an increasing rate of disregard for proper lifestyle habits during pregnancy. Also, pregnant women may be unaware of their pregnancies and may continue to consume alcohol throughout early pregnancy. Other possibilities for the increasing numbers of FASD could be due to increased awareness and self-reporting, resulting in more diagnoses. FASD is the most common, preventable cause of mental retardation in Canada and the United States (Clarke and Gibbard, 2003). In Canada, the annual cost of FASD amounts to 5.3 billion dollars (Stade *et al.,* 2009).

2.2.3 Current treatments for FASD excluding biological mechanisms

There is no treatment or cure for physical defects and central nervous system (CNS) damage resulting from foetal alcohol exposure. However, the severity of learning disabilities can be diminished. Parents and teachers can help children who have been characterized with FASD (Katarzyna, 2013).

A multi-disciplinary approach is needed to characterize deficits in children with FASD (Green *et al.,* 2009; Peadon *et al.,* 2008; Chudley *et al.,* 2005). In the same way, a multi-disciplinary approach is also needed to help a child with FASD reach his or her full

potential. Other than parents and teachers, individuals who may be involved include psychologists, social workers, and health counsellors. Techniques used by clinicians to evaluate children with FASD may involve measuring a child's growth rate and examining his or her facial features (Moore *et al.,* 2007). Psychologists, speech-language pathologists, and occupational therapists may assess CNS damage to help characterize the severity of defects resulting from prenatal alcohol exposure (Henry *et al.,* 2007). Across a population, children with FASD have highly variable IQ scores (Kodituwakku *et al.,* 2001; Mattson *et al.,* 1999; Mattson and Riley, 1998).

Individual learning profiles of each child must be developed and implemented. However, many children with FASD are ineligible for special services because their intellectual abilities often fall within the average range of intelligence (Kalberg and Buckley2007). These children — who were exposed to alcohol prenatally, but still have average intelligences — benefit from IQ and achievement measurements (Kalberg and Buckley2007). Functional classroom assessments, family information, and school assessment processes allow educators and counsellors to work together to assess the impact of environmental conditions on a child's abilities. This helps to evaluate which conditions may disrupt effective functioning and learning. The efficacy of Child Friendship Training has been reported upon; this type of training improves knowledge of appropriate social behaviour in children with FASD (O'Connor *et al.,* 2006). Another method, Cognitive Control Therapy, is often used to teach 4 progressive skill-building that focusses on the child's ability to understand his or her own learning style and challenges (Kalberg and Buckley2007).

2.2.4 Difficulty in diagnosing FASD based on phenotypes

Prenatal alcohol exposure is the most common preventable cause of cognitive disabilities in the western world (Clarke and Gibbard, 2003). However, FASD is difficult to properly diagnose, particularly because it shares traits with other complex disorders such as Attention Deficit Hyperactive Disorder (ADHD) (Peadon and Elliott, 2010). ADHD is diagnosed in up to 94% of individuals who have experienced heavy *inutero* alcohol exposure (Peadon and Elliott, 2010). Often, children with FASD have co-morbid ADHD, and can be diagnosed improperly. FASD-affected children may receive incorrect or insufficient treatment. Rasmussen and colleagues analyzed 52 children with FASD, and found that 63% received a comorbid diagnosis of ADHD (Rasmussen, *et al.,* 2009). The authors also found that children with FASD and ADHD performed worse than children with FASD but without ADHD on sensory/motor, cognitive, communication, memory, executive functioning, attention, and adaptive behaviour tasks (Rasmussen, *et al.,* 2009).

Another study found that out of 2,231 FASD-affected youth, 41% were diagnosed with ADHD, 17% were diagnosed with learning disorders, and 16% were diagnosed with oppositional defiant/ conduct disorder (Bhatara *et al.,* 2006).

Although researchers make efforts to identify behavioural characteristics differentiating children with FASD and ADHD, the psychological services are not always available (Bhatara *et al.,* 2006; Nash *et al.,* 2006). Maternal self-reported drinking as a means of predicting behavioural dysfunctions in "at-risk" children can be misconstrued (Chiodo *et al.,* 2009). Other difficulties in the characterization of FASD include absence of craniofacial malformations in children who have undergone *in utero* alcohol exposure, coupled with the unavailability of maternal drinking data (Nash *et al.,* 2006). Also, an

increase in binge drinking among women of child-bearing age, coupled with the unawareness of pregnancy early in the first trimester, could contribute to the pervasiveness and eventual mischaracterization of FASD (Kelly-Weeder, 2008; Barr *et al.,* 2006; Caetano *et al.,* 2006).

A further complication resulting in difficulty in characterization of FASD includes the inconsistency of symptoms due to dosage-specific effects of alcohol. Since the 1960's, researchers have reported on the relevance of a multitude of factors in determining the severity of FASD abnormalities; factors include: quantity, frequency, and timing of alcohol (Mulford and Miller, 1960). For instance, facial dysmophology is caused by alcohol exposure during early gestation (gastrulation) (Sulik *et al.,* 1981). Heavy drinking increases the likelihood of FASD in offspring; children prenatally exposed to high amounts of alcohol perform worse on measures important for reading and arithmetic skills than children exposed to low amounts of alcohol (Streissguth *et al.,* 1994).

Dosage- and timing specificity may be attributable to disruption of specific neurodevelopmental patterns that occur during foetal development. Though the timing- and dosage-specificity underlying FASD is not well-established, women have been advised that no amount of alcohol is safe to consume during pregnancy (Kelly-Weeder, 2008). Researchers suggest that, in addition to genetic background, the timing and dosage of prenatal alcohol exposure is an important factor in determining the variation in behavioural manifestations related to FASD (Stade *et al.,* 2009; Kelly *et al.,* 2009; Abel and Sokol, 1987).

2.3 Ethanol Exposure Can Lead to Adverse Effects in the Foetus

2.3.1 Ethanol's mechanism of action

Prenatal alcohol exposure leads to alterations in foetal brain structures that give rise to developmental and behavioural abnormalities in children (Steinhausen *et al.,* 2003). The metabolism of alcohol in the maternal liver involves the oxidation of ethanol into acetaldehyde by an enzyme called alcohol dehydrogenase (ADH); ADH is further oxidized into acetate by another enzyme, acetaldehyde-6-dehydrogenase (ALDH) (Gemma *et al.,* 2007). The intermediary compound, acetaldehyde, is harmful to the foetus (Gemma *et al.,* 2007).

Acetaldehyde leads to increased accumulation of oxygen radicals, which have harsh implications on the developing brain. For instance, DNA replication and DNA repair enzymes can be impaired (Li and Wang, 2004; Brocardo *et al.,* 2011). Tissue damage may be a direct result of the accumulation of reactive oxygen species (ROS) through the process of ethanol oxidation. Accumulation of acetaldehyde can result from ADH conversion; the ADH complex is used to convert ethanol to acetaldehyde 90% of the time (Gemma *et al.,* 2007). Acetaldehyde can result in protein adducts, which lead to cellular damage, and subsequent oxidative stress.

2.3.2 Ethanol toxicity in the developing foetus

In a developing foetus, morphological changes occur in multiple areas of the brain due to ethanol toxicity. It is well-established that prenatal alcohol exposure can alter brain development, leading to neurodegeneration in humans (Ma *et al.,* 2005; Sowell *et al.,* 2002; Bhatara *et al.,* 2002; Jones and Smith, 1975), and in mice (Hamilton *et al.,* 2009; Young and Olney, 2006; Dikranian *et al.,* 2005; Olney, 2004; Wozniak *et al.,* 2004;

Ikonomidou *et al.,* 2000; Olney *et al.,* 2000). Ethanol can lead to reductions in cerebellar volume and surface area, reductions in basal ganglia, decreases in dendritic complexity, abnormalities in grey and white matter distribution and density, and apoptosis in sensitive brain regions (Guerri *et al.,* 2009; Hamilton *et al.,* 2009; Wozniak *et al.,* 2004;).

The affected brain areas are necessary for hormone regulation, sensory information processing, learning, and memory. Ethanol (or its metabolites) can pass through the blood-brain barrier and disturb the developing foetal brain. The basis for the observed behavioural heterogeneity in FASD is unknown. Certain time points during pregnancy are critical stages of neurodevelopment, and are particularly sensitive to environmental toxins (eg. alcohol) (Guerri *et al.,* 2009). Alcohol exposure at critical stages during pregnancy may lead to the spectrum of abnormalities observed in children with FASD.

2.3.3 Binge drinking and its adverse effects on offspring

Binge drinking is defined as a pattern of alcohol consumption that raises blood alcohol concentration (BAC) to 0.08 gram percent or above, which is equivalent to the consumption of five or more drinks within a couple of hours of each drink (SAMHSA, 2006). This definition has been revised to three or more drinks per occasion; this level of drinking highly correlates with child dysmorphology and behavioural alterations (May *et al.,* 2008). Binge drinking has been identified as the most damaging form of alcohol consumption because it produces a high BAC in the mother, which can have adverse effects on the developing foetus (Livy, *et al.,* 2003; Abel, 1988). Peak BACs are higher in binge models of alcohol exposure than more moderate, continuous-

drinking paradigms; these data correlate with increased atherosclerotic plaque development in binge versus continuous models of FASD in mice (Liu *et al.*, 2011).

Alcohol-induced neuronal loss in developing rats leads to increased brain damage following maternal binge drinking (Bonthius and West1990). Binge drinking is particularly harmful to the developing foetus because alcohol takes longer to metabolize at higher BACs, particularly in women (Frezza *et al.*, 1990). Also, the specific gestational time of binge drinking may correlate with key stages of brain development, which may lead to more severe deficits in offspring. On another note, there is a strong association between binge drinking and unplanned pregnancy (Parker *et al.*, 1994). In cases of unplanned pregnancy, women may consume alcohol without knowledge of pregnancy. These women may continue to drink heavily into their pregnancies, having exposed their foetuses to the teratogenic effects of alcohol (Katarzyna, 2013).

2.3.4 Timing and dose-dependent effects of alcohol on neurodevelopment

The neurodevelopmental effects of alcohol are specific to the timing of exposure and the developmental processes that occur during specific times. One of the critical points during8 neurodevelopment occurs during the embryonic stage of gestation (first trimester) (Guerri *et al.*, 2009). Exposure to alcohol during gastrulation negatively impacts formation of germ layers and reduces neural progenitors (Rubert *et al.*, 2006). These reductions lead to long-term effects on the forebrain and brainstem (Mooney and Miller, 2007; Ashwell and Zhang, 1996). Maternal binge drinking during the early stage of embryogenesis is also associated with a high incidence of craniofacial malformations and mental disabilities (Astley *et al.*, 1999; Ernhart *et al.*, 1987).

Another critical stage during foetal development occurs in humans from 7-20 weeks of gestation (second trimester). At this stage, the foetal brain undergoes neuroepithelial cell proliferation and migration (Suzuki, 2007). Most of the areas of the nervous system (except cerebellum) begin to differentiate (Guerri *et al.,* 2009; Rubert *et al.,* 2006; Miller, 1995). Prenatal ethanol exposure can alter neuronal migration and lead to reductions in the numbers of neurons and glial cells in the neocortex, hippocampus, and sensory nucleus in the foetal brain (Rubert *et al.,* 2006; Miller, 1995). Such disturbances to brain development can lead to long-term abnormalities in brain size and likely contribute to cognitive deficits in individuals with FASD (Sowell *et al.,* 2002). Dosage is also an important factor that influences the severity of neurodevelopmental deficits associated with prenatal alcohol exposure. Alcohol may affect the developing foetus in a dose-dependent manner. Binge alcohol consumption produces more severe deficits in offspring development and behaviour than daily moderate consumption (Liu *et al.,* 2011; Livy, *et al.,* 2003). Exposure to heavy drinking (>48-60 grams ethanol/day) may cause FAS, whereas moderate drinking (between 24-48 grams ethanol/day) can result in "alcohol effects" (Ornoy and Ergaz, 2010). Binge drinking, with intakes of 4-5 drinks of ethanol per sitting (> 90 grams ethanol/day), can result in greater peak BACs than other forms of alcohol consumption (Pierce and West, 1986). It is difficult to define and characterize developmental risks associated with binge drinking in human subjects due to inconsistencies in dosage and timing (Katarzyna, 2013).

2.4 Cerebellum

2.4.1 Morphologyof the cerebellum

The cerebellum is the largest part of the hindbrain. It is dorsal to the pons and medulla, and its median region is separated from them by the fourth ventricle (Gray, 2000). It is

joined to the brain stem by three pairs of cerebellar peduncles, which contain afferent and efferent fibres. The cerebellum occupies the posterior cranial fossa, where it is covered by the tentorium cerebelli. It is roughly spherical, but somewhat constricted in its median region, and flattened - its greatest diameter is transverse (Clerke, 1996). In other words, the cerebellum is located at the bottom of the brain, with the large mass of the cerebral cortex above and the portion of the brainstem called the pons in front of it (Clerke, 1996). It is separated from the overlying cerebrum by a layer of leathery dura matter. All of its connections with the other parts of the brain travel through the pons (Clerke, 1996).

2.4.2 Cellular layers of the cerebellum

The cerebellar cortex of adults is divided into three layers. The layers from the lower to the upper include the followings as shown in Figure 2.1.

i. **The thick granular layer** which is densely packed with granule cells, along with interneurons, mainly Golgi cells and unipolar brush cells (Ghez, 1985; Bell, 2008). Their cell bodies are packed into a thick layer at the lower part of the cerebellar cortex. A granule cell emits only four to five dendrites, each of which ends in an enlargement called a dendritic claw (Linas *et al.*, 2004). These enlargements are sites of excitatory input from mossy fibers and inhibitory input from Golgi cells (Linas *et al.*, 2004).

ii. The **Purkinje layer** lies in the middle, forming a narrow zone that contains only the cell bodies of Purkinje cells (Linas *et al.*,2004). They are distinguished by the shape of the dendritic tree. The dendrites branch very profusely, but are severely flattened in a plane perpendicular to the cerebellar folds. Thus, the dendrites of a Purkinje cell form a dense planar net, through which parallel fibers pass at right angles (Bell, 2008; Linas *et al.*,2004). The dendrites are

covered with dendritic spines, each of which receives synaptic input from a parallel fiber. Purkinje cells receive more synaptic inputs than any other type of cell in the brain. The large, spherical cell bodies of Purkinje cells are packed into a narrow layer, one cell thick of the cerebellar cortex, called the Purkinje layer (Linas,*et al.*, 2004).

iii. **The molecular laye**r is thick. It contains a sparse population of neurones, dendritic arborizations, non-myelinated axons and radial fibres of the neuroglial cells. (Gray, 2000; Clerke, 1996). Purkinje cell dendritic trees extend towards the surface and spread out in a plane perpendicular to the long axis of the cerebellar folia. Purkinje cell dendrites are flattened. This outermost layer of the cerebellar cortex also contains two types of inhibitory interneurons, stellate cells, and basket cells (Linas *et al.*, 2004).

2.4.3 Functions of the cerebellum

The cerebellum performs the following function:

i. Coordination of Movement; the cerebellum controls the timing and pattern of muscle activation during movement.

ii. Maintenance of Equilibrium in conjunction with the vestibular system.

iii. Regulation of Muscle Tone and modulation of spinal cord and brain stem mechanisms involved in posturalcontrol (Linas *et al.*, 2004).

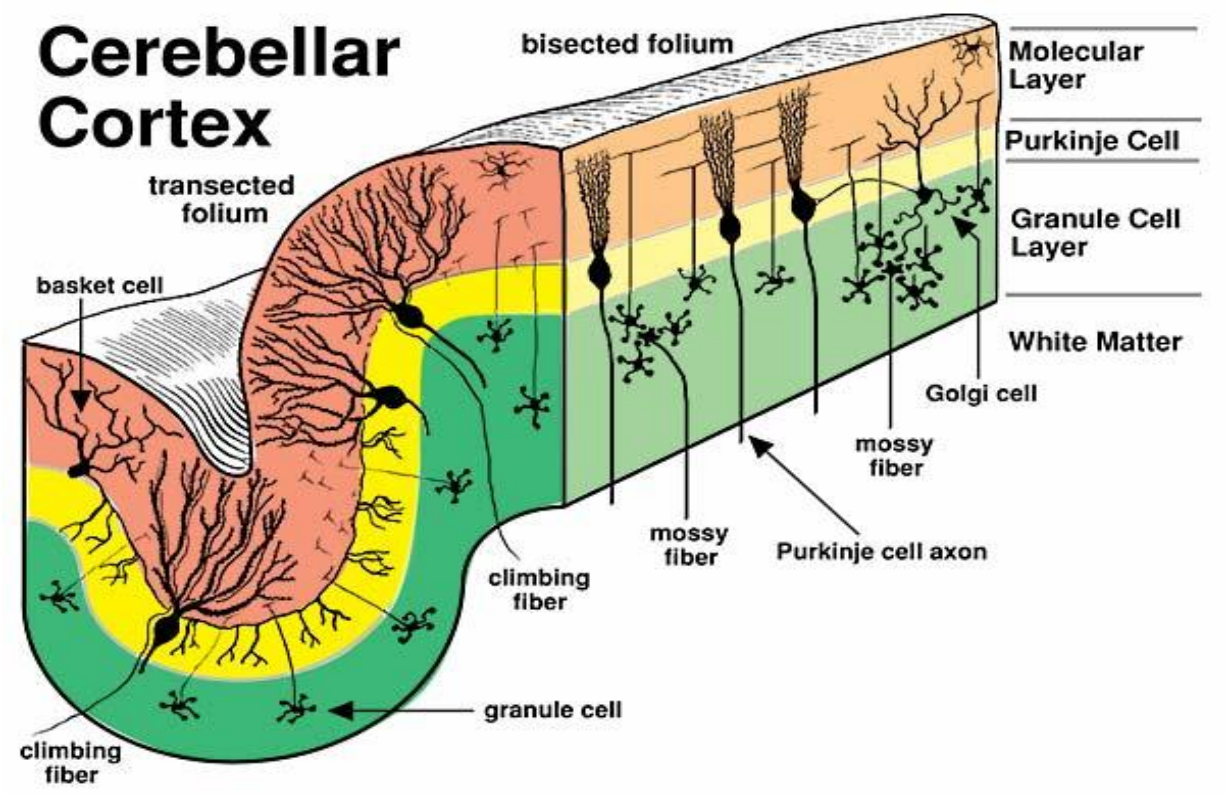


Figure 2.1: Cellular layers of the cerebellum showingthe outer molecular layer **(brown**), the purikinje cell layer **(yellow)** and the granule cell layer **(green)** (Linas *et al.*,2004).

2.5 The Cerebrum

2.5.1 The 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 2.2. The cortex is convoluted with projections called sulci and gyri. The cerebral cortex is about 1.5 - 4mm thick, and contains nerve cells, fibers, 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).

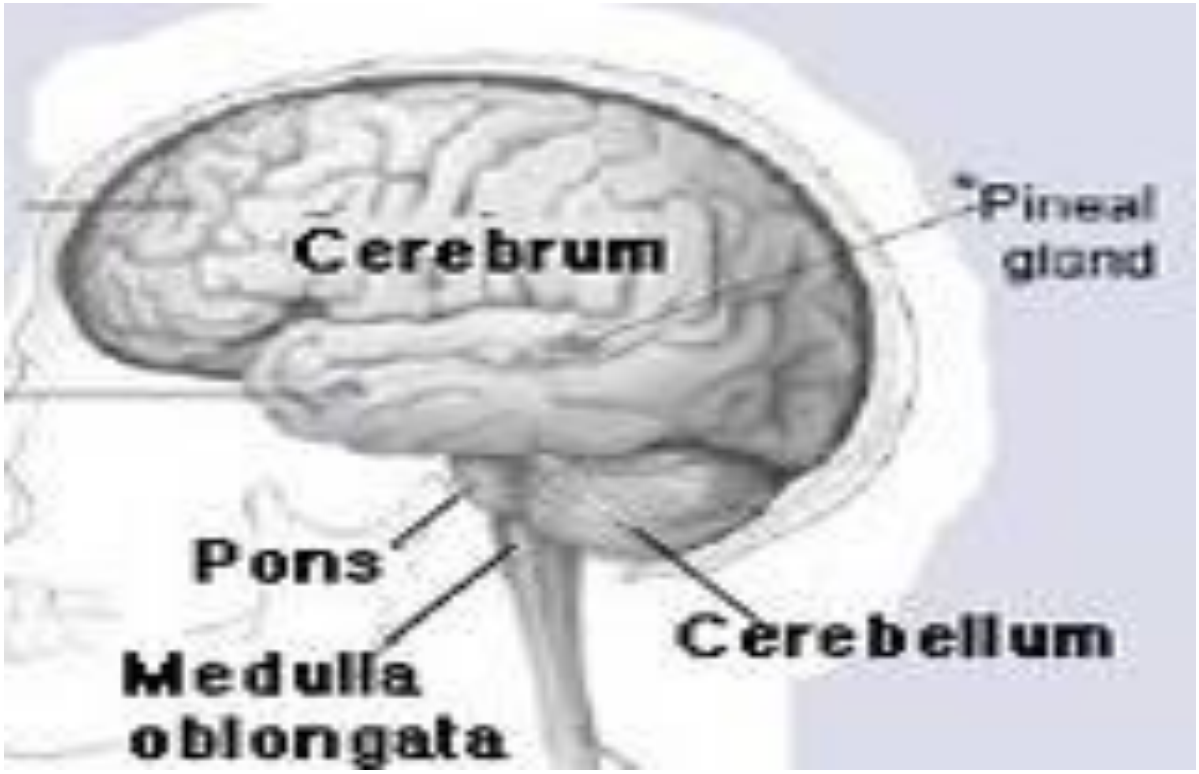


Figure 2.2: The structure of the brain showing the cerebrum (Kandel *et al.,* 2000)

2.5.2 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 features which demarcates them from each other. The neurons include the following:

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 fibers 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 been 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 2.3, according to Saladin, (2010); Shipp, (2007); Kandel*et al*., (2000).

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 (Kandel *et al.*,2000).

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 (Kandel *et al.*,2000).

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 (Kandel *et al.*,2000).

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 fibers 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 fibers 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 (Kandel *et al.*,2000). 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 2.3: The cellular layers of cerebral cortex (kandel *et al.*, 2000).

2.5.3 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 follows:

I. In the agranular cortex, the external and internal granular Laminae are inconspicuous. This type of cortex is refered 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).

2.5.4 Functions associated with 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 Broman 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 the loss of the sense of smell (Kandel*et al*., 2000).

2.6 Development of the Central Nervous System

2.6.1 Pre-differentiation stage

The pre-differentiation stage is characterised by germ cells with no distinct morphological differentiation (Suzuki, 1980). For rats, this stage is (the 'all or nothing' period) covers the time from conception to post-conception day 5. Damage during this period normally has two consequences: Either all the cells are killed and no embryo is formed (severe damage) or there are enough surviving cells to compensate for the assault and a normal embryo is formed (minimal damage) (Suzuki, 1980).

2.6.2 Embryonic stage

This stage is characterised by the mobilisation and organisation of cell and tissues to form individual organ systems (Suzuki, 1980). The periods of organogenesis are different from one mammalian specie to another. In rats, an organogenesis from

gestational day (GD) 6-15, in humans it proceeds from GD 20-55, in monkey it is from GD 20-45, and in mice from GD 7-16 (Slikker, 1994; Schardein, 1988).

2.6.3 Development of the neural tube

Nerve and glial cells of the nervous system are derived from a specialised region of the ectoderm called the neural plate (Martin, 1985). The excitation and differentiation of the cells of the ectoderm is induced by the notochord and paraxial mesoderm (Moore and Persaud, 1998). The induction is due to an embryonic protein called noggin, which has been identified as a neural inducing agent in amphibians and rodent embryos (Lamb *et al.,* 1993).

