HISTOSTEREOLOGICAL TERATOGENIC EFFECTS OF *IN-UTERO* EXPOSURE TO ALCOHOL ON THE FETAL LIVER IN ALBINO RATS (*RATTUS NORVEGICUS*)

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Histostereological Teratogenic Effects of *In-utero* Exposure to Alcohol on the Fetal Liver in Albino Rats (*Rattus norvegicus*)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this thesis to my parents, Onesmus Musa and Monica Musa and also my daughter Juliet Pauline for their encouragement and prayers.

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ABBREVIATIONS AND ACRONYMS

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ANOVA	One way analysis of variance
bwt	Body weight
CE	Coefficient of error
CG	Control group
cm	Centimeters
COHES	College of health science
CRL	Crown rump length
CYP 2E1	Cytochrome P450 enzyme
DPX	Dis-tyrene plasticizer and xylene
FAEEs	Fatty acid ethyl esters
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorder
GD	Gestational day
GIT	Gastro intestinal tract
gm	Grams
H&E	Hematoxylin and eosin
HAG	High alcohol group
HC	Head circumference
HD	High dose
IUGR	Intra uterine growth retardation
JKUAT	Jomo Kenyatta University of Agriculture and Technology
kg	Kilo gram
mm^3	Millimeters cubed
LAG	Low alcohol group
LD	Low dose
LD50	Lethal dose 50%
MAG	Medium alcohol group
MD	Medium dose
mg	Magnification

ml	Mils
NAD	Nicotinamide adenine dinucleotide
ROS	Reactive oxygen species
SAFARI	Small animal facility for research and innovation
SEM	Standard Error of the Mean
SPSS	Statistical Package of Social Scientist
SURS	Systematic uniform random sampling
TM_1	Trimester one
TM_2	Trimester two
TM_3	Trimester three
WHO	World Health Organization
WIM	Water immersion method

DEFINITION OF TERMS

Ethanol	A form of alcohol contained in alcoholic beverages including beer and wine among others which is simply referred as alcohol
Histomorphometry	The use of histology to study the cellular morphology, cellular densities and connecting stromal tissues using a microscope
Liver cirrhosis	Disease in which the healthy liver tissues are replaced with scar tissue
LD50	A statistically derived dose at which 50% of the rats die due to exposure to a chemical agent. This is obtained from acute toxicity study.
Steatosis of the liver	Abnormal accumulation of fat in the liver due to alcohol
Steato-hepatitis	The inflammation of the liver with concurrent fat deposition
Stereology	Is a three dimensional analysis and interpretation of two dimensional cross section of material or tissue to extract quantitative information

ABSTRACT

In-utero exposure to alcohol has been shown to perturb the normal development of the fetal liver. Though data exist on the alcohol teratogenicity to the fetal viscera, there is paucity of data on the anatomical histo-stereological effects of alcohol when administered in varying doses and at different gestational periods. The broad objective of this study was therefore to evaluate the histo-stereological effects of prenatal exposure to varying doses of alcohol on the fetal liver when exposed at different gestational periods in albino rats. In carrying out the study, animal experimentation was done in SAFARI animal house while tissue processing was done in the Department of Human Anatomy, JKUAT. A static-group experimental study design was adopted where 30 albino rats (dams) weighing between 200 to 230gm from a pure colony were used as the experimental model. These 30 dams were broadly organized into two study groups namely control (n=3) and experimental (n=27) group. The 27 rats in the experimental group were further assigned into three study groups of nine (9) rats each according to study dosages as (i) low alcohol group (2g/kgbwt), (ii) medium alcohol group (3.5g/kgbwt) and (iii) high alcohol group (5g/kgbwt). These 9 rats in each of the three study groups were further subdivided into three study groups with 3 rats each according to trimesters as (i) 3 rats in TM_1, (ii) 3 rats in TM_2 and (iii) 3 rats in TM_3. All rats in trimester 1(TM_1) study groups received alcohol treatment from gestational day (GD) one all through to gestational day 20 (GD 1-20), those in TM_2 study category received alcohol treatment from GD 7-20 while those in TM_3 received alcohol treatment from GD 14-20. In addition, all the rats received standard rodent pallets and water *ad libitum* throughout the gestational period and were humanly sacrificed on the 20th day of gestation. A total of 90 fetuses (three fetuses having the lowest, median and highest fetal weight from each animal) had their livers harvested, fixed in 10% formaldehyde and processed for histological and stereological analysis. Data was collected using structured datasheets and photomicrographs. The data was then analyzed using STEPnizer software and SPSS version 23 where One-way Analysis of Variance (ANOVA), followed by Tukey's post hoc multiple comparison tests were done. The results were expressed as mean ± standard error of the mean (SEM) for all values. The findings were then presented in form of bar graphs and tables. A significant reduction ($P<0.05$) in the fetal weights, CRL and head circumference were observed. Varying histo-morphological changes of the liver including constriction of the central vein, dilatation of the liver sinusoids and reduction in hematopoietic tissues among others were observed too. A significant increase ($P=0.001$) in total liver volume and volume densities were also observed. In conclusion, the present study revealed that alcohol consumption during pregnancy is teratogenic and has a wide array of fetal teratogenic outcomes. In addition, alcohol caused hepatomegaly in time and dose dependent manner. It is therefore recommended that expectant mothers should abstinence from alcohol consumption.