The neural plate indents and forms the neural groove from which the lateral lips close to create the neural tube. In rats, the neural tube is formed around GD 11 (Martin, 1985). Damage during this early period results in either anencephaly (closure of the neural tube at the rostral level) or spina bifida (caudal portion of the neural tube fails to close).

2.6.4 Start of neurogenesis

The epithelial cells lining the neural tube are called neuro-epithelium. The neuro-epithelium is the source of all neurons and glial cells of the central nervous system. The nerve cells of the peripheral nervous system (dorsal nerve root ganglion cells and post-ganglionic neurons of the autonomic nervous system) originate from the neural crest cells, a population of cells whose cell bodies lie outside the neural tube (Bayer and Altman, 1995).

The precursors of nerve cells, the neuroblast, divide repeatedly but do not proliferate uniformly along the length of the neural tube. The neuro-epithelium appears to be 'spatio-temporal mosaic' where the neuroblasts seem to be committed to generate specific neuron populations according to strict timetables (Moore and Persaud, 1998). In strictly time-dependent sequence, programmed cell proliferation occurs at specific sites in the neuro-epithelium which gives rise to the occurrence of three primary brain vesicles called the rostral prosencephalon (primitive forebrain), the mesencephalon (primitive midbrain) and the caudal rhombencephalon (primitive hindbrain). This is known as the three-vesicle stage (Singh, 2003; Moore and Persaud, 1998; Bayer and Altman, 1995). More programmed cell proliferation leads to an enormous expansion of the primary brain vesicles with a further sub-division.

2.6.5 Foetal stage

This stage is characterised by a significant increase in brain size and also by enhanced cellular proliferation and differentiation. The prosencephalon divides into (from rostral to caudal) the telencephalon with the cerebral cortex and the basal ganglia and the diencephalon with the thalamus and hypothalamus. The mesencephalon remains undivided, with the tectum and tegmentum (Singh, 2003; Bayer and Altman, 1995). The rhombencephalon divides into the metencephalon with the cerebellum and pons and the myelencephalon with the medulla oblongata. This is known as the five-vesicle stage (Singh, 2003; Bayer and Altman, 1995). In the caudal part of the neural tube, programmed cell proliferation forms the spinal cord, which contains sensory and motor neurons and many inter-neurons (Singh, 2003; Moore and Persaud, 1998; Bayer and Altman, 1995).

2.6.6 Pre and postnatal Stage

This stage is characterised 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 cell population is produced is produced in the postnatal period (Krinke and Eisenbrandt, 1994). According to Bayer and Altman (1995), central nervous system proliferation and differentiation continues after birth in many species.

2.7 Teratogenicity

2.7.1 Background on teratology

Teratology from the Greek word *teras*, meaning "marvel" or "monster" is the science dealing with the causes, mechanisms, and manifestations of developmental deviations of either structural or functional nature otherwise known as congenital anomalies or malformations. These structural or functional abnormalities are present at birth although they may not be diagnosed until later in life. They may be visible on the surface of the body or internal to the viscera. Congenital malformations account for approximately 20% of deaths in the perinatal period (Debus and Wolfe 2010; Shepard and Miller, 1976). Hence, this necessitates interest in the knowledge about the effect of maternal environmental factors on the conceptus during development, as one or combination of these factors may act to derail the normal course, leading to aberrations. Such factors responsible for this deviation are known as teratogens (Adebisi, 2011).

A teratogen therefore, is a drug, chemical, virus, infectious agent, physical condition, excess or deficiency that, on foetal exposure, can alter foetal morphology or subsequent function in postnatal life. However, teratogenicity depends upon the ability of the agent

to cross the placenta; for instance, certain medications such as heparin cannot (Bánhidy *et al.*, 2005; Czeizel, 1993; Czeizel and Dudás, 1992).

However, some exposures when tested could be categorized as potent (proven) teratogens (Adebisi, 2009; TSPAC, 1994); or probable (possible) teratogens (Beasley, 2010; Chung, 2010), based on the following criteria:

• A recognizable pattern of anomalies

A statistically higher prevalence of a particular anomaly in patients exposed to an agent than in appropriate controls

• Presence of the agent during the stage of organogenesis of the affected organ system

• Decreased incidence of the anomaly in the population prior to the introduction of the agent

• Production of the anomaly in experimental animals by administering the agent in the critical period of organogenesis.

2.7.2 Principle of teratogenicity

According to Wilson (1959), the following six principles normally apply to teratogenesis:

• Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors

• Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence. There are critical periods of susceptibility to agents and organ systems affected by these agents.

• Teratogenic agents act in specific ways on developing cells and tissues to initiate sequences of abnormal developmental events

• The access of adverse influences to developing tissues depends on the nature of the influence. Several factors affect the ability of a teratogen to contact a developing conceptus, such as the nature of the agent itself, route and degree of maternal exposure, rate of placental transfer and systemic absorption, and composition of the maternal and embryonic/foetal genotypes.

• There are four manifestations of deviant development (death, malformation, growth retardation and functional defect).

• Manifestations of deviant development increase in frequency and degree as dosage increases from the no observable adverse effect level (NOAEL) to a dose producing 100% lethality (LD100).

2.7.3 Mechanisms of teratogenicity

Medical science cannot always predict how exposure to a teratogenic drug will affect a foetus. The potential to harm depends on a range of factors as stated by Adebisi, (2011). Other factors, such as maternal diet, maternal age, Rh factor, and physical condition such as stress or distress, illness – all these could singly or jointly play a role. Some teratogens are associated with recognizable patterns of malformations: for example, thalidomide produces limb phocomelia, while valproic acid and carbamazepine produce neural tube defects, alcohol with foetal alcohol syndrome, phenytoin with foetal hydantoin syndrome and coumarin anticoagulants with foetal warfarin syndrome (Czeizel, 2004; Rockenbauer *et al.*, 2001).

In any case, some of the pathways by which teratogens could possibly potentiate their toxic effects have been well examined and succinctly highlighted as being through any of the following: folate antagonism - such as aminopterin; enzyme-mediated

teratogenesis; oxidative stress – such as nutritional deficiencies, hypoxia or environmental chemicals; functional disruptions in the neural crest cells; disruption in the vascular system or disturbed endocrine systems – such as cortisone, progestin. Teratogens could also trigger off the disruption of carbohydrate metabolism in maternal diabetes (Adebisi, 2011; Van Gelder *et al.*, 2010).

2.7.4 Transfer of teratogens across the placenta

On the ability and rate of transfer of teratogens across the placenta, earlier reviews include those of Mirkin and Singh (1976) and Waddell and Marlowe (1976). These authors reported the differences in transfer as a combined function of route of administration of the materials and the animal models in use, since rapidity and extent of crossing the placenta into the foetus by drugs and chemicals are by no means measures of the toxic action on the foetus or the persistence of the compound in the foetal tissues (Miller, 1977). The rate of transfer of a chemical across the placenta depends on the net sum of many factors: molecular size, lipid solubility, protein binding, pH gradients *etc*. Small molecules, less than 600 molecular weight and low ionic charge cross by simple diffusion, active transport, pinocytosis or perhaps also by leakage (Adebisi, 2011).

Lipophilic chemicals are known to cross the placenta and other membranes more readily than other compounds (Wilson, 1979). It now seems that the rate as determined by size, charge, lipid solubility, affinity to complex with other chemicals and so on, all play a significant role in placenta permeability. For instance, ethanol with a molecular weight of 46.07 has been shown to pass across the placental barrier and that its concentration in the foetus is almost as high as in the mother (Jones and Smith, 1973).

2.7.5 Site of action of teratogens

Unfortunately, knowledge of the certainty of the specific site of action of the teratogenic agents within the maternal-placenta-foetal unit is yet obscure. All too frequently, the naive assumption is made that the administered agents find their way to the foetus and directly interfere with the growth and differentiation of these cells (Adebisi, 2011). Not only is such evidence not available, but a large number of clues actually indicated that these chemicals do not act directly on foetal cell. For instance, it had been pointed out that the concentration of the teratogen, cortisone was not higher at its site of teratogenicity in the foetus than it was in any other foetal tissues and its site of action, and that its concentration in all the tissues was lower than in the maternal tissues (Waddell and Marlowe, 1976). Comparison of maternal foetal concentration ratios of variety of chemicals with low or high teratogenic potential revealed that the tendency was considered to be that, the potent teratogens have high foetal maternal ratios (Waddell and Marlowe, 1976).

Hence, clearly the earlier naive assumption is untenable that 'the greater the amount of chemical reaching the foetus, the more likely the production of foetal anomalies. In light of this puzzle, the problem has now become a search for the sites within the entire maternal-placenta unit (Adebisi, 2011). Lately, the predominant direction of reports are on the action that produces anomalies by their effects on the placenta, and that the direct effects of the agents on the mother may be as frequent as those acting directly on the foetus. However, the total dose of a chemical reaching the conceptus is a product of interaction of many variables, some relating to the maternal functional capacity, others undoubtedly reflecting the complex characteristics of the placenta. The possible

interruption of utero-placental blood flow by the chemical has also been suggested (Danielsson *et al.*, 1990; Jones and Cunnings, 1978).

2.7.6 Nutritional role in teratogenesis

The role of maternal nutrition in the potentiation of the toxicity or teratogenicity of an agent cannot be undermined. For a long period of time, the association of malnutrition of the mother with malformations of the foetus was subjected to debate. This brings us to consider and justify the proponent of the foetal origin hypothesis, which opined that the postnatal health may be influenced by prenatal factors (HMIC, 2006; Barker, 1995). This hypothesis argues that the environment experienced during the individual's prenatal life 'programs' the functional capacity of the individual's organs, and this has a subsequent effect on the individual's health (Adebisi, 2011).

For instance, when the foetus experiences a poor nutritional environment, it develops its body functions to cope with this, with the idea that the environment experienced prenatally is the one that it expects to continue experiencing, and thus, its body develops to cope with it - a predictive adaptive response mechanism (Adebisi, 2011; Barker, 1995).

Moreover, some workers have argued against nutritional deficiency as a possible mediator or potentiator of teratogenicity. According to these investigators, there was no evidence that the nutrition of women giving birth to infants with foetal alcohol effect (FAE) or foetal alcohol syndrome (FAS) is any worse than those women whose infants do not have these problems even though their mothers drank equally heavily (Adebisi, 2011). However, it appears that enough evidence is available to establish the fact that

maternal nutritional deficiency is a possible teratogenic potentiator and this has been amply demonstrated in the rats (Adebisi, 2011; Preedy *et al.*, 1990) and in humans (Barker, 1995).

2.7.7 Genetic role in teratogenesis

The genetic make-up of the developing organism is the setting in which induced teratogenesis occurs. Differences in the reaction to the same potentially harmful agents by individuals, strains and species are presumed to depend on variations in their biochemical or morphological make-up, which are in turn determined by genes (Collins, 2010). The fact that the mouse embryos are usually susceptible to cleft palate induction by glucocorticoids whereas most other mammalian embryos are resistant to these agents can be interpreted to mean that mice possess inborn chemical or anatomical features which make them more vulnerable (less resistant) to these agents than are other animals and these are at least to some extent genetically determined (Adebisi, 2011).

Thus, the same sort of determinants that gives rise to individuals, strains and species their distinctive similarities and dissimilarities in normal structure and function probably give them varying degree of susceptibility to adverse influences (Wilson, 1977). To this end, it has been suggested that the occurrence of anomalies is in part a measure of the inability of genetic and other regulatory mechanisms to overcome the adverse localized sensitivity of the embryo to an external intrusion. This could be explained from the fact that chromosomal aberrations induced in somatic cells are the cause of malformation following exposure to teratogens (Adebisi, 2011).

In the light of this knowledge, differences between model systems and man emphasize some of the reasons for the perceived low level of predictability of animal tests for man. Therefore, the level of confidence in the ability of these animal studies to be predictive for man remains far from high. Only when the mechanism of teratogenesis and the factors affecting species differences in teratogenic response are understood will we have confidence in the predictive accuracy of animal studies (Adebisi, 2011).

Although major new teratogenic drugs in humans have been predicted from animal studies, there are problems in extrapolating animal data to humans, and this has since, been a matter controversy (Bremer, 2007; Bailey *et al.*, 2005; Brent, 2004). For instance, animals have a different "gestational clock" to humans, there is marked interspecies variability in susceptibility to teratogens and no experimental animal is metabolically and physiologically identical to humans (Wilson, 1977). In any case, animal studies are important because, in some instances, they have shed light on mechanisms of teratogenicity and moreover, when an agent causes similar patterns of anomalies in several species, human teratogenesis should also be suspected (Adebisi, 2011).

Above all, for obvious ethical considerations no studies of teratogenicity are conducted during embryogenesis in humans (Wilson *et al.*, 1979). On the other hand, reports have shown that in some cases, genetic vulnerability is related to the sex of the developing organism. Male embryos and foetuses are at a greater risk than female in that more male embryos are more often aborted spontaneously; new-born boys have more birth defects, and older boys have more learning disabilities and other problems caused by

behavioural teratogens (Vallance, 1996; Schardein, 1985; Hill, 1983; Braun *et al.*, 1982).

In any case, whether it is the maternal or foetal component of genetics that is more important in determining the extent of the effect of teratogens is not yet well understood. It appears, for instance, that the rate of maternal alcohol metabolism could modify the effects of alcohol on the foetus (Mirkin and Singh, 1976), although it has been reported that not all the offsprings of alcoholic women manifest the characteristic features of FAS and hence, the rate of maternal alcohol metabolism could not have modified the effects of alcohol metabolism on the foetus (Adebisi, 2011).

2.7.8 Effective dosage of teratogens

Considering the dosage of an agent, not all dosage levels of known teratogens are potent enough to trigger-off any response. There are lower (sub threshold) dosages, which apparently do not affect the normal development of an embryo and even the mother, and lethal dosages, which will cause death of both the foetus and even the mother. Between these two extremes is a narrow 'teratogenic zone' in which the dosage is sufficient to interfere with specific development without destroying the whole embryo (Adebisi, 2011). In addition, the frequency of administration of teratogens determines more or less the extent of its action (Adebisi, 2011).

It could therefore be concluded that an appropriate dosage of a known teratogen administered at the appropriate time of development in a given species will cause developmental disturbances. Low doses of cytotoxic agents may produce levels of cell death that can be replaced through restorative hyperplasia of surviving cells, resulting in

the formation of small but morphologically normal foetuses. High doses that cause damage to too many cells and organ systems to be compatible with life result in embryo lethality (Adebisi, 2011).

2.7.9 Effects of teratogens on DNA synthesis

Some cytotoxic agents at lower dosage suppress DNA synthesis and cell division without causing cell death. Depressed proliferative activity in itself may contribute to teratogenesis by reducing the number of cells available for the formation of tissues during the organogenesis. Many agents that depress DNA synthesis are known to be teratogenic. Yet it is not clear that depression of DNA synthesis alone can lead to birth defects. The cell deaths that accompany inhibition of DNA synthesis are believed to be more important correlates to dysmorphogenesis. The relationship between depression in DNA synthesis and teratogenesis has been examined in a study of cysteine arabinoside (Ara – C) induced birth defects. Further study suggested that the teratogen action was not inhibition of DNA synthesis alone but rather the cytotoxicity that accompanied it. In a similar study with hydroxyurea (HU), Ara-C and aminothiadiazole (ATD), the same relationship between the depression of DNA synthesis and cell death was observed (Hill, 1983).

So far as the depression in DNA synthesis and cell death is concerned in embryonic as well as adult tissues treated with teratogenic agents, cytotoxicity can be assumed to be a common biological property of these agents. Whether or not birth defects results from the cytotoxic response of the embryos, teratogenesis depends upon gestational time of treatment (proliferative and differentiative state of the target organ), and the extent of cell death (Adebisi, 2011).

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

Light Microscope, Absolute ethanol (99.9 % purity), Distilled water, Amscope, Computer, Bouin's fluid, Beakers, Specimen bottles, Weighing balance, 1ml and 5ml syringes and injecting needles, Dissecting tray, Chloroform and Dissecting kit.

3.2 Experimental Animals

Wistar rats were purchased from Faculty of Medicine, Department of Human Anatomy, Ahmadu Bello University, Zaria, housed in wire mesh cages under controlled environmental conditions at temperature 22ºC and 12 hour reversed day-night cycle and maintained on standard rodent pellets and water ad *libitum*. The animals were allowed to acclimatize for 2 weeks prior to commencement of the experiment.Forty two (42) rats were used, comprising of 28 females and 14 males. The males weighed (200g-220g) indicative of their maturity. Young virgin females weighing 120g-140g were used in the experiment.

3.3 Experimental Design

Pregnant dams were randomly divided into seven (7) groups, each group comprising of four (4) females. The dose and the duration is summarised using the table below:

Table 3.1 showing the groupings, dosages, timing and the frequency of ethanol administration

SN	GROUPS	AMOUNT AND % OF ETHANOL	FREQUENCY	DURATION
1	A	Control (distilled water)	Once daily	20 days
2	B	0.5ml of 20%	Once daily	7 days (1^{st} trimester)
3	C	0.5ml of 20%	Once daily	14 days (1^{st} and 2^{nd} trimesters)
4	D	0.5ml of 20%	Once daily	20 days (1^{st}, 2^{nd} and 3^{rd} trimesters)
5	E	0.5ml of 30%	Once daily	7 days (1^{st} trimester)
6	F	0.5ml of 30%	Once daily	14 days (1^{st} and 2^{nd} trimesters)
7	G	0.5ml of 30%	Once daily	20 days (1^{st}, 2^{nd} and 3^{rd} trimesters)

The route of administration was oral.

3.3.1 Route of ethanol administration and basis for the choice of the concentration

30% v/v as adopted by Adebisi (2003) was used in addition to lower dose of 20% v/v. 0.5ml as used by Allam and Abdulhamid, (2013) of the respective doses of the ethanol was administered via oral intubation using insulin syringe with the needle covered with a scalp vein set tube to prevent harming the animals. The percentage and the duration is homology of binge drinking pattern. These doses are low if compared to the doses used by Maier and West (2001), which were 2.5, 4.5 & 3.6 g/kg/day, or to the dose (6.6 g/kg/day) used by Thomas *et al*. (1998). However, it is relatively close to the dose used by Allam and Abdulhamid, (2013), 33% ethyl alcohol, which is equivalent to 700 mg/kg.

3.3.2 Cycling of the animals

Oestrous cycle in Wistar rats last 4-5 days and consists of four main oestrous phases namely: pro-oestrous, oestrous, metoestrous and diestrous as shown on plate 3.1. These phases are characterised by the morphology of the cells found in the vaginal smear. The pro-oestrous phase is predominantly composed of nucleated epithelial cells, the oestrous precedes the pro-oestrous and it is predominated by irregular anucleated cornified cells, whereas the metoestous preceding the oestrous is composed of three different types of cells, these are: nucleated epithelial cells, irregular anucleated cornified cells, and leucocytes, then the last phase which is di-oestrous is predominated by leucocytes (Adebisi, 2009; Hubscher *et al*., 2005; Marcondes *et al*., 2002; Long and Evans, 1922) (plate 3.1).

The oestrous phases were determined by collecting the vaginal smear using a soaked cotton bud in distilled water, the soaked cotton bud was inserted slightly into the rat's vagina and the posterior vaginal wall was gently scratched to remove some of its contents. The content was then smeared on a clean glass slide and allowed to dry for 1 minute, the dried smear was then stained for the period of 5-10 minutes using 10% giemsa stain that was diluted in exactly 9 parts of water.

The stained slides were then washed with Distilled Water and allowed to dry and viewed under light microscope. Following determination of the oestrous phases on each day, pro-oestrous females were mated with matured males overnight in ratio 1:2 depending upon the number of pro-oestrous females on that day. The next morning, vaginal smear was taken and the presence of protein coagulate confirmed mating and

day zero of pregnancy was designated on each day (Adebisi, 2009; Long and Evans, 1922).

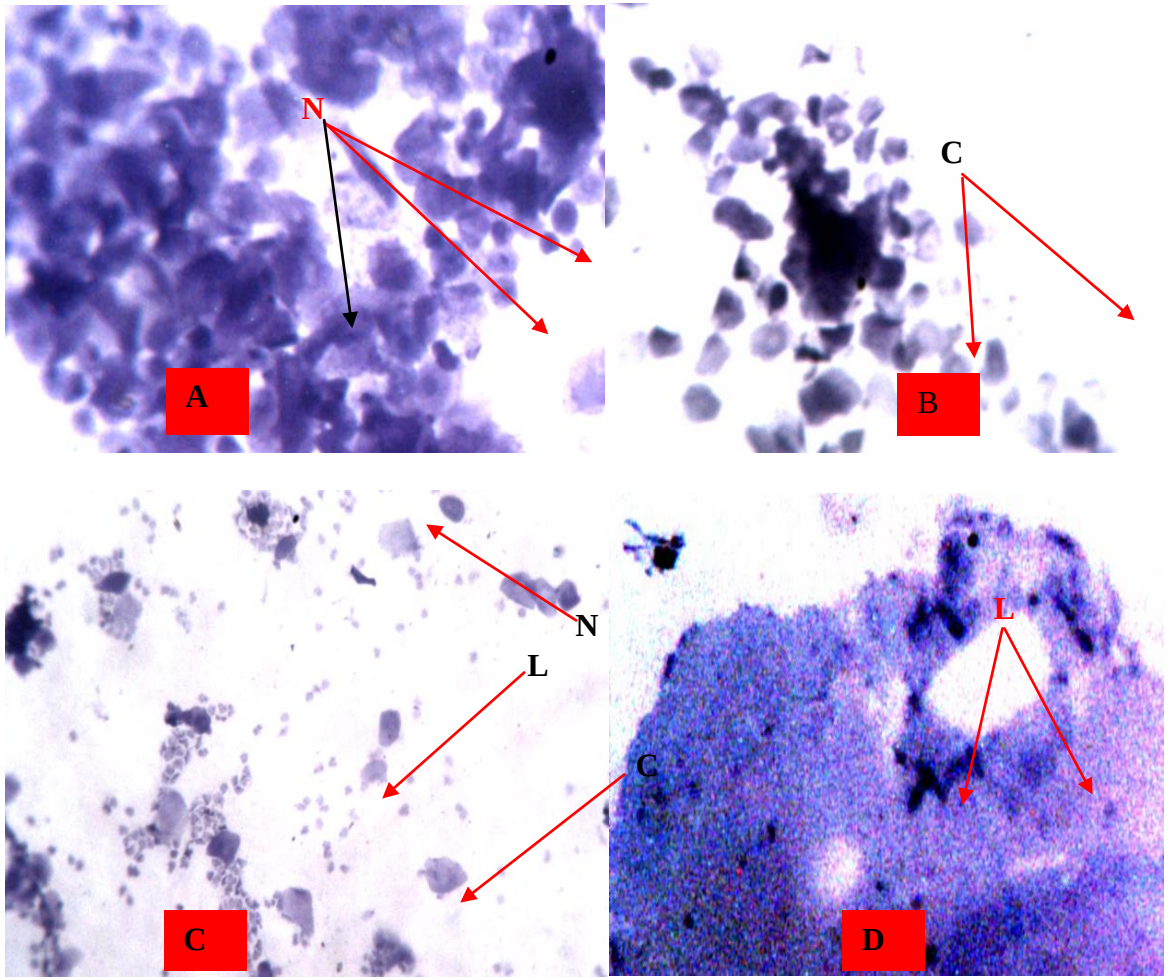


Plate 3.1: Vaginal smear demonstrating oestrous phases of Wistar rats: Prooestrous (A), Oestrous (B), Metoestrous (C) and Dioestrous (D); showing nucleated cells (N), Cornified cells (C) and Leucocytes (L) (Giemsa Stain X100).

3.3.3 Giemsa stain preparation

50mls of giemsa stain was prepared in the histology laboratory, Department of Human Anatomy, A.B.U, Zaria by making a solution in the following proportions: Giemsa powder: 0.38g, Glycerol: 25mls , Methanol: 25mls. The stain was prepared to stain the vaginal smear for the study of the cytology of the smear. The stain was diluted in distilled water in ratio of 1:9, i,e 10% giemsa stain before staining the smear to enhance stain absorption and permit more light penetration through the smear from the microscope light source, this was done according to modified Iliya technique (2008).

3.3.4 Confirmation of pregnancy

Daily examination of increase in weight of the animals was observed using digital weighing machine to asses for remarkable weight gain**.** In addition, towards the end of the first trimester, vaginal smear was taken consecutively for three days to observe if there was persistent di-oestrous phase, which is the undisputed indication of cessation of ovulation, hence, confirming pregnancy (Iliya, 2008).

3.3.4 Ethanol Preparation

100mls of both 20% and 30% ethanol were prepared by diluting 20mls of absolute ethanol in 80mls of distilled water and 30mls of absolute ethanol in 70mls of distilled water respectively. This was prepared based on the calculated amount of the respective percentages needed throughout the whole period of the administration.

3.3.5 Animal sacrifice

On the expected day of delivery (GD 21), some of the litters of treated and control groups were randomly selected and sacrificed using chloroform inhalation by placing the pups into a closed container containing a soaked chloroform cotton wool and humanely sacrificed, while some of the pups were randomly selected and allowed to lactate for pre-weaning neuro-behavioural tests (Allam and Abdulhamid, 2013). The morphometric indices of the foetuses were taken prior to the sacrifice. The brain was then dissected out and fixed immediately in Bouin's fluid (Bancroft and Gamble, 2008).

3.3.6 Tissue processing

The tissues were allowed to stay in the fixing fluid for 48hrs to enable efficient fixation. The tissues were then processed routinely and stained using Haematoxylin and Eosin (Hand E) and Cresyl Fast Violet Stain.

3.3.7 Routine paraffin sectioning

The fixed tissues were removed from the bouin's fluid and dehydrated using ascending grades of alcohol. This method involved dehydration of tissues in 2 changes of 70% alcohol and 2 changes in 90% alcohol, 3 changes of 95 % alcohol and finally 3 changes of absolute alcohol, each of the stages lasted for 30mins. The dehydrated tissues were then cleared in 2 changes of chloroform for 2hrs. The clearing helped in removing the opacity from the dehydrated tissues thereby making them transparent (Bancroft and Gamble, 2008).

The cleared tissues were infiltrated by immersion into molten paraffin wax and allowed to solidify. The embedded tissues were blocked in rectangular block and then tissue

sections were cut using rotary microtome at 5µm per section (Bancroft and Gamble, 2008). The tissue sections were allowed to float in water bath at 30ºC to help in spreading the paraffin ribbons. Clean slides were then used to pick the tissues from the warm water bath (Bancroft and Gamble, 2008). The slides were left to dry and later stained using H and E and Cresyl Fast violet.

3.4 Staining Methods

3.4.1 Haematoxylin and Eosin (H & E) staining methods

The method of H and E staining technique involved hydrating the tissue sections in descending grades of alcohol from 100%, 95%, 90% and finally 70%. Each of these steps lasted for 15 minutes and the tissues were then washed in running tap water. The tissue was then stained with haematoxylin for 25 minutes, washed with water and then differentiated in acid alcohol. The tissue was then counter stained with eosin and blued in Scott water. The tissues were then hydrated in ascending grades of alcohol and cleared in xylene for three changes in 5 minutes each. The tissues were subsequently mounted with cover slips using a mounting media (Bancroft and Gamble, 2008). The tissues were finally viewed under light microscope and the photomicrographs were taken using digital amscope with model number (MD 900).

3.4.2 Cresyl fast violet staining (Nissl Stain)

Cresyl Violet stain was used to stain Nissl substance in the cytoplasm of the neurons. This technique employs basic aniline dye to stain RNA blue and was used to highlight important structural features of the neurons. The nissl substance (rough endoplasmic reticulum) appeared dark blue due to the staining of the ribosomal RNA, giving the

cytoplasm a mottled appearance. DNA present in the nucleus will be stained a similar colour (Bancroft and Gamble, 2008).

3.5 Atomic Absorption Spectrophotometry

Varian Fast Sequential Atomic Absorption Spectrophotometry with model number AA240FS was used to quantify the amount of Iron, Copper, Zinc and Manganese in the cerebrum and cerebellum of the neonatal Wistar rats. This was done at Multi-user Science Research laboratory, Chemistry Department, Ahmadu Bello University Zaria. The tissues were homogenised with Phosphate Buffer Solution (PBS) prior to the meta-analysis. It was then digested with nitric acid, heated and allowed to cool, this was done to remove organic matrix from the tissue homogenate (EHD, 1995).

Subsequent to digestion, the samples were diluted with deionised water and aqueous sample containing the metal analyte was aspirated into an air-acetylene flame, causing evaporation of the solvent and vaporization of the free metal atoms (EPA, 2007). This process is called atomization. A line source (hollow cathode lamp) operating in the UV-visible spectral region was used to cause electronic excitation of the metal atoms, and the absorbance is measured with a conventional UV-visible dispersive spectrometer with photomultiplier detector (EPA, 2007). The narrow spectral lines of atomic samples necessitate the use of a line source as well as a high-resolution monochromator. This helps to prevent interference from adjacent spectral lines of other atomic species present in the sample matrix (EPA, 2007). In this experiment, AAS in conjunction with flame atomization was used to determine specific metals (Fe, Zn, Cu, Mn) in aqueous sample. The availability of a spectrometer equipped with a lamp turret (allowing several line

sources to be used in sequential fashion) facilitated the measurement of multiple metals in the sample (EPA, 2007).

3.6 Morphometric Analysis

The crown rump length, and the weight of the pups were taken and the average for each group was recorded using digital vernier caliper. The crown rump length was measured in centimetre (cm) as a distance between the peak of the skull to the tip of the tail. The weight of the pups and their heads was measured in grams (g) using electronic digital weighing machine.

3.7 Photomicrography

The photomicrographs were snapped using an instrument called amscope, a microscope digital camera with a model number MD900. The microscope's eyepiece was replaced by the amscope's eyepiece. The amscope has an extension cable that was connected to a laptop through a USB port. The tissue was viewed through the computer, and the area that best reveals the tissue and what is targeted was captured, and the photomicrograph captured was printed out using colour printer.

3.8 Neuro-Behavioural Studies

The neuro-behaviour of both the ethanol-treated and control pups were assessed for the achievement of developmental milestones. Achievement was based on the ability of a pup to display a positive response within the stipulated time. For each milestone, the time in seconds to reach the milestone was recorded. Testing commenced at 10:00 am each day, with the dam placed in a separate cage throughout the testing procedure.

3.8.1 Surface righting reflex on postnatal day 5 (PND 5)

This test was conducted to measure the development of the vestibular reflex and motor coordination. It assesses the ability of the pups to co-ordinate the necessary movement to roll over from its back onto its paws. The rats were placed in supine position and the time taken in 30seconds to turn over to prone position and place all four paws in contact with the surface was recorded. Each animal was tested in 2 trials on post-natal day five (PND 5). Positive responses were noted when pups were able to complete the task. The maximum time allowed per trial was 30secs (Kihara *et al.*, 2001; Kihara, 1991; Vorhees *et al.*, 1979a,b; Fox, 1965). The number of litters with successful responses under 2 trials were recorded (figure 3.1).



Figure 3.1: Surface Righting, showing the pup placed in supine position

3.8.2 Cliff avoidance (PND 6)

On PND 6, the pup's ability to avert a cliff was assessed. Pups were placed on the end of a 5-cm-high platform with their noses and fore-digits suspended over the edge of the simulated cliff. The latency to back away from the edge of the cliff, turn, and crawl away was measured. This test is considered an evaluation of sensory and motor coordination development. Positive responses were noted when pups completed the task in < 30 seconds. Each pup was tested in 1 trial. The maximum time allowed per trial was 60secs (Kihara *et al.*, 2001; Kihara, 1991; Vorhees *et al.*, 1979a,b; Fox, 1965) (figure 3.2).



Figure 3.2: Cliff Avoidance, showing the pup on the cliff

3.8.3 Negative geotaxis (PND 7)

This test was conducted to assess motor co-ordination of the pups when challenged on a sloped surface. Latency to rotate the body is considered a measurement of vestibular and postural reflexes. The animals were placed with their nose pointed downward on an inclined grid (45º) of 30cm. The hindlimbs of the pups were placed in the middle of the grid. The time taken to complete a 180º turn was measured. Each animal was given one trial at a maximum of 60seconds (Kihara *et al.,* 2001; Kihara, 1991; Vorhees *et al.,* 1979a,b; Fox, 1965) (figure 3.3).



Figure 3.3: Negative Geotaxis, showing the pup turning 180º against the slope

3.9 Statistical Analysis

The data was analysed using SPSS software version 20. The data were expressed as Mean ± SEM (Standard Error of Mean). One-Way Analysis of Variance (ANOVA) was used to compare the mean difference between and within the groups and a P-value of <0.05 was considered to be statistically significant. Bonferroni corrected post-hoc test was used where there was significant difference between and within the groups to find where the significant lies. Charts were produced using Microsoft Excel 2013.

CHAPTER FOUR

4.0 Results

4.1 Physical Observation

There was no physical deformity observed in both the control and the ethanol-treated pups. However, the pups of the ethanol-treated dams that were exposed to 30% ethanol in utero throughout the gestation period were smaller in size when compared to the Control and other groups. It was also observed that Group G showed delayed parturition extending to 22nd day of gestation.

4.2 Morphometric Analysis

The result showed a high birth weight in the Control Group when compared to the other treated groups which is not statistical significant at $P< 0.05$(Figure 4.1). Following examination of weight of the pups on postnatal day 7, ethanol-treated Groups B, C, D and E that were exposed prenatally to 0.5ml 20% ethanol for 7, 14, 20 days and 0.5ml 30% ethanol for 7days respectively showed a recuperative increase in body weight higher than the Control Group, but statistical significance was only met with Group D (0.5ml 20% ethanol 20days) at $P<0.001$ (Figure 4.1). Groups F and G that received 0.5ml 30% ethanol for 7 and 14 days respectively exhibited non-statistically significant low increase in weight on comparison with the Control Group (Figure 4.1).

On the other hand, the crown-rump length of the Control Group at birth was higher than that of the ethanol-treated Groups but it was not statistically significant with the exception of Group E that met statistical significance at $P<0.05$ as shown in Figure 4.2. Moreover, the crown-rump length at postnatal day 7 of the Control Group A is slightly higher than that of ethanol-treated Groups C, F and G which was not statistically

significant (Figure 4.2). The crown-rump length on postnatal day 7 was slightly higher in ethanol-treated Groups B, D and E when compared to Control Group A, but does not meet statistical significance (Figure 4.2).

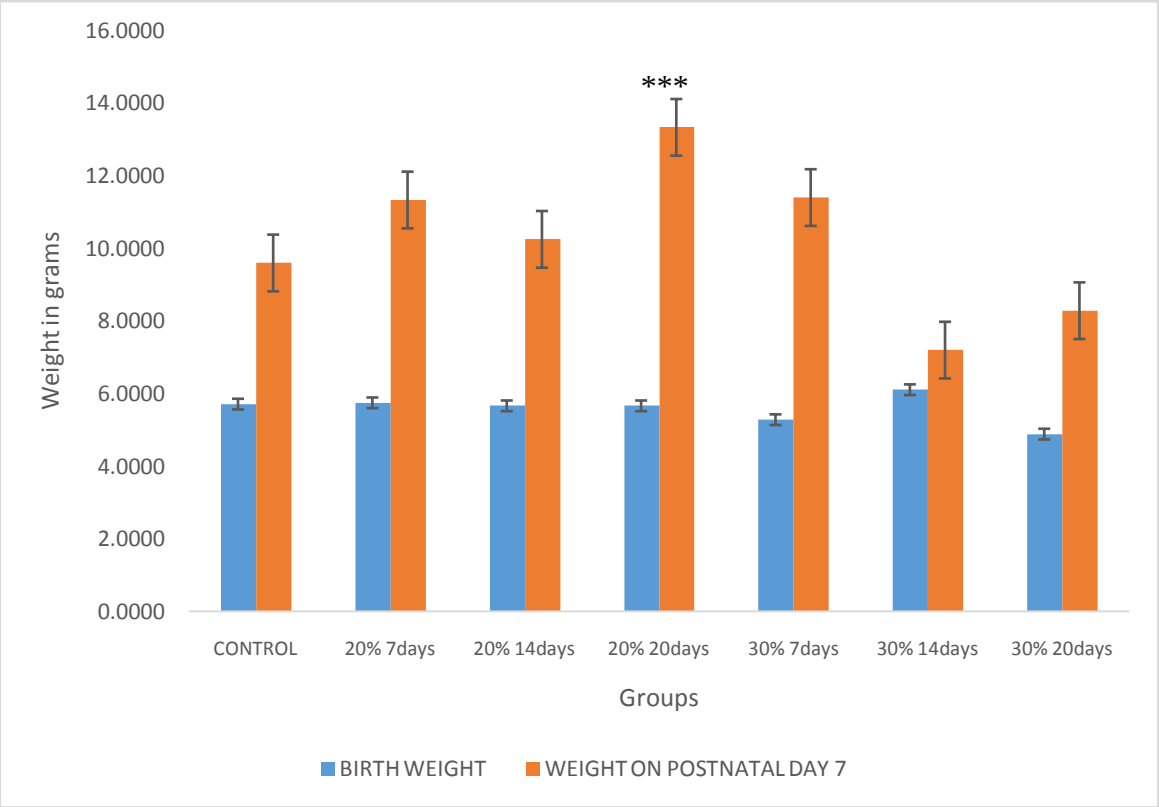


Figure 4.1: Comparison of the neonatal Wistar rat's mean birth weight and weight on postnatal day 7. * $P < 0.05$; *** $P < 0.001$.

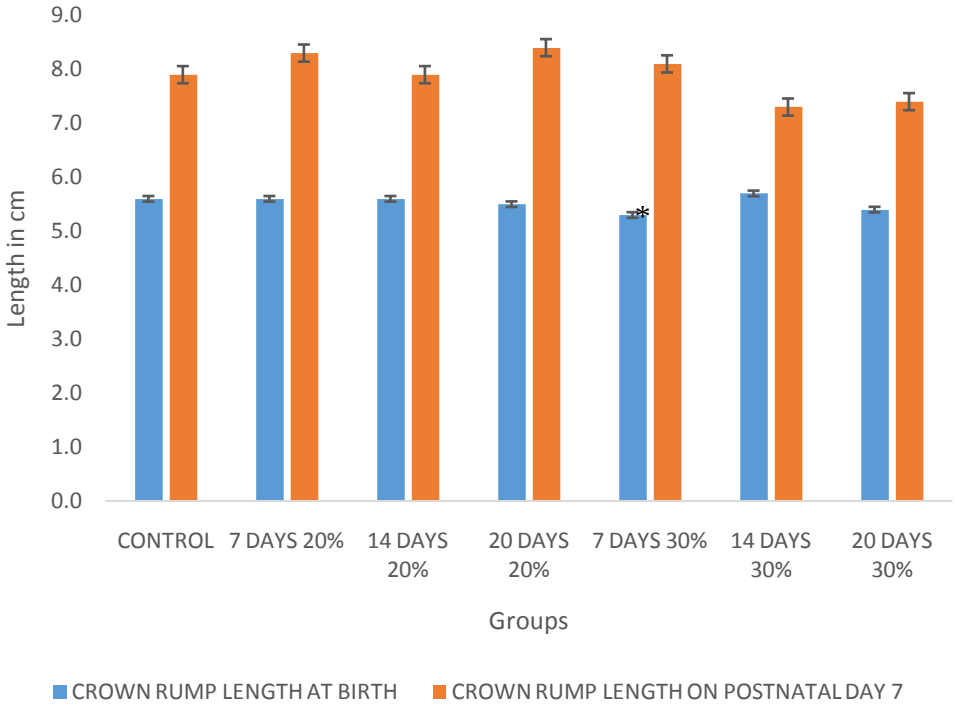


Figure 4.2: Comparison of neonatal Wistar rat's mean crown rump length at birth and on postnatal day 7. * $P < 0.05$.

4.3 Pre-weaning Neuro-Behavioural Battery Tests

The results of the pre-weaning battery tests as described below and illustrated in figure 4.3 revealed a neuro-behavioural effects on the ethanol-treated neonates. However, the effect did not show a dose-dependent pattern of effects.

4.3.1 Surface righting reflex

The control group A showed a quick reflex latency when compared with the ethanol-treated groups especially groups B, C, F and G although the difference does not meet statistical significance at $P<0.05$. Groups D and E showed relatively close timing response compared to the control group, this also however was not statistically significant at $P<0.05$ (figure 4.3).

4.3.2 Negative geotaxis

The control group A showed a statistically significant difference in the timing response compared to the ethanol-treated Groups E at $P<0.05$, F at $P<0.001$ (highly statistical significance) and G at $P<0.01$ as shown in figure 4.3. The control group A also showed a quick reflex latency when compared with treated Groups B, C and D, although this does not meet statistical significance at $P<0.05$ (figure 4.3).

4.3.3 Cliff avoidance (Visual cliff)

The Control Group A, showed a quick latency in avoiding the cliff compared to ethanol-treated Groups B, E, F, and G but only Groups B and E met statistical significance at $P<0.001$ and $P<0.05$ respectively as shown in Figure 4.3.

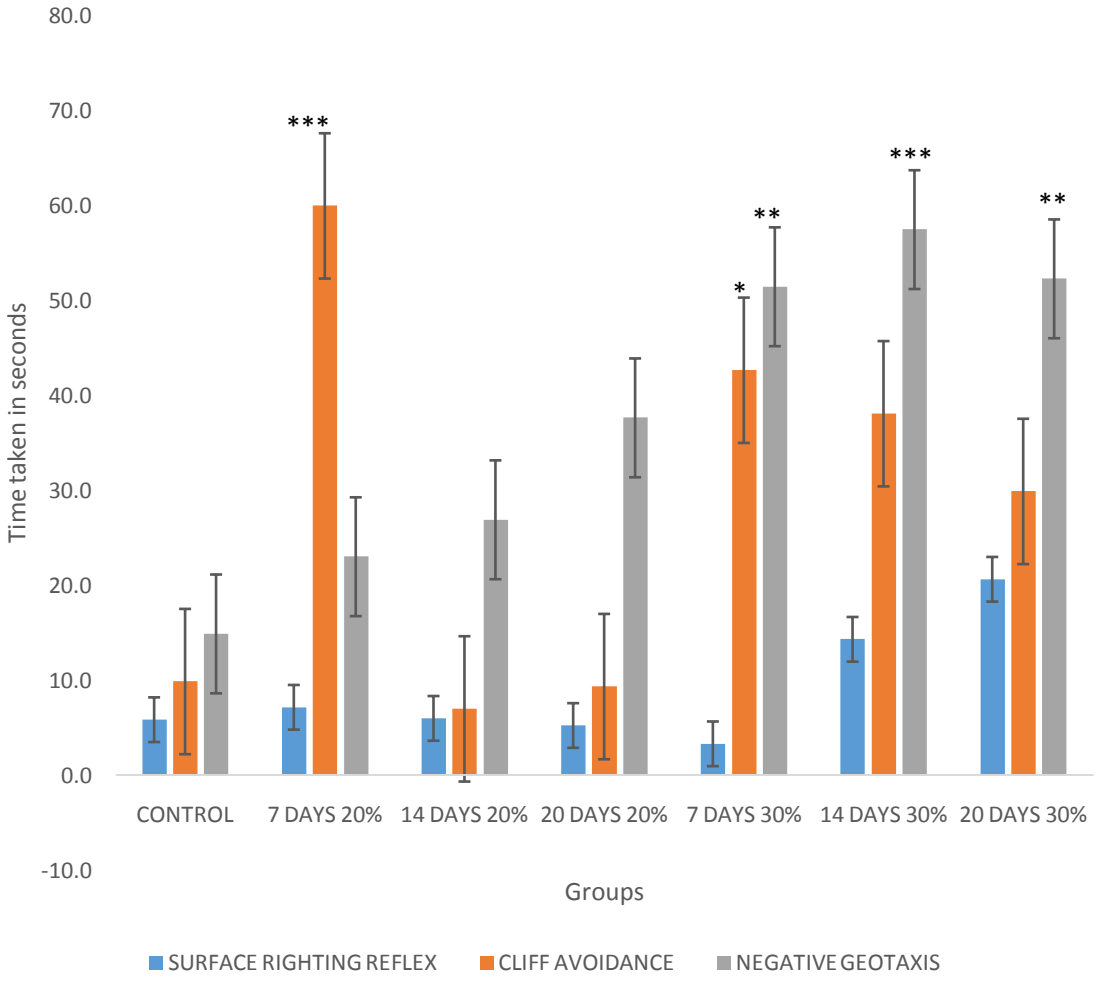


Figure 4.3: Comparison of the effects of intrauterine ethanol exposure on neuro-behaviour of the pups. * P < 0.05; ** P < 0.01; *** P < 0.001

4.5 Histologic Findings

The result from histological observations showed varying degrees of histopathological presentations in the treated groups. The cytoarchitecture of the cerebral and cerebellar cortices were explored using Haematoxylin and Eosin (H&E) and Cresyl Fast Violet staining techniques to enable an in-depth histopathological evaluation of the intra-uterine ethanol exposure on the tissues at varying percentages and periods of development.

4.5.1 Histology of the cerebral cortex

Plate 1 showed a photomicrograph of the cerebral cortex of neonatal Wistar rats of the Control Group A on postnatal day 7 using Hand E stain, it was shown to have the normal cellular architecture revealing different types of neural cells including Pyramidal Cells, Fusiform cells, cells of martinoti and stellate cells.Plate 2 shows a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group B that received 0.5ml 20% ethanol for 7 days showing karyorhexic cells, Pyknotic cells, Degenerated cells and clumped cells. Plate 3 showed a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group C that received 0.5ml 20% ethanol for 14 days showing clumped cells and numerous pyknotic cells. Moreover, Plate 4 showed a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group D that received 0.5ml 20% ethanol for 14 days showing Pyknotic cells, Degenerated cells and clumped cells.Plate 5 is a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group E that received 0.5ml 30% ethanol for 7 days showing karyorhexic cells, Pyknotic cells and clumped cells. Plate 6 is showing a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group F that received 0.5ml 30% ethanol for 14 days showing karyorhexic cells, Pyknotic cells and clumped

cells. Plate 7 is a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group G that received 0.5ml 30% ethanol for 20 days showing Vacoulations, Pyknotic cells and clumped cells. The ethanol treated neonates exhibited different forms of histopathological appearance when compared to the Control Group. The effects seen did not follow a dose-dependent pattern but rather a time of exposure dependence, this was shown in the 14 days exposed neonates with a characteristic feature of numerous clumped cells.

4.5.2 Histology of cerebellar cortex

On the other hand, H and E stain as shown in plates 8 to 14 of the cerebellar cortices showed a unique presentation of neonatal cerebellum which shows distinction from the adult cerebellum. The distinction is as a result of dense granulation of the outer molecular layer of the neonatal Wistar rat cerebellum as opposed to the adult rat cerebellum that is devoid of granular cells. Plate 8 is a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Control Group A that received normal saline showing the foetal arrangement demonstrating Purkinje cells, Granular Cells, Purkinje cell layer, Outer Molecular Layer and Precursor Granular cells.

Ethanol exposure is shown to affect the purkinje cellular density and arrangement. Plate 9 is showing a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group B that received 0.5ml 20% ethanol for 7 days showing some Degenerated Purkinje Cells, Karyorhexic Cells and Pyknotic Cells. Plate 10 showed a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group C that received 0.5ml 20% ethanol for 14 days showing some Karyorhexic Cells, Pyknotic Cells and clumping of the purkinje cells. Plate 11 is a section of the cerebellar cortex of neonatal

Wistar rat on postnatal day 7 for Group D that received 0.5ml 20% ethanol for 14 days showing some Karyorhexic purkinje Cells and Pyknotic purkinje Cells. Plate 12 showed a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group E that received 0.5ml 30% ethanol for 7 days showing some Karyorhexic purkinje Cells and Pyknotic purkinje Cells.Plate 13 is showing a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group F that received 0.5ml 30% ethanol for 14 days showing some Degenerated Purkinje Cells, clumped pyramidal cells and Pyknotic Cells. Plate 14 showed a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group G that received 0.5ml 30% ethanol for 20 days showing some Degenerated Purkinje Cells and Ferrugination. Clumping of purkinje cells was also observed only in 14 days treated neonates revealing the much significance of time of exposure more than the percentage of ethanol as a function of its teratogenicity.

4.5.3 Histochemistry of cerebral cortex

Plates 15 demonstrated the cerebral cortex of the Control Group using cresyl fast violet, the Control Group was shown to have high density of Nissl substance while the treated groups exhibited a varying levels of Nissl substance degradation, a feature referred to as neurocyte chromatolysis. Plate 16 showed a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group B that received 0.5ml 20% ethanol for 7 days showing moderately stained nissl substance with Central Chromatolysis. Plate 17: A section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group C that received 0.5ml 20% ethanol for 14 days also showing moderately stained nissl substance with Central Chromatolysis. Plate 18 is a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group D that received 0.5ml 20% ethanol for 20 days showing faintly stained nissl substance with Central Chromatolysis. Plate 19 is

a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group E that received 0.5ml 30% ethanol for 7 days also showing faintly stained nissl substance with Central Chromatolysis. Plate 20 also showed a section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group F that received 0.5ml 30% ethanol for 14 days showing moderately stained nissl substance with Central Chromatolysis. Plate 21: A section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group G that received 0.5ml 30% ethanol for 20 days showing faintly stained nissl substance with severe neurocyte chromatolysis was also observed with reduced apical dendrite in the treated groups, distortion in the nuclear outline and neural degeneration in the treated groups was observed when compared to the control.

4.5.4 Histochemistry of the cerebellar cortex

Plates 22 to 28 demonstrated the cerebellum using cresyl fast violet. Plate 22 is the Control Group showing nissl substanceand finely arranged purkinje cells while the treated groups exhibited a varying levels of Nissl substance degradation (chromatolysis) and distortion in the nuclear outline of the purkinje cells.Plate 23 is a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group B that received 0.5ml 20% ethanol for 7 days showing degenerated purkinje cells with distorted nuclear outline. Plate 24 is also a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group C that received 0.5ml 20% ethanol for 14 days showing Degenerated Purkinje cells. Plate 25 is showing a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group D that received 0.5ml 20% ethanol for 14 days showing purkinje cells with distorted nuclear outline as a result of nissl substance degradation. Plate 26 is a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group E that received 0.5ml 30% ethanol for 7 days showing

Degenerated Purkinje Cells and Central Chromatolysis. Plate 27 represents a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group F that received 0.5ml 30% ethanol for 14 days showing Degenerated Purkinje Cells. Plate 28 is a section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group G that received 0.5ml 30% ethanol for 20 days showing Degenerated Purkinje Cells and degradation of nissl substance.

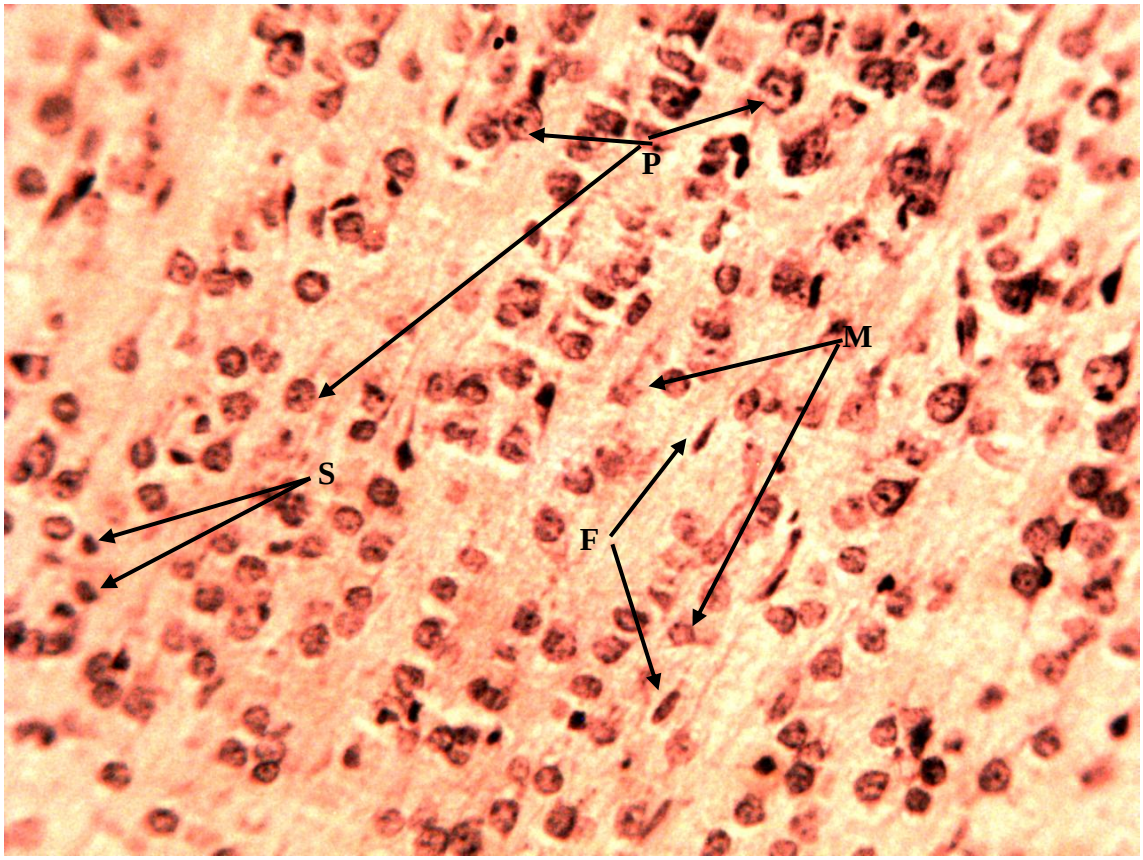


Plate 1: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for the Control Group that received normal saline showing normal architecture with Pyramidal cells (P), Martinotti cells (M), Fusiform cells (F) and Stellate cells (S) (H and E X250)

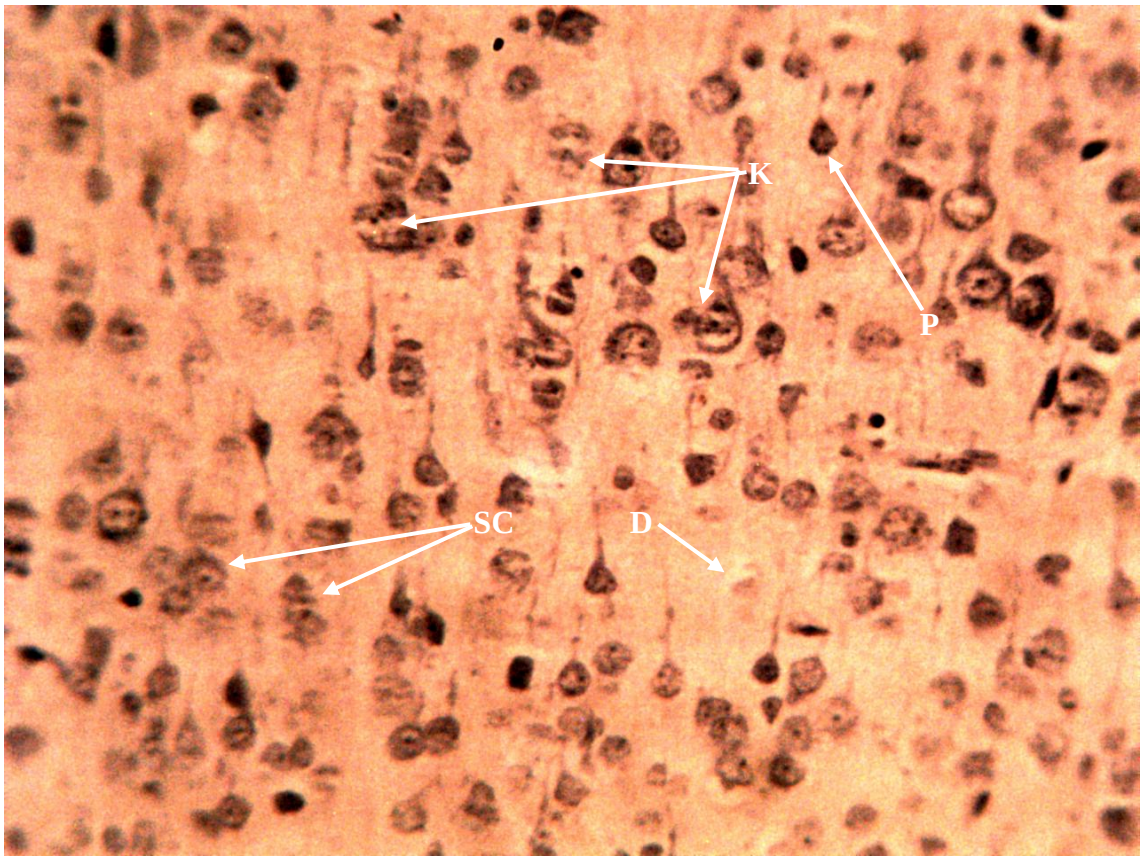


Plate 2: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group B that received 0.5ml 20% ethanol for 7 days showing karyorhexic cells (K), Pyknotic cells (P), Degenerated cells (D) and a feature of Satellitosis (SC) (H and E stain X250)

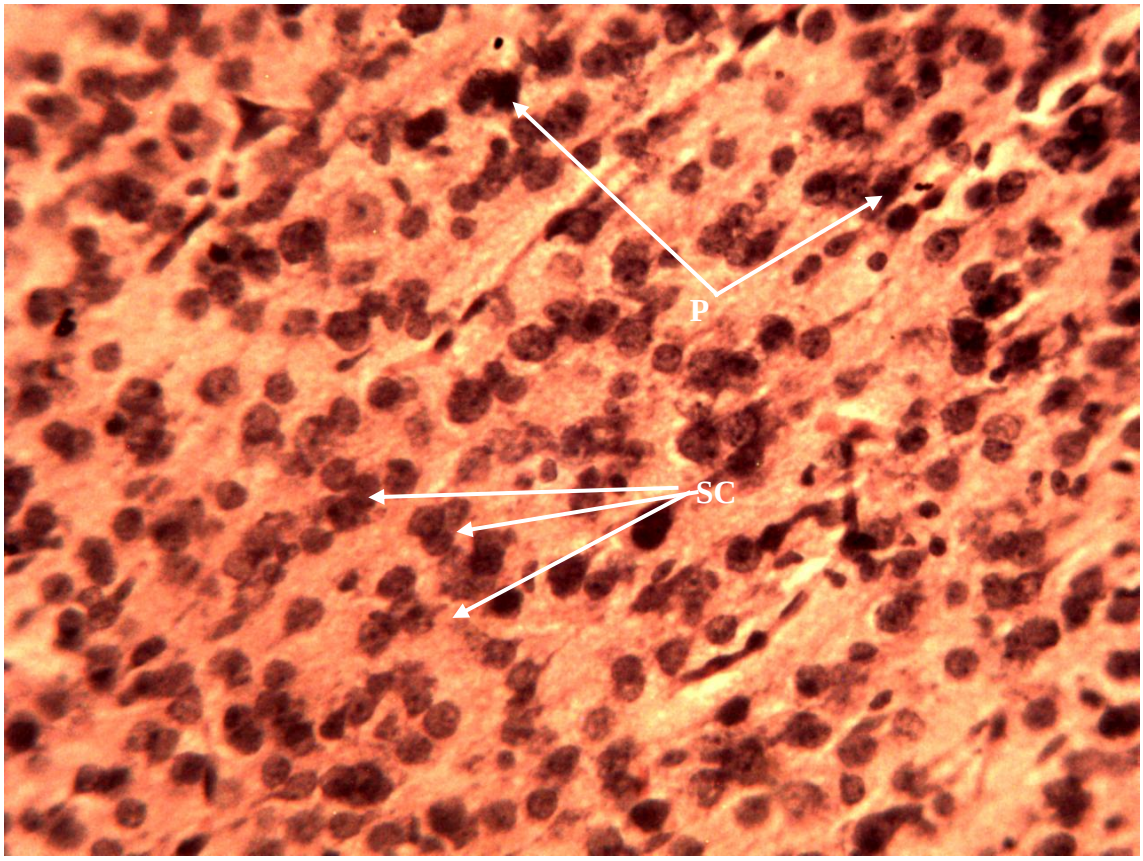


Plate 3: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group C that received 0.5ml 20% ethanol for 14 days showing a feature of Satellitosis (SC) and numerous pyknotic cells (P) (H and E stain X250)

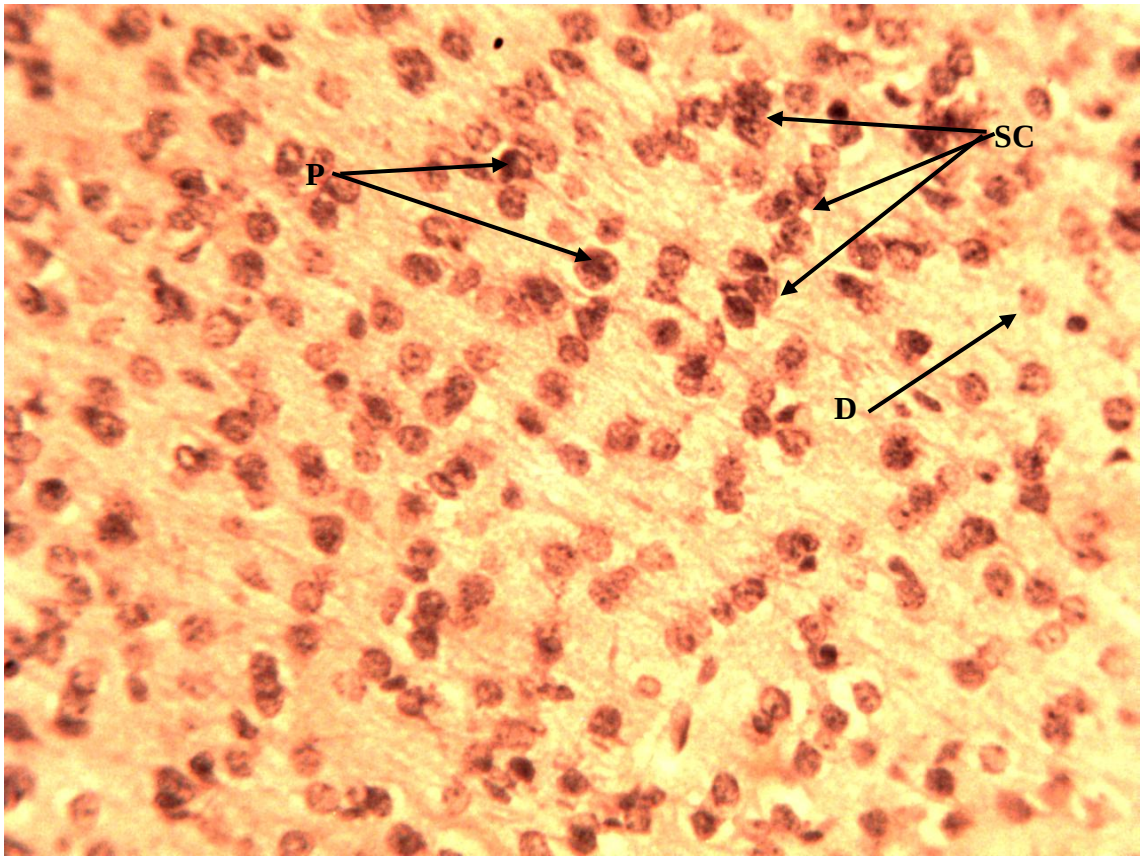


Plate 4: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group D that received 0.5ml 20% ethanol for 14 days showing Pyknotic cells (P), Degenerated cells (D) and a feature of Satellitosis (SC) (H and E stain X250)

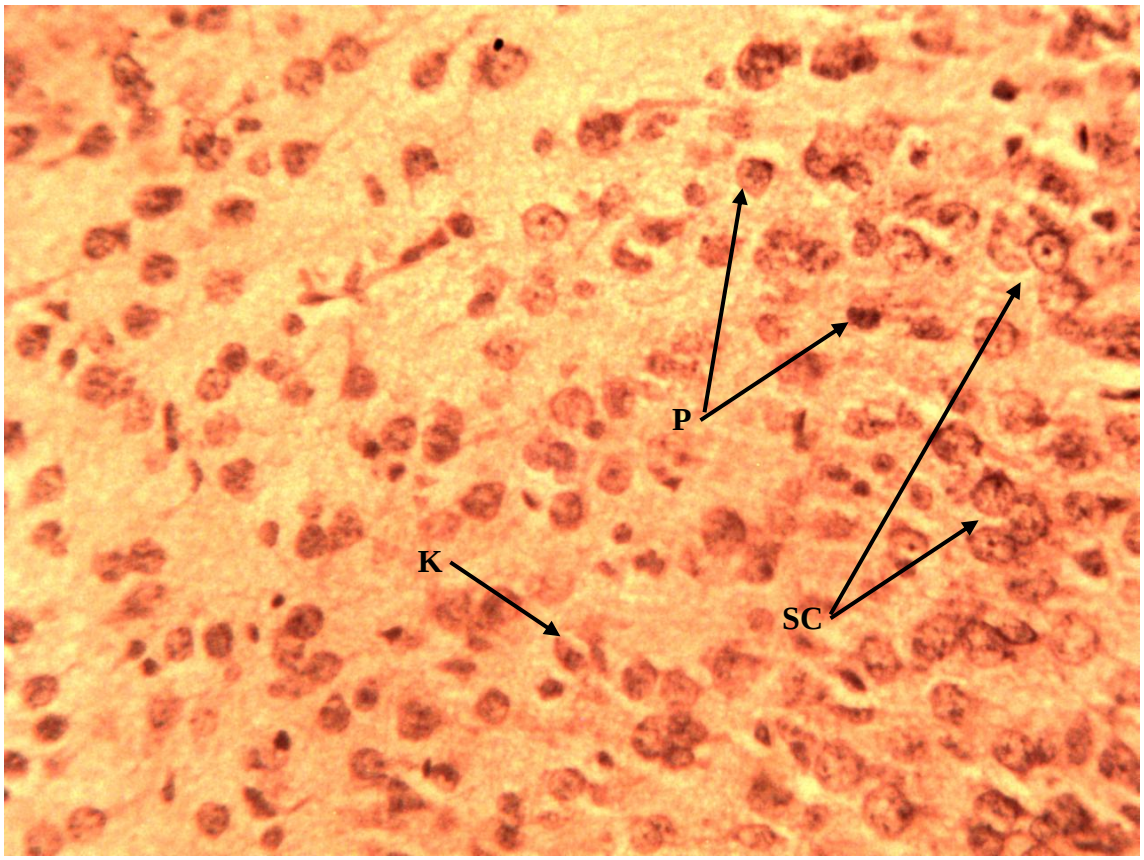


Plate 5: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group E that received 0.5ml 30% ethanol for 7 days showing karyorhexic cells (K), Pyknotic cells (P), and a feature of Satellitosis (SC) (H and E stain X250)

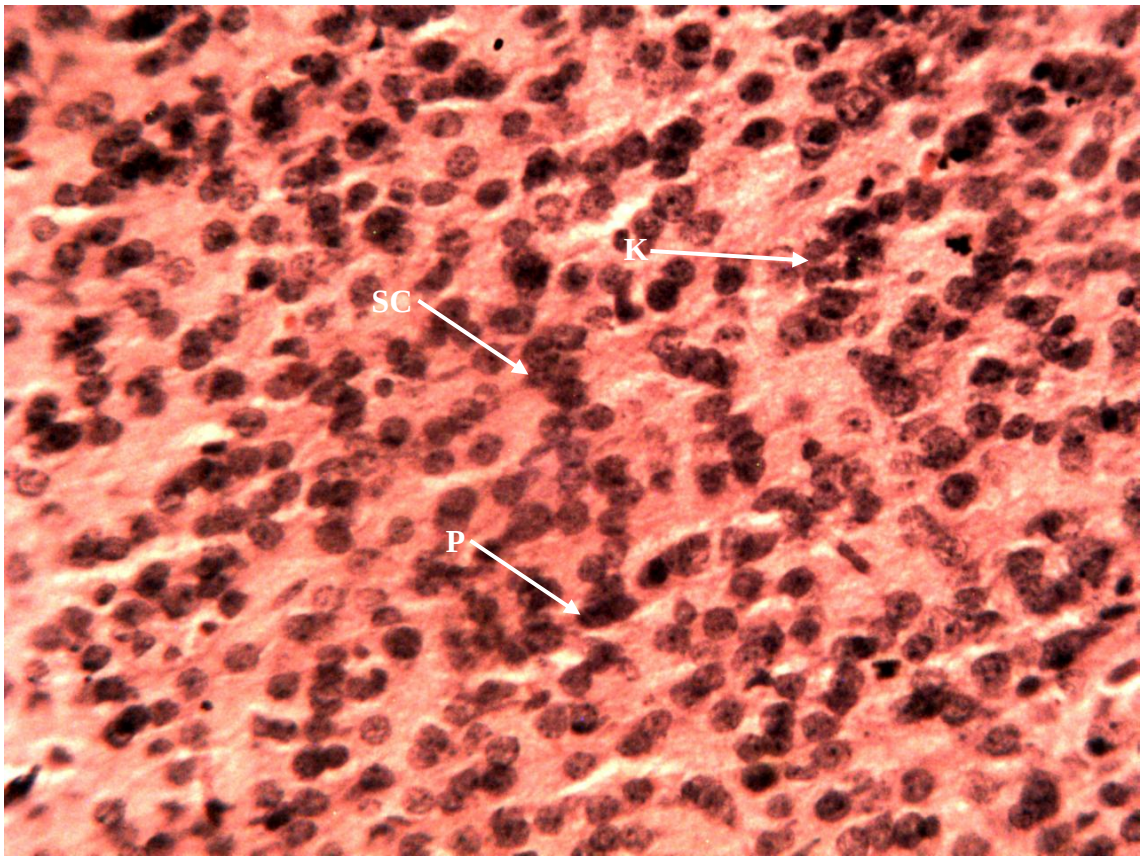


Plate 6: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group F that received 0.5ml 30% ethanol for 14 days showing karyorhexic cells (K), Pyknotic cells (P), and a feature of Satellitosis (SC) (H and E stain X250)

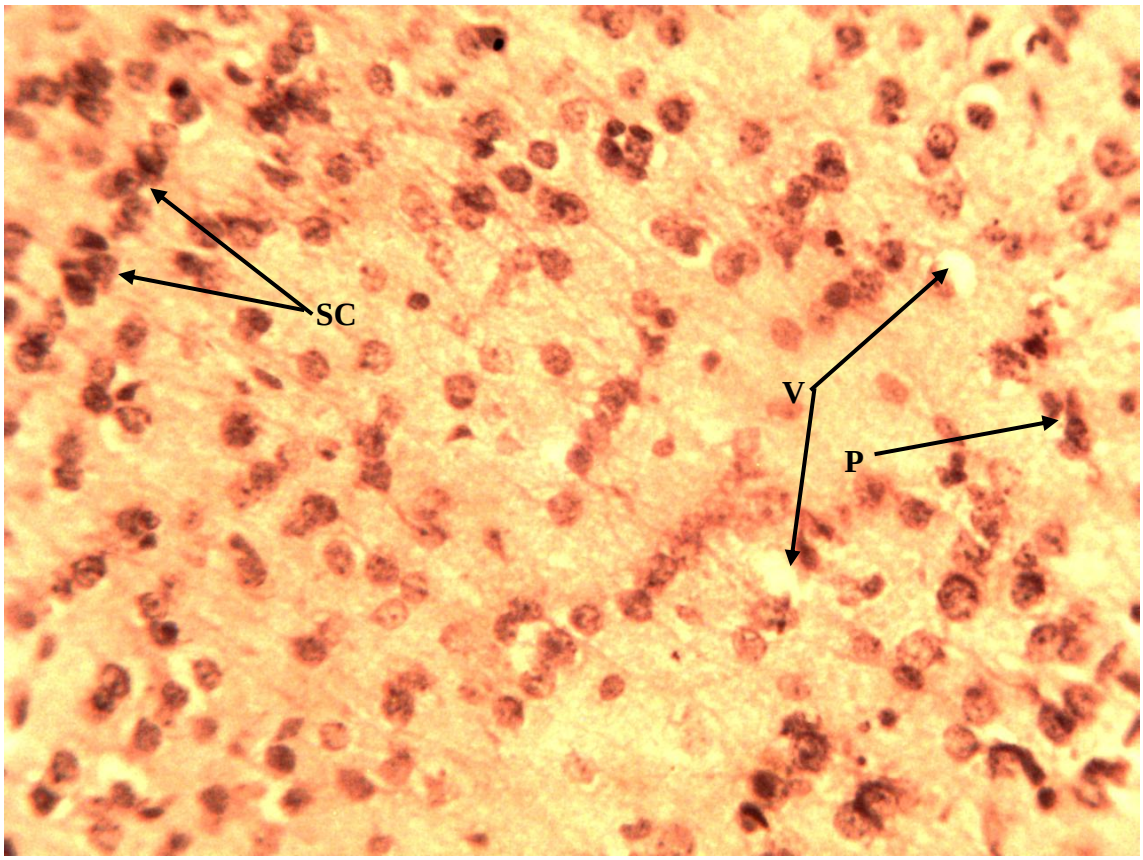


Plate 7: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group G that received 0.5ml 30% ethanol for 20 days showing Vacoulations (V), Pyknotic cells (P), and a feature of Satellitosis (SC) (H and E stain X250)

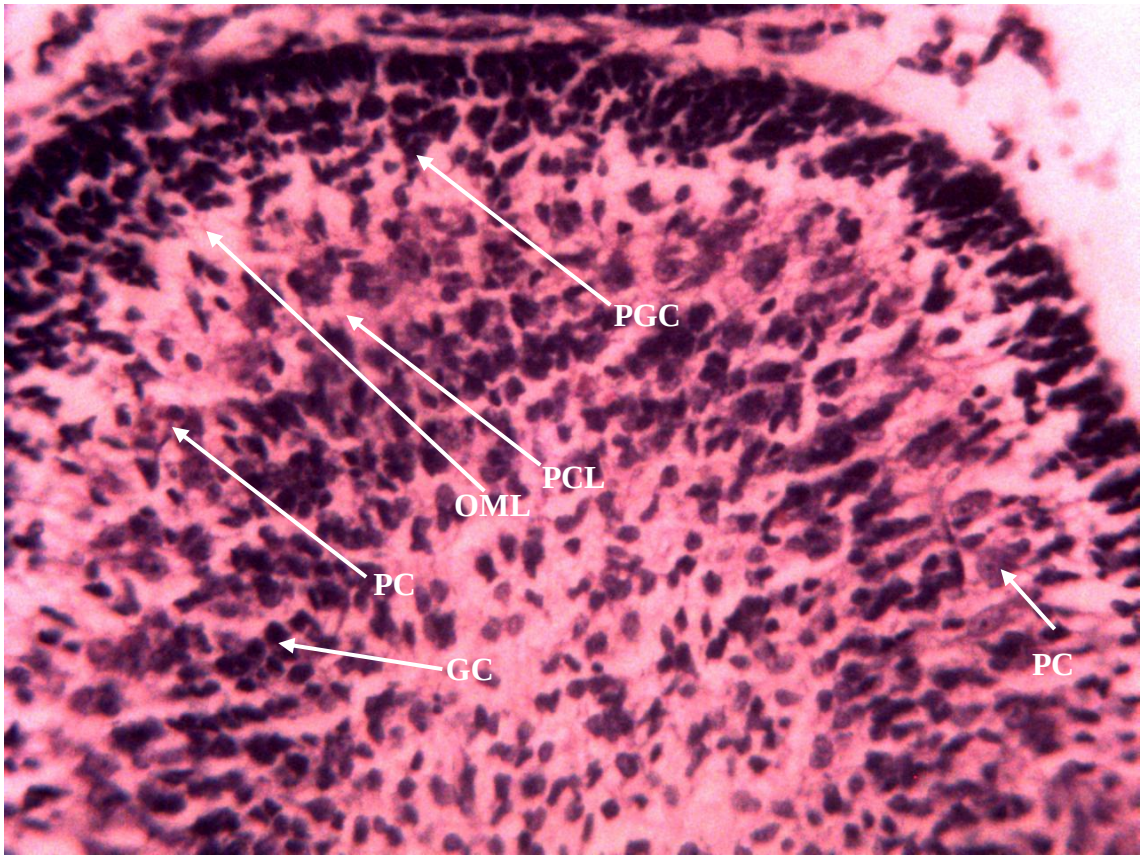


Plate 8: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Control Group A that received normal saline showing the foetal arrangement demonstrating Purkinje cells (PC), Granular Cells (GC), Purkinje cell layer (PCL), Outer Molecular Layer (OML) and Precursor Granular cells (PGC) (H and E stain X250).

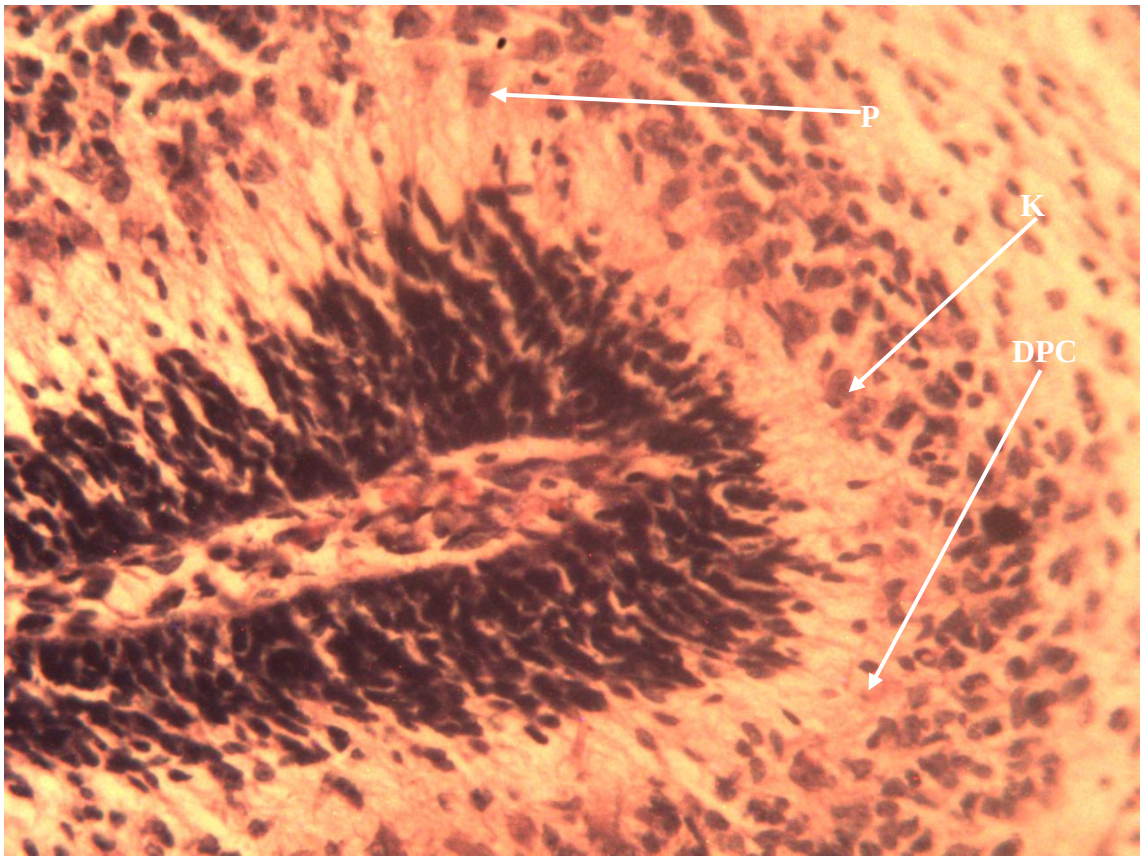


Plate 9: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group B that received 0.5ml 20% ethanol for 7 days showing some Degenerated Purkinje Cells (DPC), Karyorhexic Cells (K) and Pyknotic Cells (P) (H and E stain X250)

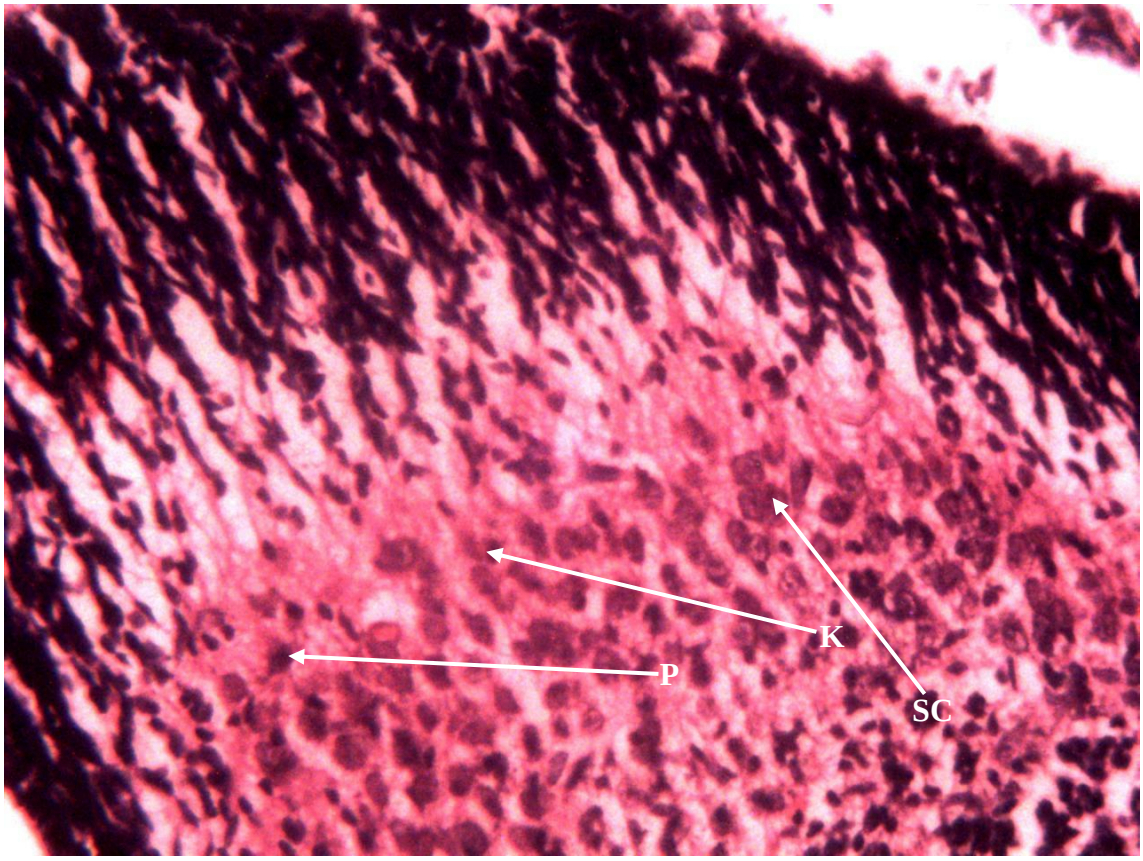


Plate 10: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group C that received 0.5ml 20% ethanol for 14 days showing some Karyorhexic Cells (K), Pyknotic Cells (P) and a feature of Satellitosis of the purkinje cells (SC) (H and E stain X250)

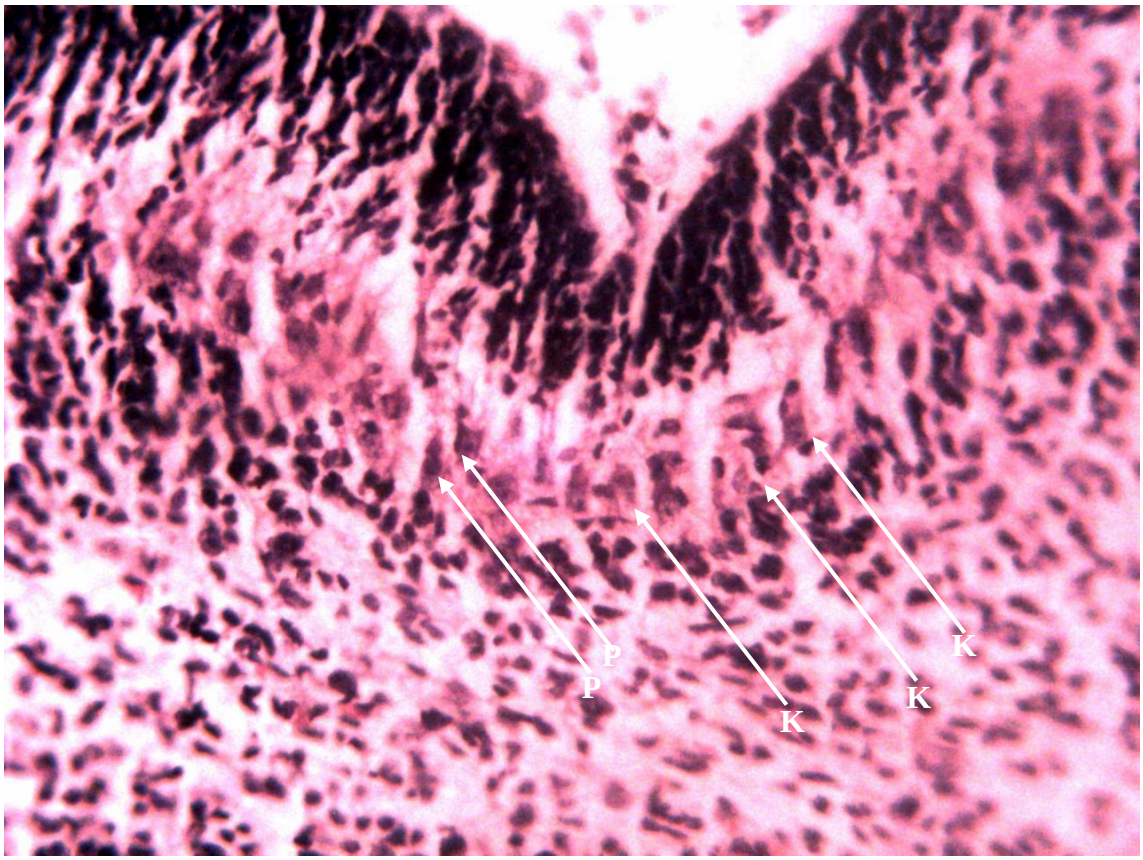


Plate 11: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group D that received 0.5ml 20% ethanol for 14 days showing some Karyorhexic Cells (K) and Pyknotic Cells (P) (H and E stain X250).

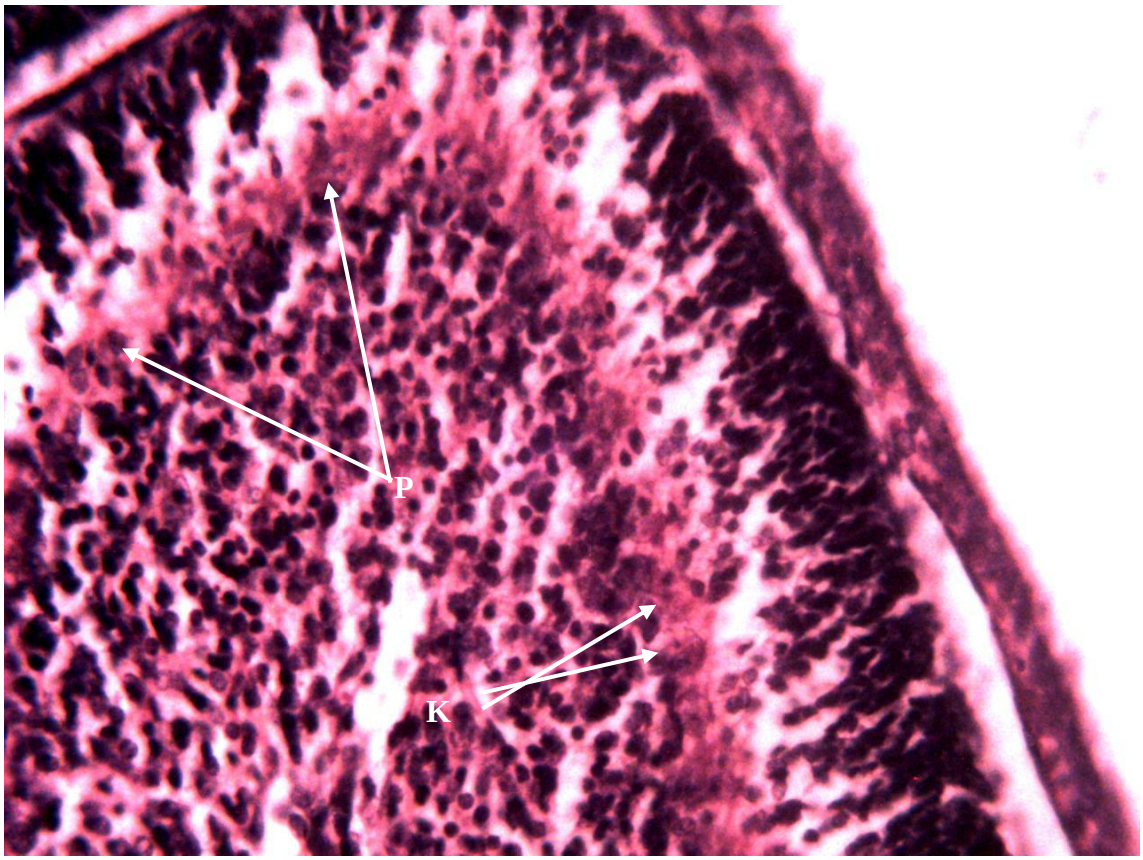


Plate 12: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group E that received 0.5ml 30% ethanol for 7 days showing some Karyorhexic Cells (K) and Pyknotic Cells (P) (H and E stain X250)

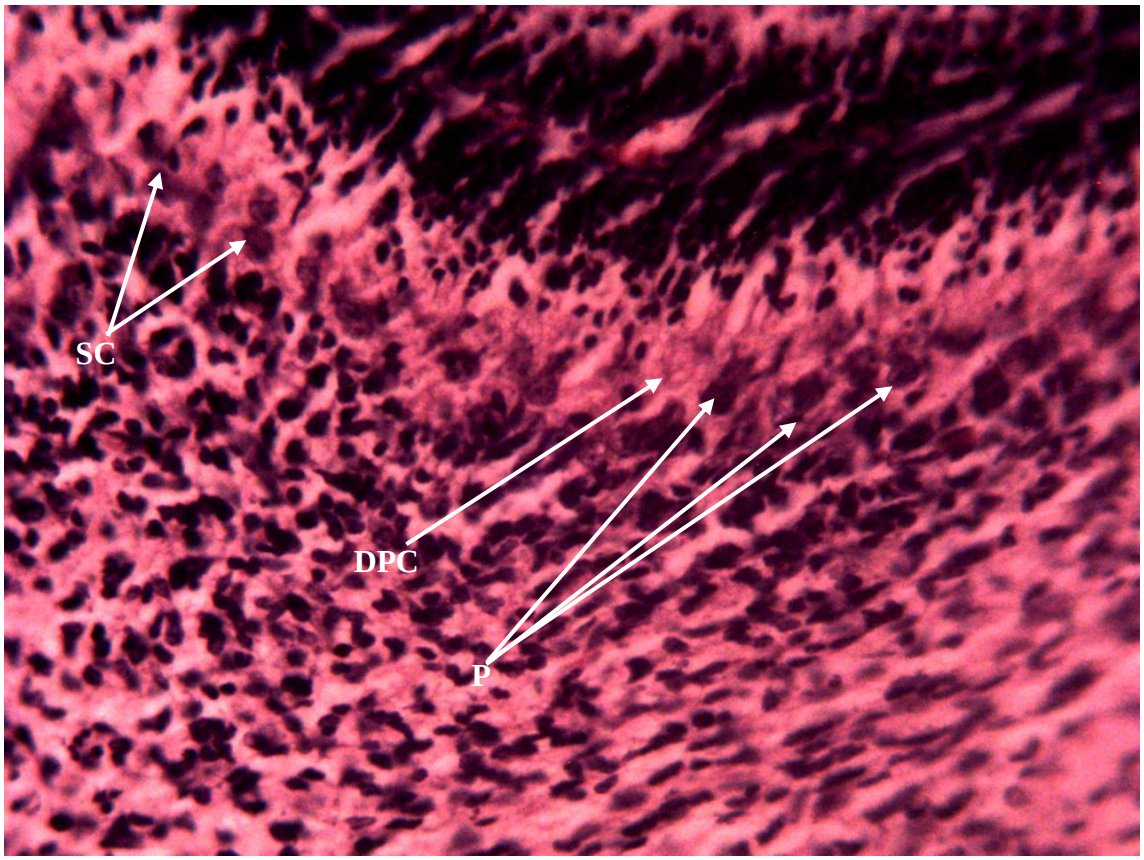


Plate 13: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group F that received 0.5ml 30% ethanol for 14 days showing some Degenerated Purkinje Cells (DPC), Satellitosis (SC) and Pyknotic Cells (P) (H and E stain X250)

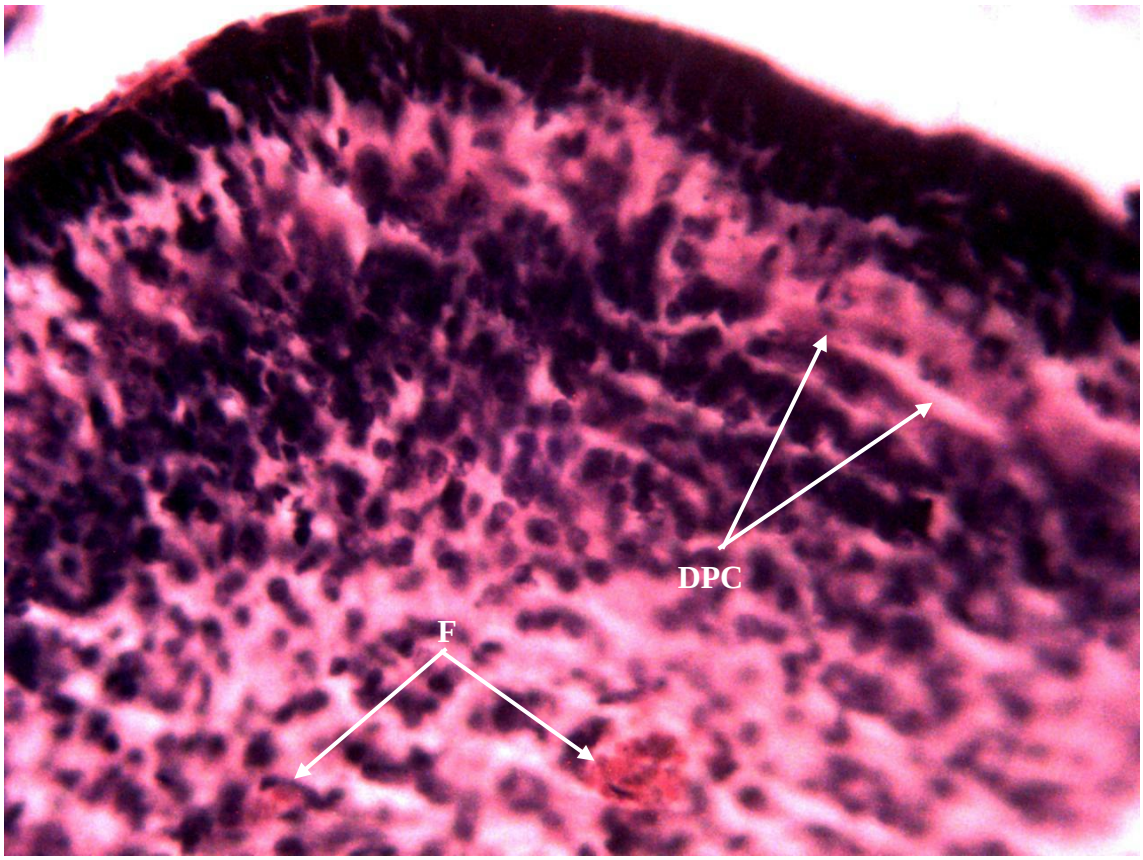


Plate 14: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group G that received 0.5ml 30% ethanol for 20 days showing some Degenerated Purkinje Cells (DPC), and Ferrugination (F) (H and E stain X250).

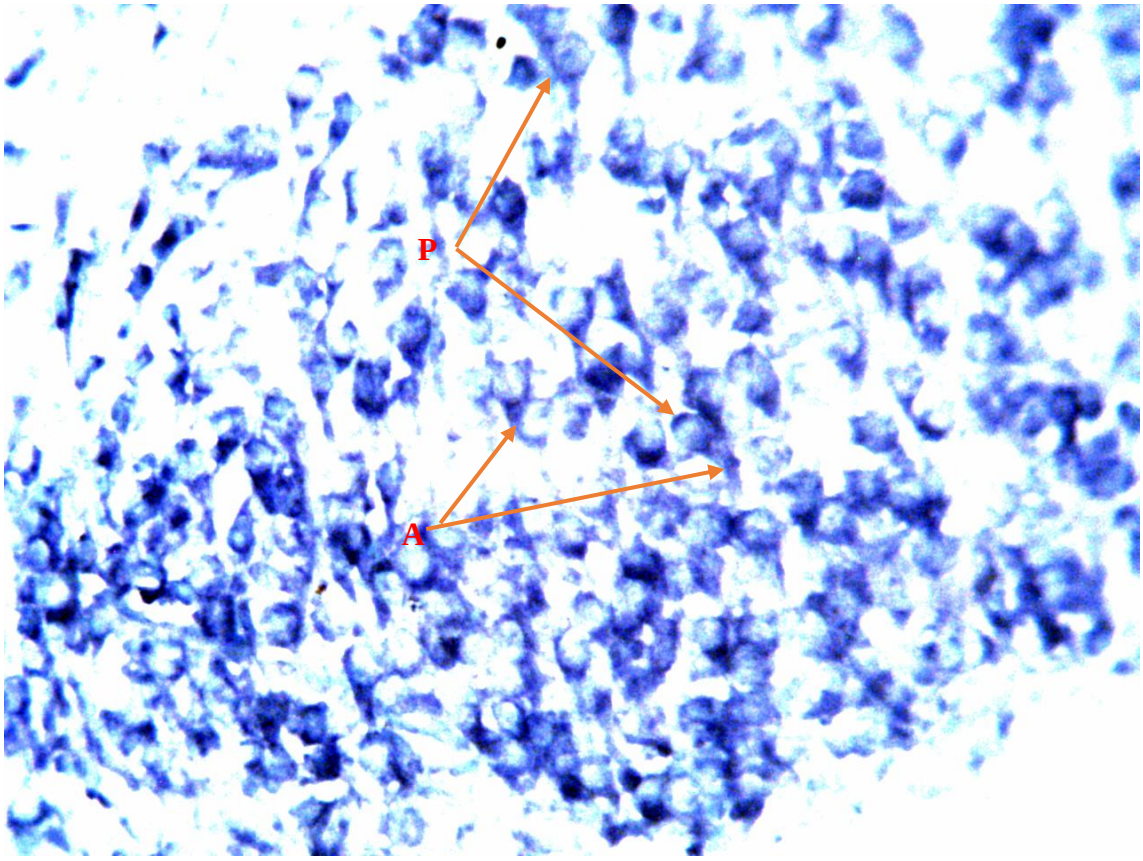


Plate 15: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Control Group A that received normal saline showing pyramidal cells (P) and Apical dendrite (A) (Cresyl Fast Violet stain X250)

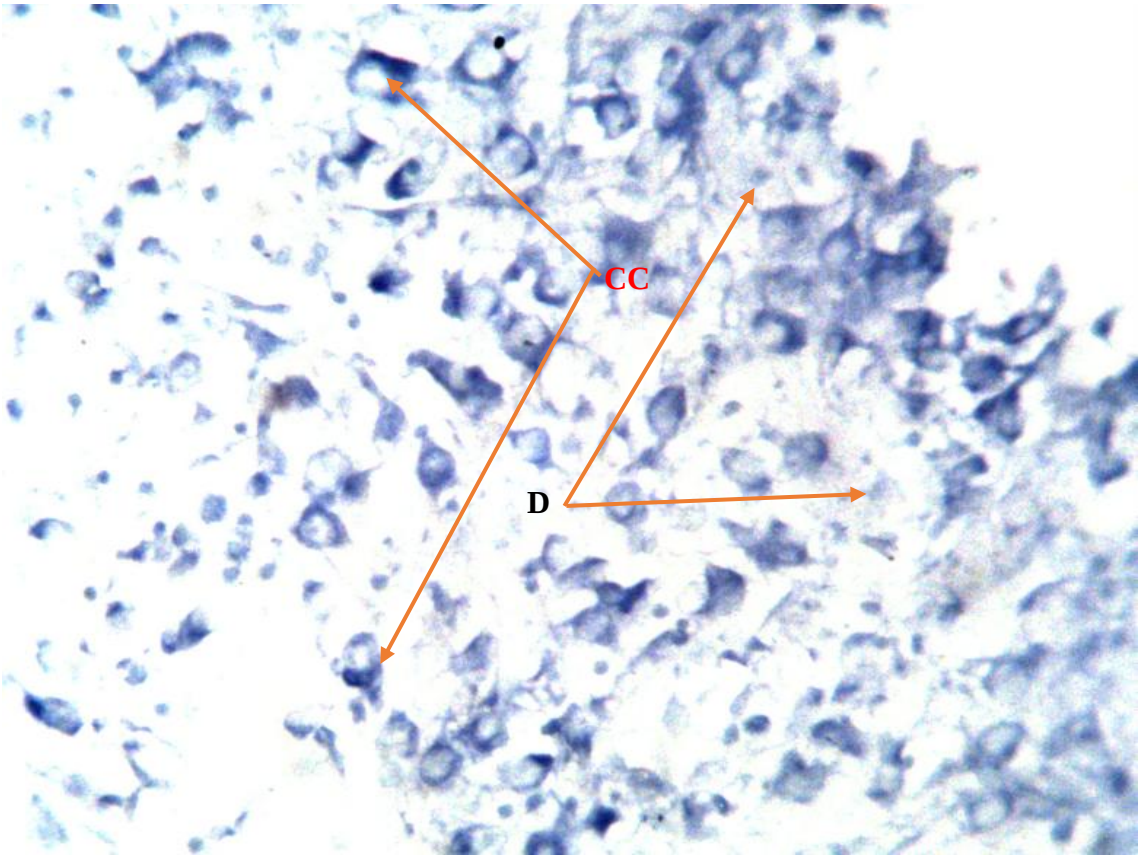


Plate 16: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group B that received 0.5ml 20% ethanol for 7 days showing moderately stained nissl substance with Central Chromatolysis (CC) and Degeneration of cells (D) (Cresyl Fast Violet stain X250)

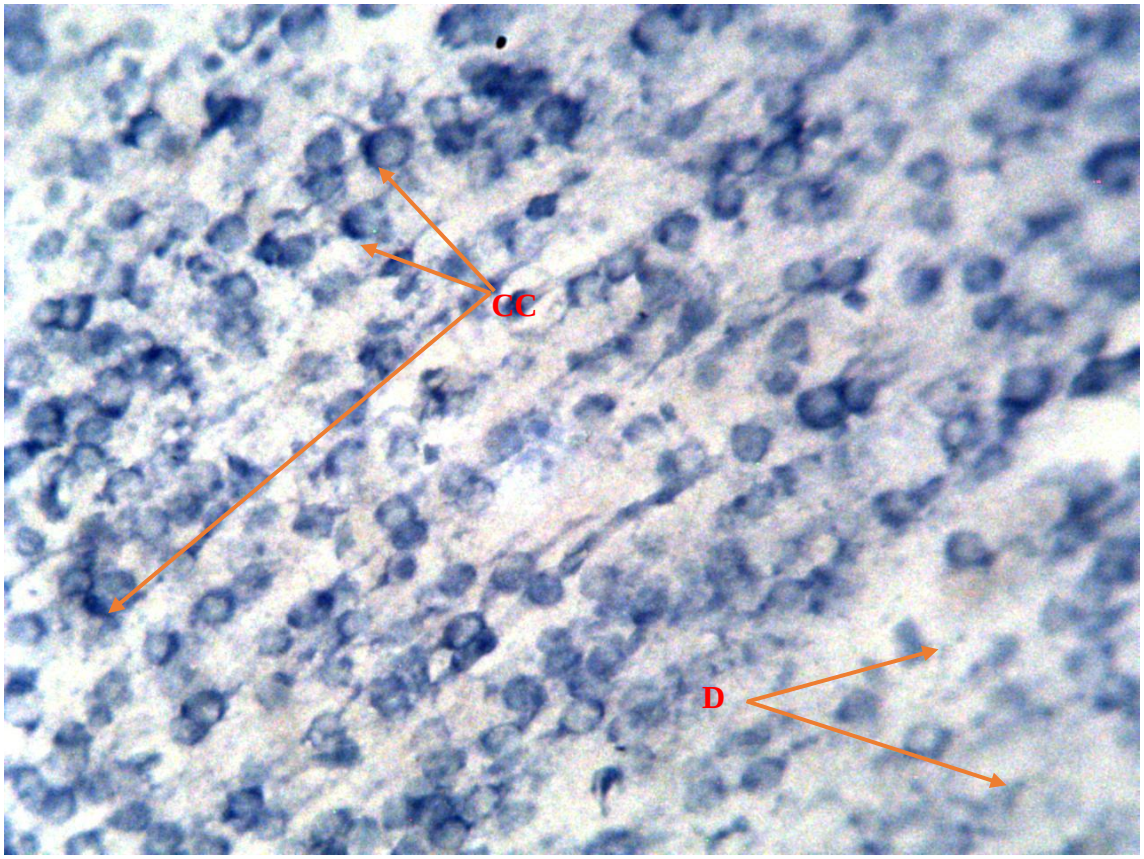


Plate 17: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group C that received 0.5ml 20% ethanol for 14 days showing moderately stained nissl substance with Central Chromatolysis (CC) and Degeneration of cells (D) (Cresyl Fast Violet stain X250)

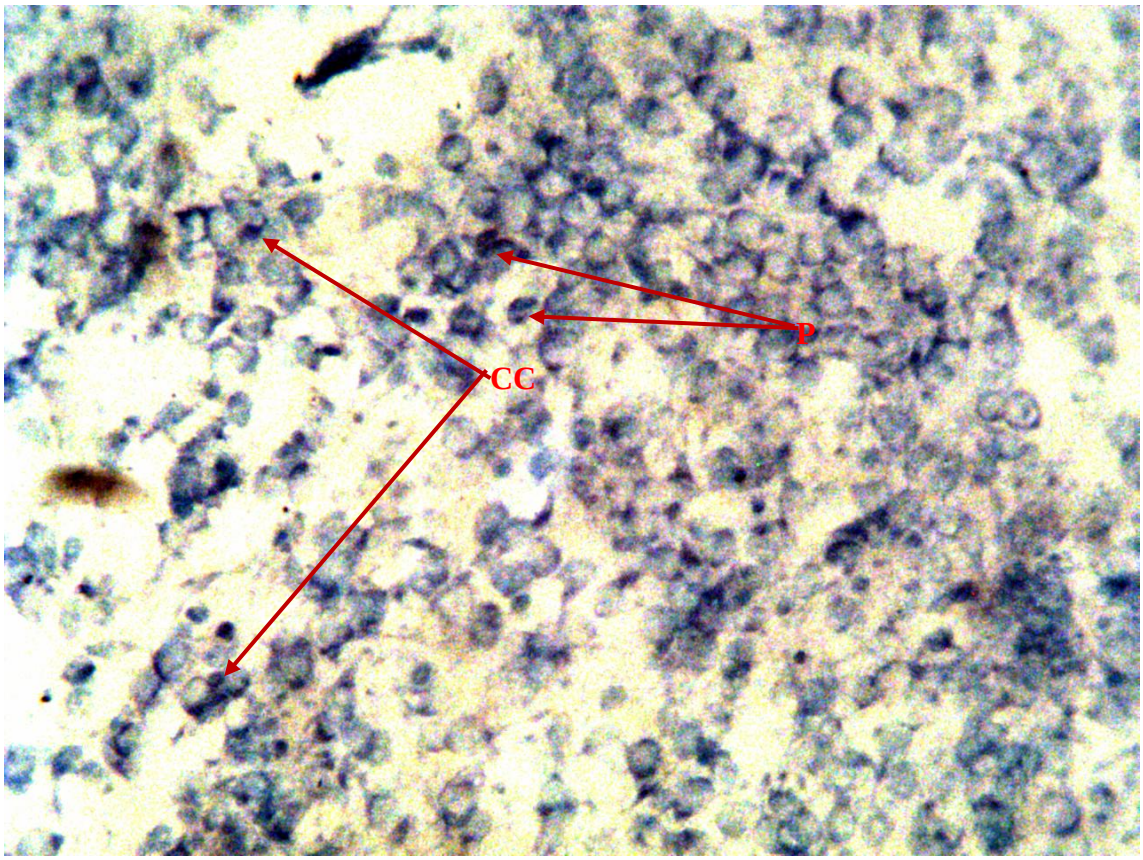


Plate 18: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group D that received 0.5ml 20% ethanol for 20 days showing faintly stained nissl substance with Central Chromatolysis (CC) and pyknotic cells (P) (Cresyl Fast Violet stain X250)

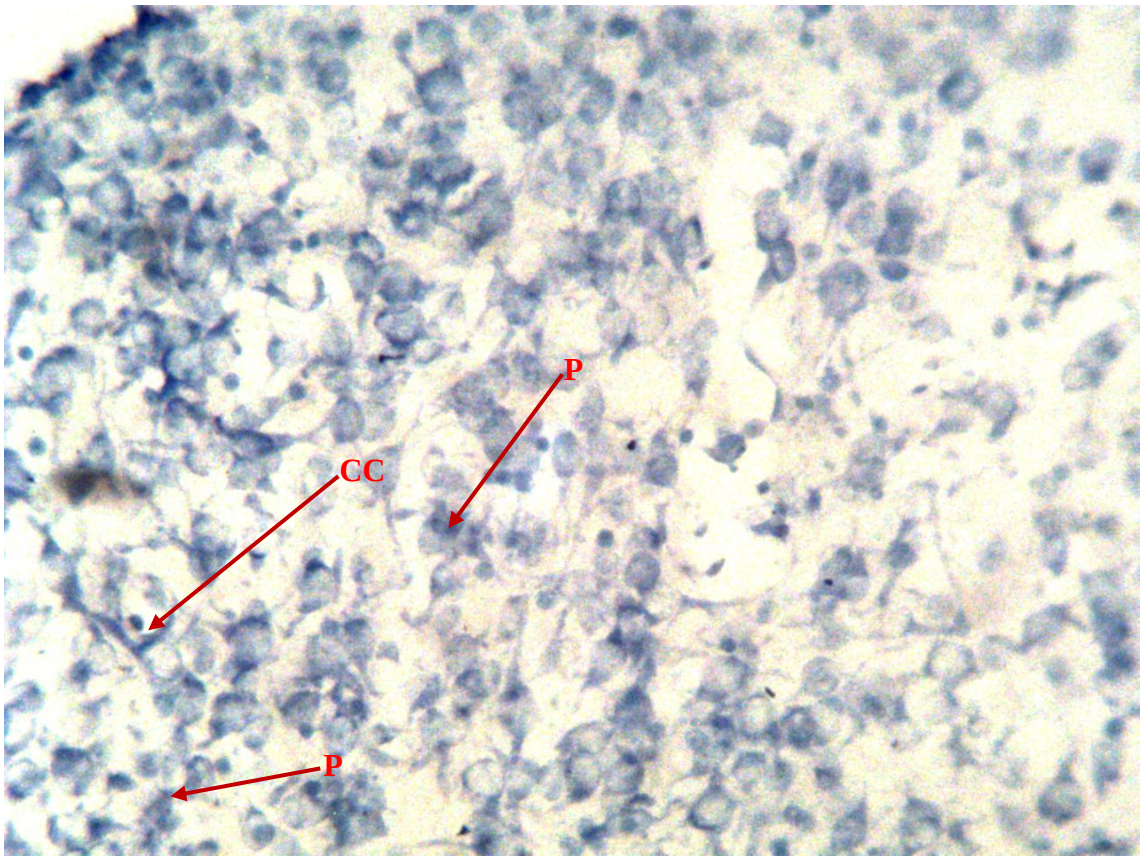


Plate 19: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group E that received 0.5ml 30% ethanol for 7 days showing faintly stained nissl substance with Central Chromatolysis (CC) and pyknotic cells (P) (Cresyl Fast Violet stain X250)

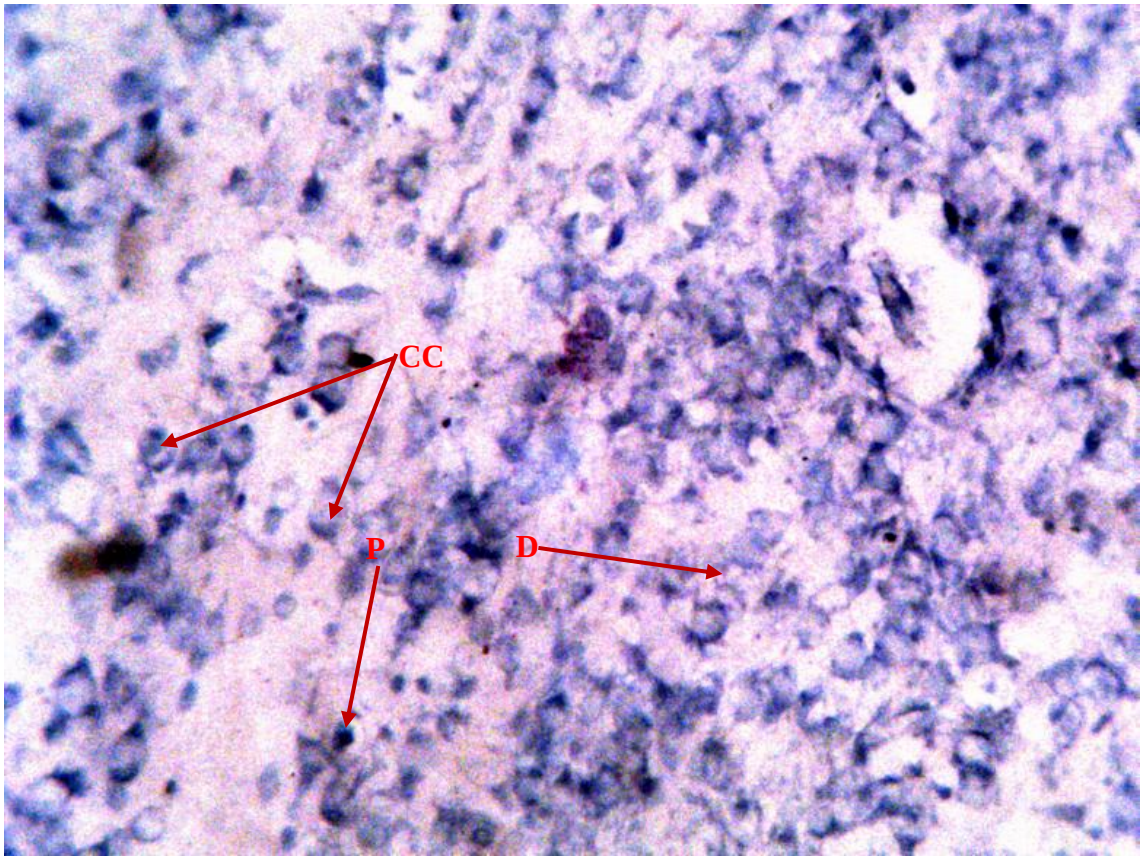


Plate 20: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group F that received 0.5ml 30% ethanol for 14 days showing moderately stained nissl substance with Central Chromatolysis (CC), pyknotic cells (P) and Degeneration of cells (D) (Cresyl Fast Violet stain X250)

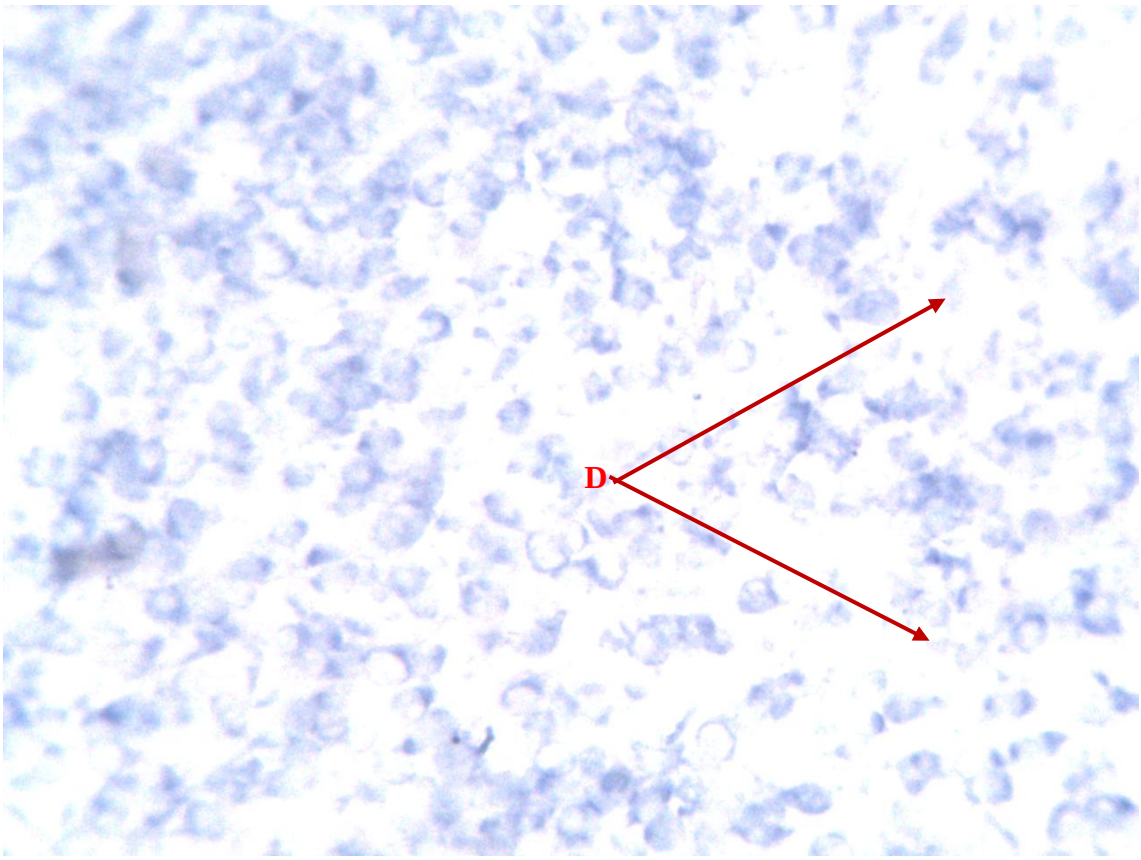


Plate 21: A cross section of the cerebral cortex of neonatal Wistar rat on postnatal day 7 for Group G that received 0.5ml 30% ethanol for 20 days showing faintly stained nissl substance with Central highly Degenerated cells (D) (Cresyl Fast Violet stain X250)

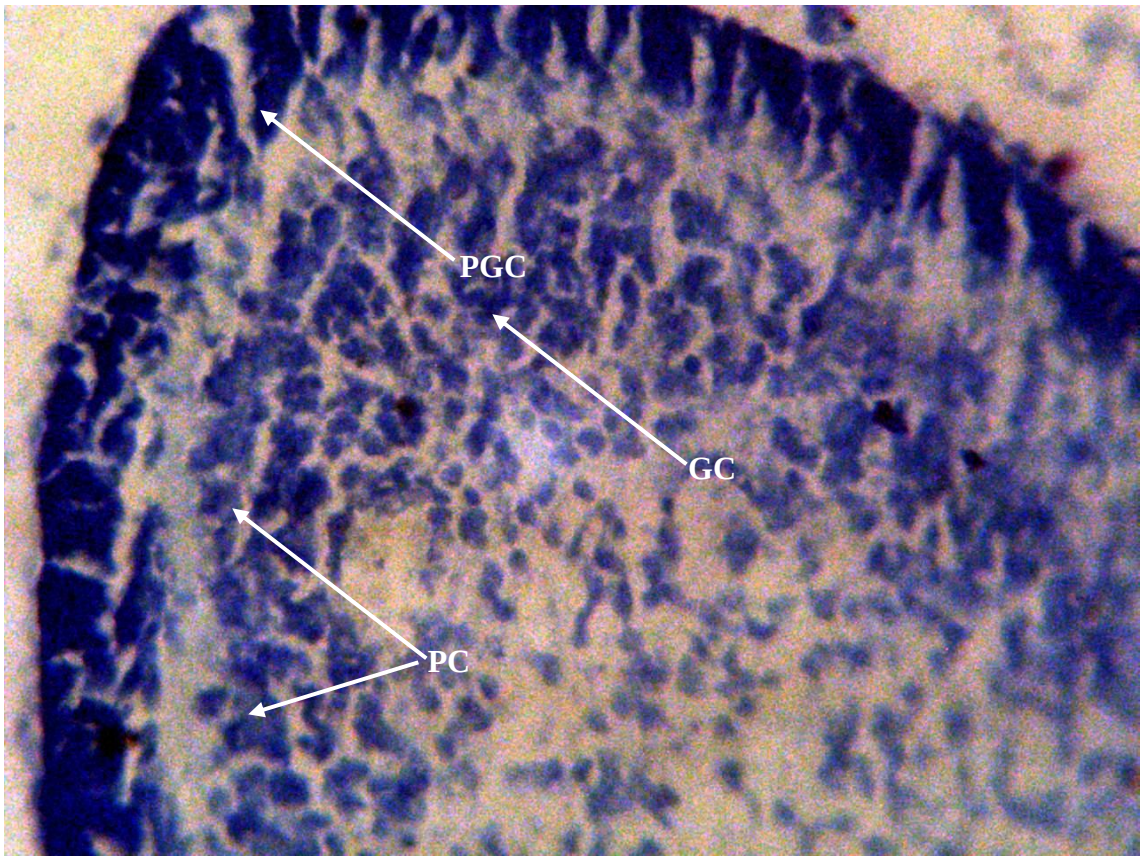


Plate 22: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Control Group A that received normal saline showing the foetal arrangement demonstrating Purkinje cells (PC), Granular Cells (GC) and Precursor Granular cells (PGC) (Cresyl Fast Violet stain X250).

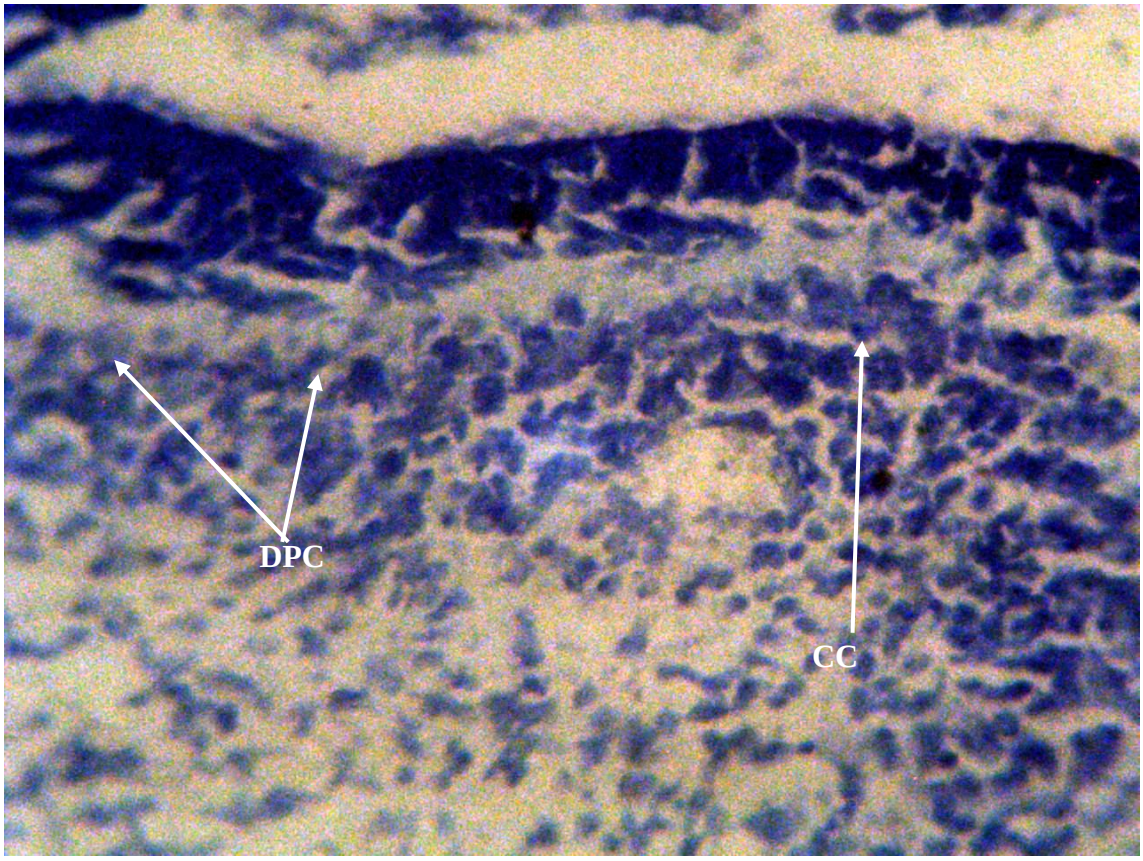


Plate 23: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group B that received 0.5ml 20% ethanol for 7 days showing Degenerated Purkinje cells (DPC) and Central Chromatolysis (CC) (Cresyl Fast Violet Stain X250)

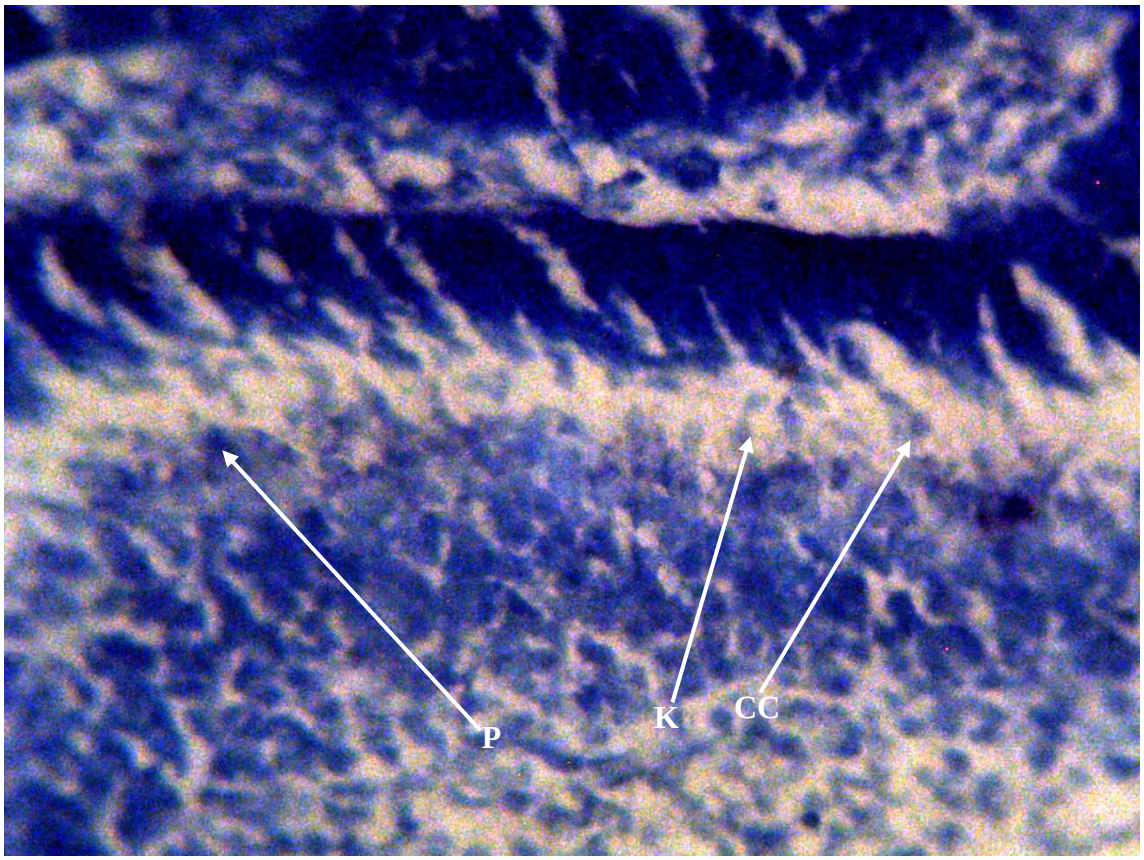


Plate 24: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group C that received 0.5ml 20% ethanol for 14 days showing Degenerated Purkinje cells (DPC) and Central Chromatolysis (CC) (Cresyl Fast Violet Stain X250).

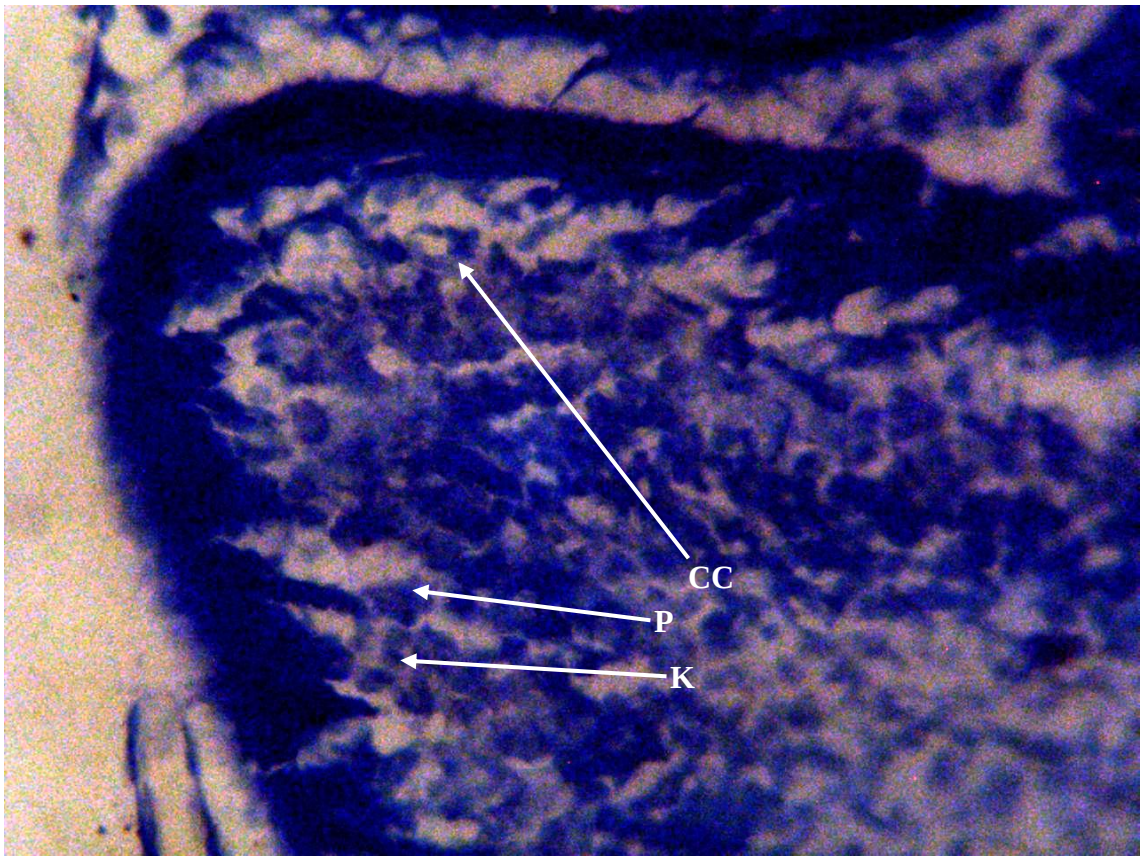


Plate 25: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group D that received 0.5ml 20% ethanol for 14 days showing Pyknotic cells (P), Karyorhexis (K) and Central Chromatolysis (CC) (Cresyl Fast Violet Stain X250).

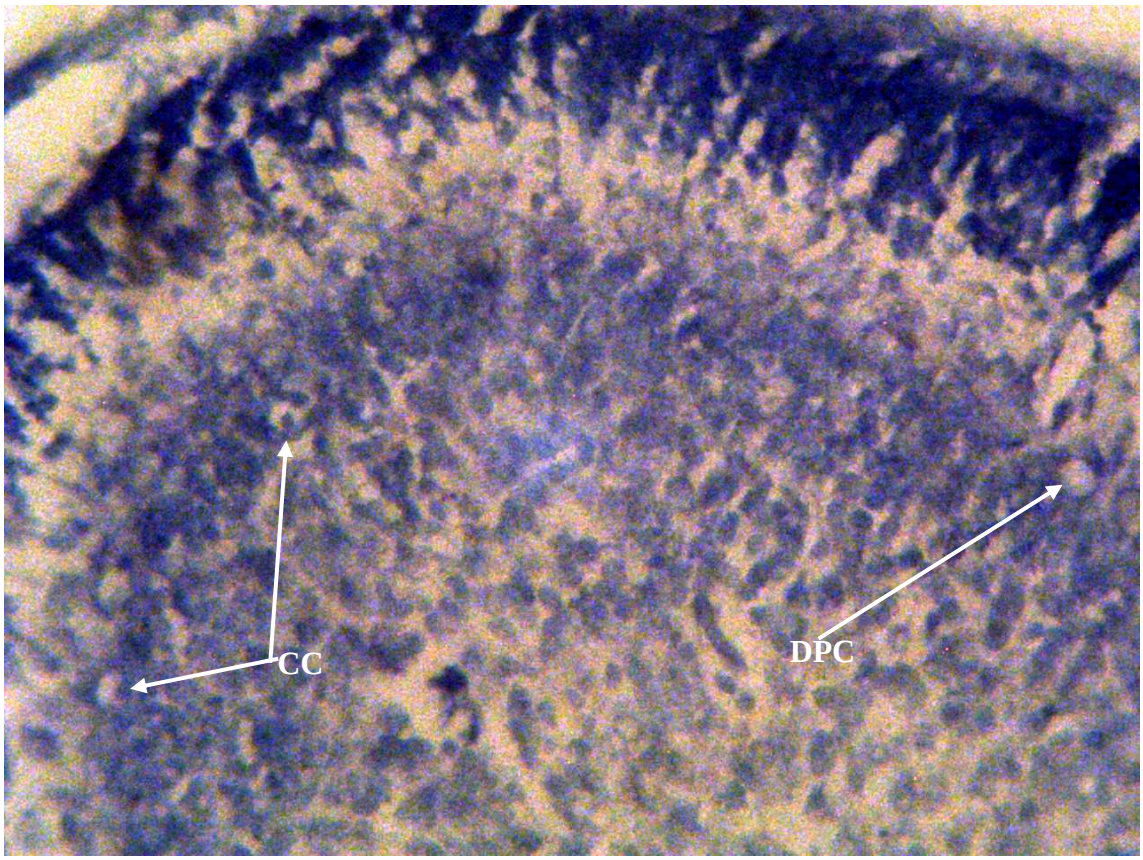


Plate 26: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group E that received 0.5ml 30% ethanol for 7 days showing Degenerated Purkinje Cells (DPC) and Central Chromatolysis (CC) (Cresyl Fast Violet Stain X250).

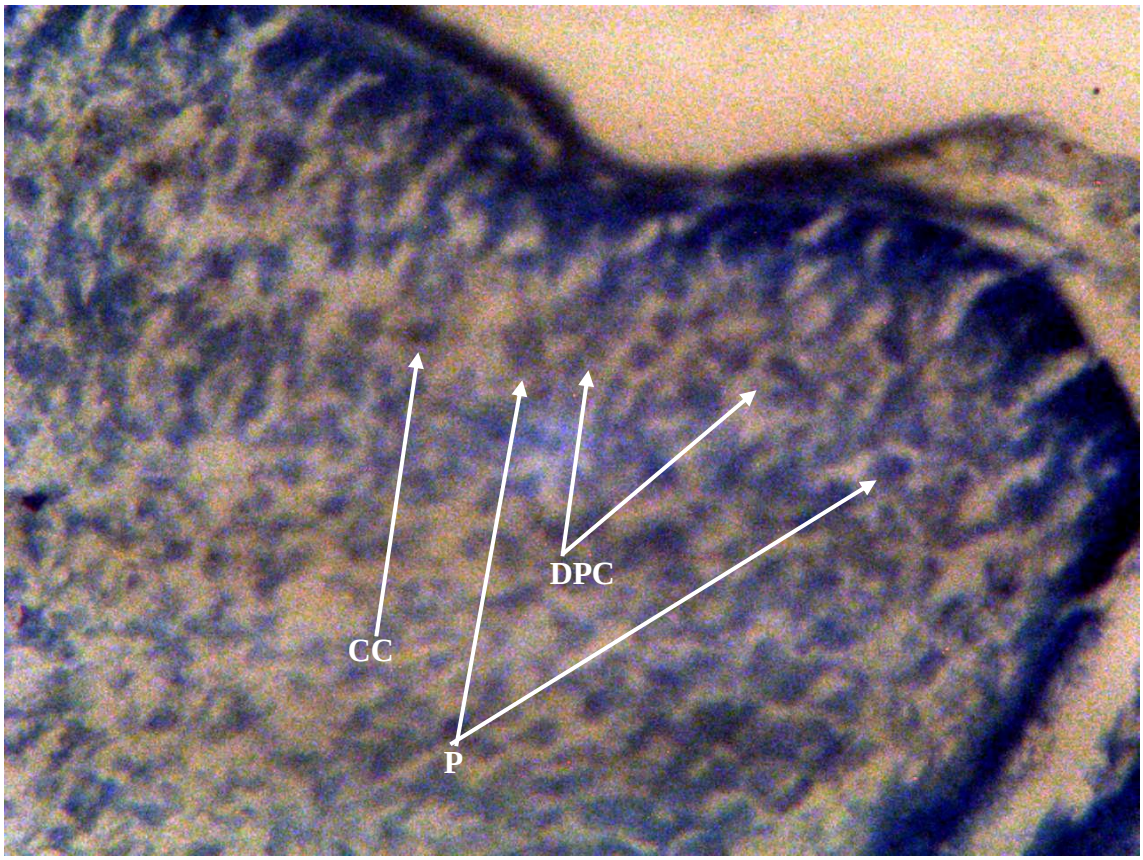


Plate 27: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group F that received 0.5ml 30% ethanol for 14 days showing Pyknotic cells (P), Degenerated Purkinje Cells (DPC) and Central Chromatolysis (CC) (Cresyl Fast Violet Stain X250).

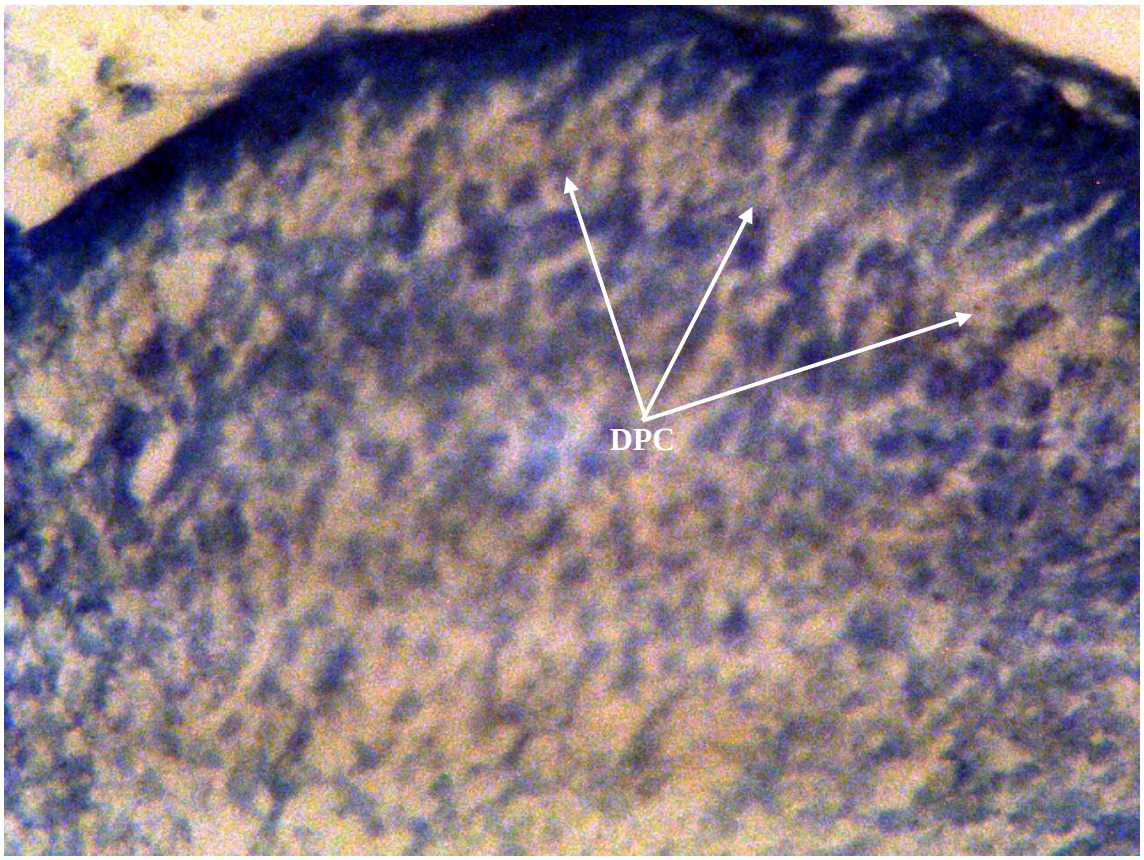


Plate 28: A cross section of the cerebellar cortex of neonatal Wistar rat on postnatal day 7 for Group G that received 0.5ml 30% ethanol for 20 days showing highly Degenerated Purkinje Cells (DPC) (Cresyl Fast Violet Stain X250)

4.4 Atomic Absorption Spectrometry (AAS)

The result of the trace element analysis using AAS in cerebrum and cerebellum of the foetal rats showed the presence of Zinc (Zn), Copper (Cu), Iron (Fe) and Manganese (Mn).

Iron has the highest amount of 10 ≥mg/kg weight of the tissues in both the cerebrum and cerebellum of neonatal Wistar rats. The amount of Iron in the cerebrum of the treated groups is depleted when compared with the amount found in the Control Group. This however, does not meet statistical significance at $P<0.05$. On the other hand, intra-uterine ethanol exposure led to the elevation in concentration of Iron in the cerebellum of the treated groups which met statistical significance with only Groups C ($P<0.001$), D ($P<0.01$) and E ($P<0.01$) in comparison with the Control Group.

Elevation and depletion in the amount of Manganese in the cerebrum of the treated groups was observed. Groups B (0.5ml 20% ethanol 7days), D (0.5ml 20% ethanol 20days) and E (0.5ml 30% ethanol 7days) showed elevated amount that does not meet statistical significance while the other treated Groups C (0.5ml 20% ethanol 14days), F (0.5ml 30% ethanol 14days) and G (0.5ml 30% ethanol 20days) showed non-statistically significant decrease in Manganese level in the cerebrum in comparison with the control Group. Consequently, non-significant elevated Manganese level was observed in the cerebellum of the treated groups when compared to the amount in the Control Group with the exception of Group F (0.5ml 30% ethanol 14days), but no statistical significance was met as well.

Moreover, the amount of Copper in the cerebrum of the treated groups was depleted when compared with the Control Group, but the depletion only met statistical significance with Group B (0.5ml 20% ethanol 7days) at $P<0.05$. Interestingly, the amount of Copper in the cerebellum of the treated groups was elevated, but statistical significance is only met in the treated Groups D ($P <0.01$), E ($P<0.05$) and F ($P <0.001$) when compared to the Control Group.

Amount of Zinc in the cerebrum of the treated groups was depleted when compared with the Control Group, this depletion showed highly statistical significance at $P <0.001$ with all the ethanol-treated Groups. The amount of Zinc in the cerebellum also showed non-statistically significant depletion in the treated groups when compared with the Control Group, except Group G that was slightly higher than the Control Group, although statistical significance was not met.

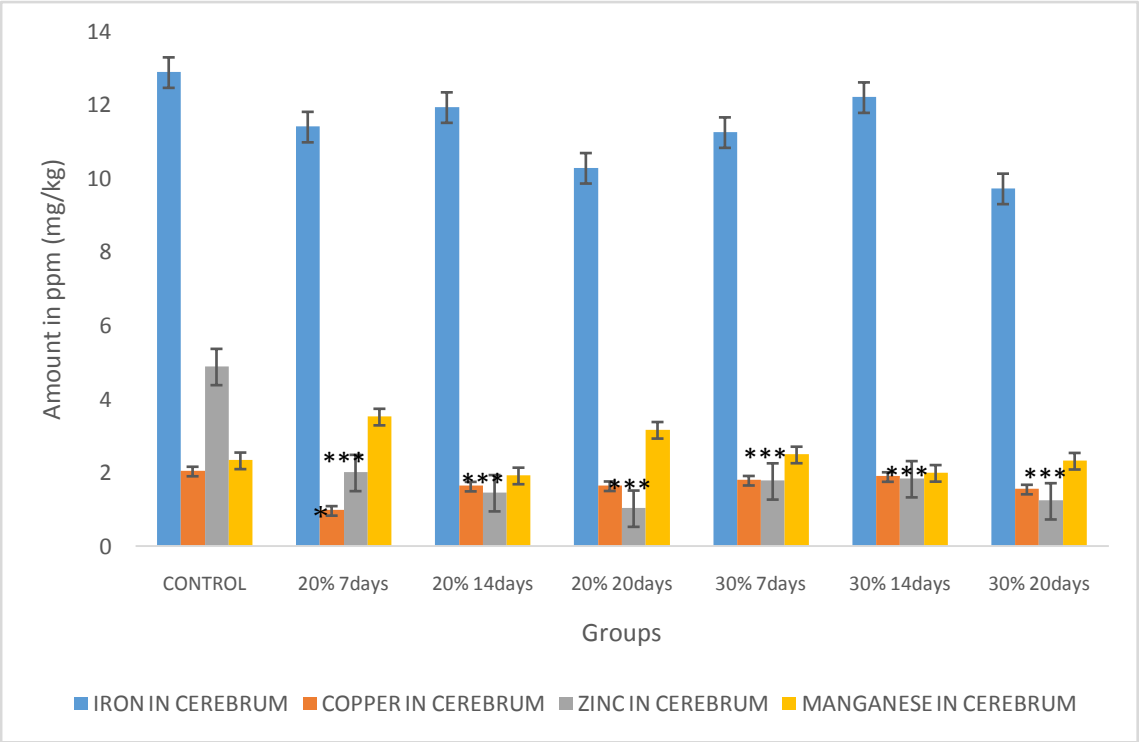


Figure 4.4. Comparison of the mean concentration of some trace elements in Cerebrum of neonatal Wistar rats. *P<0.05, **P<0.01, ***P<0.001

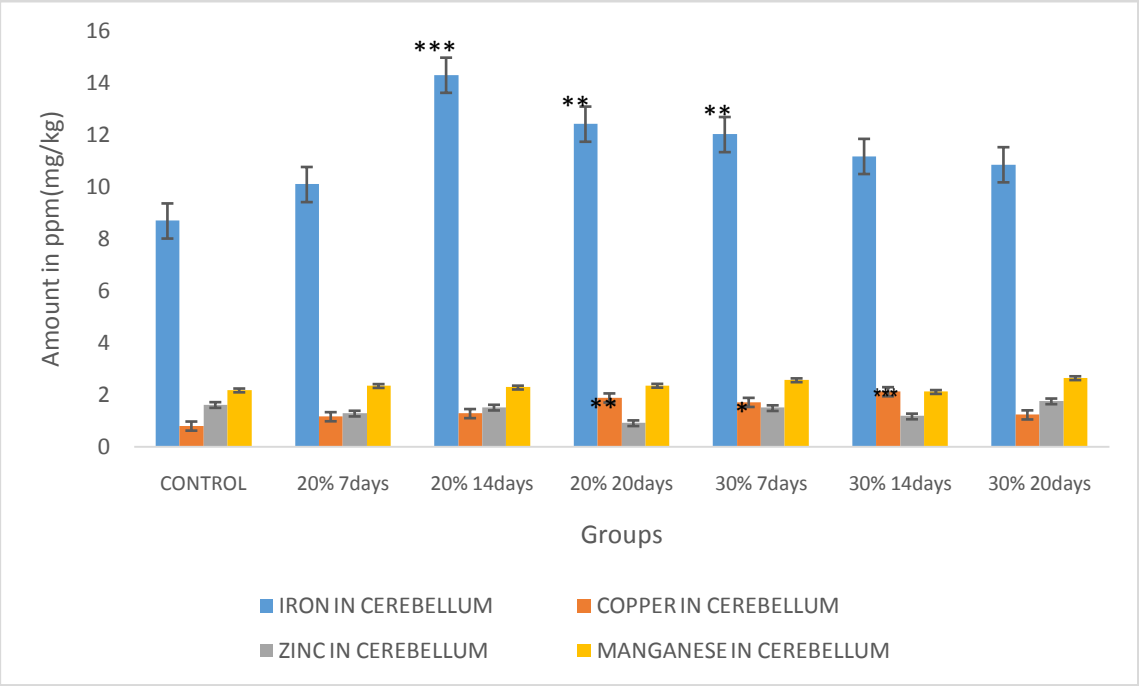


Figure 4.5: Comparison of the mean concentration of some trace elements in Cerebellum of neonatal Wistar rats. *P<0.05, **P<0.01, ***P<0.001

CHAPTER FIVE

5.0 Discussion

5.1 Morphometric Analysis

The prenatal ethanol exposure did not show any significant difference in the crown rump length and birth weight of the pups when compared with the control, although the latter is shown to have higher values of the above stated parameters in comparison with the former. This indicated that prenatal ethanol exposure could affect the morphometric indices of the affected neonates, this is in line with the findings of Adebisi (2003) which showed that ethanol treated foetuses have a statistically significant low birth weight when compared with the control. This also agrees with the work of Allam and Abdulhamid (2013) who also observed a decreased birth weight in prenatally ethanol treated foetuses.

However, on postnatal day (PND) 7, some of the ethanol treated foetuses showed a recuperative increase in morphometric variables, this could be attributed to the nutrition as a result of lactation or genetic constitution of the foetuses. The ethanol treated foetuses were also shown to have reduced crown rump length when compared to the control, this could be explained by the finding of Abebisi (2003) who reported a significantly lower osteometric values in foetuses exposed to ethanol during prenatal life.

5.2 Histological Analysis

In this study, several histopathological presentations were observed in the cerebral and cerebellar cortices of the neonatal Wistar rats exposed to ethanol during embryonic life.

The observed histopathological manifestations include pyknosis, cellular or neural degeneration, chromatolysis and decrease in cellular density as well as ferrugination. Interestingly, the cerebellum of neonatal Wistar rats has a different cytoarchitecture with the adult Wistar rats. The outer molecular layer that is supposedly devoid of granule cells has a very dense granule cells, these are termed as precursor granular cells that migrate from the molecular layer through the purkinje cell layer to the granular layer during early postnatal periods of rodents (Hans *et al.*, 2006).

The cerebellar cortex of the ethanol treated foetuses showed varying forms of histopathological manifestations depicting some neurodegenerative effects that could affect the normal functions of the cerebellum. The resulted effects include, ferrugination which is an effect occasionally seen as haemorrhagic infarcts around dead neurons as a result of basophilic mineral deposits, chiefly Iron and calcium salts. Other features seen include chromatolysis which caused eccentric displacement of the purkinje cell nuclei and margination of the nissl substance in the ethanol treated groups. Massive infiltration of the granule cells into the white matter especially in the 30% ethanol treated foetuses could also affect the normal architecture of the fibres in the cerebellum, specifically the parallel fibres formed by the axons of the granule cells that end on the dendrite of the purkinje cell.

The density and the orientation of the purkinje cells were also seen to be altered in the ethanol treated foetuses. Intrauterine ethanol exposure also led to the depletion of nissl granules in the cerebellar purkinje cells, this could lead to depletion in protein synthesis which is the function of the ribosomes that are very rich in the nissl substance. The resulting effect of the above mentioned histomorphological alterations could lead to

interference with the function of the purkinje cells which are the only neural cells in the cerebellum that connect with other parts of the brain through the cerebellar nuclei situated in the white matter of the cerebellum. The disorientation of the purkinje cells could affect the motor activity and other motor functions such as loss of fine movement, loss of grasping power, disturbance in maintenance of equilibrium and loss of regulation of muscle tone which are modulated by the spinal cord and brain stem through the cerebellar nuclei. This is in line with the work of Allam and Abdulhamid (2013) who showed that prenatal ethanol exposure led to cellular degeneration, pyknosis, depletion in nissl substance as well as chromtatolysis in the cerebellar cortex.

The cerebral cortex of the ethanol treated neonates revealed varying forms of histopathological and neurodegenerative effects. The effects seen such as degeneration of pyramidal cells, pyknosis, chromatolysis, satellitosis and decreased synaptic density are a characteristic feature of neurodegenerative disorder that could result to effects on functional areas of the cerebral cortex that deal with sensory integration, information analysisand initiation of motor responses.

The satellitosis was more conspicuous in both 20% and 30% ethanol exposed neonates during first through second trimester equivalent, this was seen as clumping of cells around dying cells. The present study is in agreement with the work of Allam and Abdulhamid (2013) which showed that prenatal ethanol exposure led to neurocyte chromatolysis, perivascular oedema, and pyknosis in the foetuses. The effects seen in the present study did not follow a dose-dependent manner, however, the time of the exposure to the teratogen (ethanol) is shown to play a role as reporteded by Adebisi

(2011) who stated that the potency of a teratogen depends on the exposure to the teratogen at the critical period of development of the target organ.

The present study showed that both 20% and 30% ethanol exposed neonates during first through second trimester equivalent have similar pattern of teratological insult in both the cortices studied. They are shown to have high density of clumping cells around dying neurons (satellitosis), while the intrauterine ethanol exposed neonates during the whole gestation period have less cellular density and reduced clumped cells. The effects seen in both 20% and 30% first trimester equivalent ethanol exposed neonates have less severe histological assault in both the cortices.

5.3 Trace Element Analysis Using Atomic Absorption Spectrophotometry (AAS)

The meta-analysis of trace elements namely: Iron, Zinc, Copper and Manganese in the cerebrum and cerebellum of neonatal Wistar rats was conducted using Fast Sequential Atomic Absorption Spectrophotometry (FS-AAS). These trace elements play vital role in the synthesis of numerous neurotransmitters as well as serving as co-factors for several enzymes and proteins. Excess or deficiency of these trace elements play a significant role in the aetiology of several neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's Disease, Menkes Disease and Wilsons Disease.

The role of the above stated elements in neural cells justifies the exploration of these vital players in the current studies to reveal the effects of intra-uterine ethanol exposure on the distribution of the trace elements in cerebellum and cerebrum of the neonates, this will help in elucidating one of the many mechanisms through which ethanol

mediates its foeto-toxicity. The result from the present study showed alterations in the amount of Iron, Copper, Zinc and Manganesein the cerebrum and cerebellum of the treated groups when compared to the amount in the Control Group. Iron has the highest amount in both the cerebrum and cerebellum, this indicates that brain has the highest amount of Iron compared to other trace elements.

Interestingly, the present study established a relationship between Iron and Copper in the cerebrum and cerebellum. The amount of both Iron and Copper in the cerebrum is shown to have a reciprocal relationship with the amount in the cerebellum. Prenatal ethanol exposure was shown to cause depletion in both Copper and Iron in the cerebrum while simultaneously resulting to elevation in the amount of Copper and Iron in the cerebellum. This can be attributed to the fact that the uptake mechanism of the two metals interact, resulting to an interplay between the two metals (Tina *et al.*, 2012). Both shortage and excess of Iron and/or Copper can affect the brain by affecting either neurotransmitter and/or protein synthesis (Tina *et al.*, 2012).

The transition metals, Iron and Copper are cofactors for a variety of proteins that are vital for the normal function of cells. In the brain, Iron and Copper are important cofactors for enzymes involved in the neurotransmitter synthesis and myelin formation (Gaggelli *et al.*, 2006; Lutsenko *et al.*, 2010). When in excess, they both participate in the Fenton reaction – a series of chemical reactions initiated by transition metals and hydrogen peroxide – which leads to the formation of unstable free radicals that significantly affect DNA, proteins, and lipids and cause deleterious damage to the cells (RiveraMancia *et al.*, 2010). Therefore a strict regulation of these metals is vital for normal cellular function and survival. Hence, the histopathological effects of

intrauterine ethanol exposure observed in the present study can be attributed to the generation of free radicals leading to neural death especially in the cerebellum.

Copper is essential for normal CNS function. It is required as a cofactor for dopamine beta monooxygenase, peptidylglycine alpha amidating monooxygenase, superoxide dismutase, and a large number of other enzymes. Furthermore, it is required for mitochondrial respiration and oxidation of Iron, leaving the brain vulnerable in case it gets deprived ofCopper. On the other hand too much Copper is toxic. Brain Copper accumulation observed in Wilson disease results in Parkinsonian symptoms such as dystonia and dysarthria (Tina *et al.,* 2012).

Therefore, excess Copper amount observed in the cerebellum of the ethanol treated neonates can lead to excitotoxicity of doperminernic neurons in the cerebellum as a result of interference with the neuromodulatory effect of Copper in the doperminergic neurons. Tina *et al.,* (2012) reported that cerebral Copper deficiency can lead to decreased neuronal cytochrome Coxidase activity that consequently alters mitochondrial function and brain energy metabolism. Hence, the resulting Copper deficiency in the cerebrum of the ethanol-treated neonates could be attributed to the pyknosis and degeneration of the pyramidal cells as observed in the histological tissues of the cerebrum.

The present study was able to establish that intrauterine ethanol exposure was able to disturb Zinc homeostasis in the brain.The study revealed that ethanol is capable of depleting the amount of Zinc in both the cerebrum and cerebellum, which could affect the neuromodulatory effect of Zinc on $GABA_A$ receptor.This will lead to inactivation of

GABA, resulting to inhibition of neurotransmission. Zinc and Iron are proposed as endogenous modulators of the $GABA_A$ receptor complex (Zerrouk*et al.,* 2012).

The present study is supported by the work of Bailey *et al.,* (2001), who stated that chronic prenatal exposure to ethanol throughout gestation, via maternal ethanol administration, resulted in an increase in $GABA_A$ receptor number in the adult guinea pig cerebral cortex. Zinc deficiency is also shown to impair developmental neurogenesis via reductions in neuronal precursor proliferation. There was also early evidence suggesting that Zinc deficiency may impair neuronal differentiation during development (Cathy and Deborah, 2014). It could therefore be inferred that the depleting Zinc effect seen in the ethanol-treated neonates could affect neuronal proliferation and differentiation.

Intrauterine ethanol exposure was shown to affect Manganese homeostasis in both the cerebrum and cerebellum by either increasing or decreasing the amount in the tissues. Manganese is largely bound to mangano-proteins especially glutamine synthetase in astrocytes. A portion of Manganese exists in the synaptic vesicles in glutamatergic neurons, suggesting that Manganese is concurrently released with glutamate from neuron terminals. This is dynamically coupled to the electrophysiological activity of the neurons.

Therefore, Manganese release into the synaptic cleft may influence synaptic neurotransmission (Atsushi, 2003). Excess or shortage of Manganese can therefore affect glutamatergic neurotransmission by either excitotoxicity or reduced activity of the excitatory neurotransmitter, glutamate respectively. The findings in the current

study suggest that, the alteration in the amount of Manganese seen in the ethanol-treated foetuses either by depleting or elevating the concentrations could affect the glutamate action mechanism in the brain which is the major excitatory neurotransmitter in the brain. Moreover, glutamate can become an excitotoxin when its message is overwhelming leading to generation of reactive oxygen species and eventually cause neuronal death.

5.4 Neuro-Behavioural Study

The present study shows that intrauterine ethanol exposure has effect on the development of vestibular, postural and motor coordination of the affected neonates, although this does not meet statistical significance. In the surface righting reflex test, the ethanol-treated neonates were shown to have a high latency response to the challenge, the development of vestibular and motor co-ordination of the pups can be said to be affected by intrauterine ethanol exposure.

On the other side, cliff aversion test for the evaluation of the sensory and motor reflexes of the neonates was also shown to be affected in the ethanol-treated groups. Moreover, negative geotaxis evaluation of the postural and motor coordination of the pups was also shown to be affected by maternal ethanol exposure. This can be attributed to the neurodegenerative effects seen on the histology of the tissues studied, especially the cerebellum that has the primary function of regulation of motor activity, the histological evaluation has supported the neuro-behavioural defects seen in the ethanol-treated groups.

The role of trace elements in neurotransmission can be attributed to the neuro-behavioural effects seen. For instance, the role of Copper in modulating the activity of

the neurotransmitter, dopamine which is important in motor function activity can be said to be compromised due to elevation and depletion of Copper in cerebellum and cerebrum of the in-utero ethanol exposed foetuses respectively. Zinc is also shown to play role in proliferation and differentiation of neural cells, therefore, the depleted Zinc levels seen in the ethanol-treated neonates could be responsible for causing the delayed or impaired development of the motor neurons as demonstrated by the neuro-behaviour of the ethanol-treated pups. The altered level of Manganese which is a neuromodulator for glutamate could play role as a result of possible excitotoxicity by the glutamate in the brain of the ethanol-treated foetuses.

CHAPTER SIX

6.0 Conclusion and Recommendations

6.1 Conclusion

It can be concluded from the current study that intrauterine ethanol exposure during first trimester, first through second trimester and during the whole gestation period has deleterious effects on the morphometry, histology, neurochemistry and neuro-behaviour of the affected neonates.

In utero ethanol exposure is shown to affect birth weight and crown rump length of the affected neonates by exhibiting lower weight and crown rump length values in the ethanol treated neonates. In addition, intrauterine ethanol exposure at varying periods of development is also shown to affect the development of numerous brain functions such as postural reflex, vestibular reflex, motor and sensory reflexes as a result of increased latency response observed in the ethanol-treated foetuses in surface righting, cliff avoidance and negative geotaxis neuro-behavioural battery tests. Moreover, varying forms of histopathological presentations were observed in the ethanol exposed foetuses, these include: pyknosis, neurodegeneration, decreased synaptic density and ferrugination in the cerebral and cerebellar cortices.

Interestingly, another mode through which ethanol mediates its foeto-toxicity is explored in the current study. This was done by analysing the amount trace elements in the cerebral and cerebellar cortices of the neonatal Wistar rats. The finding in this study established a relationship between the amounts of Iron and Copper in the cerebrum and cerebellum, this reveals a reciprocal relationship between the amount of Iron and Copper in the cerebrum

and cerebellum as discussed earlier in chapter four of the study. The amounts of Zinc and Manganese were also shown to be affected as a result of intrauterine ethanol exposure. The altered amounts of the trace elements is sought to play role in the aetiology of alcohol-induced neurotoxicity such as Foetal Alcohol Spectrum Disorders (FASD).

Finally, the current study is able to authenticate the fact that no known amount of alcohol is safe for the foetus irrespective of the period of the pregnancy.

6.2 Recommendations for Further Studies

1. Further molecular studies should be carried out on the foetuses such as Gel electrophoresis to study the extent of DNA damage in the foetal brain, gene expression studies to explore some proteins that play role in the aetiology of some neurodegenerative diseases.

2. Trace elements analysis should be carried out concurrently together with assays for oxidative stress markers to quantify some enzymes such as catalase in the brain and if possible the serum of the neonates.

3. Trace elements analysis should also be married with the neurotransmitter assays to better understand the alteration in the amount of trace elements and consequent effects on neurotransmitter mechanisms in the brain of intrauterine ethanol exposed foetuses.

6.3 Contribution to knowledge

1. Establishment of differential pattern of intrauterine effects of ethanol exposure as a function of time and duration, irrespective of the dosage on cerebral cortex of neonatal wistar rats, thus supporting the literature that states that potency of ethanol in inducing teratogenicity is more dependent on time of exposure than the dosage.

2. The current study was able to establish a reciprocal relationship in Iron and Copper homeostatic mechanisms of cerebrum and cerebellum of neonatal Wistar rats exposed to ethanol in-utero by causing a depletion in the amount of copper in the cerebrum of the ethanol treated groups while simultaneously elevating the amount in the cerebellum. The observed amount of Copper and Iron in parts per million in the cerebellum of the control group is 0.8mg/kg and 8.68mg/kg respectively, while the elevated amount of Copper and Iron observed in the ethanol treated groups was as high as 1.88mg/kg and 14.28mg/kg, and the level of significance is$P<0.001$.

On the other hand, The observed amount of Copper and Iron in parts per million in the cerebrum of the control group is 2.04mg/kg and 12.88mg/kg respectively, while the depleted amount of Copper and Iron observed in the ethanol treated groups was as low as 0.97mg/kg and 10.28mg/kg, and the level of significance is $P<0.05$.

3. The role of some trace elements namely, Iron, Copper, Zinc and Manganese in affecting the cytoarchitecture of the cerebral and cerebellar cortices of neonatal Wistar rats exposed to ethanol in-utero was also linked.

4. The role of some trace elements namely, Iron, Copper, Zinc and Manganese in the impairment of neuro-behavioural developmental milestones in neonatal Wistar rats following intrauterine ethanol ingestion was also correlated and there was statistical significance at $P<0.05$.

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