CHAPTER ONE

INTRODUCTION

1.1 Background information

In-utero exposure to alcohol, also known as ethanol has been shown to perturb the normal development of the fetal liver. At the same time, cases of maternal alcohol consumption are concurrently reported to be on the increase (WHO, 2018). Further, cases of liver related deaths emanating from excessive alcohol consumption are on the increase globally (Papova *et al*., 2017). For instance, 50% mortality rate in the global population has been associated with acute alcohol hepatitis in the expectant mothers and this has become a major public health problem (Papova *et al*., 2017;Willmore *et al*., 2017). On the other hand, the prevalence of alcohol related liver conditions that includes alcoholic hepatitis, liver cirrhosis, steatosis, steato-hepatitis among others during pregnancy has been estimated to be 10% globally and between 3.4%-20.5% in East Africa (WHO, 2018; Popova *et al*., 2017). Studies have also projected that with the current rates of alcohol consumption, cases of liver failure may continue to increase globally. Though studies have shown that alcohol has injurious effects to the developing fetal liver, there is paucity of data on its anatomical histo-stereological effect on the fetal liver following prenatal exposure to varied doses of alcohol. Data on the most vulnerable periods and the critical doses of alcohol teratogenicity to the fetal liver is also generally lacking.

The normal morphogenesis of the fetal liver has been shown to be disrupted by the intrauterine exposure to alcohol (Ebugosi *et al*., 2017). This is based on the fact that alcohol and its toxic metabolites (acetaldehyde) readily crosses the blood placental barrier, therefore accumulating in the developing fetal tissues (Marek & Kraft, 2014). This ability of alcohol to cross the blood placental barrier has been associated with its amphiphile properties and low molecular weight (46g/mol) (Zelner *et al*., 2013). The specific teratogenic injurious mechanism of alcohol to the developing fetal liver has been shown to occurs through the generation of reactive oxygen species (ROS), depletion of the normal antioxidants and cellular hypoxia (Mansouri *et al.,*

2018). Consequently, cellular differentiation is interfered with leading to distortion of the histo-cytoarchitecture of the developing fetal organs including the liver also occurs(Elshennawy *et al.*, 2015).

Studies have further indicated that injury to the parenchymal tissue of the adult liver following alcohol consumption commences around the central vein and extends progressively outward towards the portal triad. (Gupta *et al*., 2017; Lobo, 2014). As a result, this injures the three zones of the hepatic parenchyma namely periportal zone, intermediary zone and centrilobular zone (Gupta *et al*., 2017). These injurious effects on the liver consequently leads to sinusoidal dilatation, fat deposition, hepatocyte inflammation as well as hepatocyte degeneration among others (Andrade *et al*., 2019; Fung & Pyrsopoulos, 2017)). (Cardoso *et al*., 2018;

Increase in liver cellular volume as well as reduction in hepatocyte nuclear-cytoplasm ratio have been reported in alcoholics (Andersen *et al*., 2000). This injurious effects to the liver have been associated with the generation of oxygen free radicles (OFR) during the oxidative metabolism of alcohol (Asuzu *et al*, 2015). This OFR further causes oxidative stress to the cellular component of the liver and as a result, the normal functioning of the organ is interfered with (Andersen *et al*., 2000). In another study, a dose dependent increase in the liver cellular volume was observed following administration of 0.5g/kgbwt and 1g/kgbwt of *aspira africanas* leaf extract to rats (Eweka, 2006).

1.2 Statement of the problem

In-utero exposure to alcohol has been associated with perturbations in development of a wide range of fetal viscera including the liver, commonly grouped together as fetal alcohol syndrome (FAS) (Nakhoul *et al*., 2017). Globally, it is estimated that 1 in every 67 expectant women give birth to child/children with FAS (Popova *et al*., 2017). This is likely to be on increase based on the fact that there is reported increase in alcohol usage by expectant mothers during pregnancy worldwide (Popova *et al*., 2017). Perturbations of *in-utero* liver development following prenatal exposure to teratogens like alcohol may explain the *in-utero* origin of some of the adult causes of liver failure that are also reported to be on the increase today. Though there is a lot

of data on the teratogenic effects of alcohol on the developing fetal organs when exposed prenatally, there is paucity of data on its histo-stereological effects on the fetal liver when exposed *in-utero*. Data to show whether the effects are dose and or time dependent following varying doses of maternal alcohol exposure has not been well established. Therefore this study sought to establish the histostereological effects of alcohol on the developing fetal liver when exposed in different gestational periods at varying doses.

1.3 Justification

Lack of histostereological data on the effects of prenatal exposure to alcohol on the developing fetal liver including the most vulnerable period and the most effective teratogenic doses was vital. Such data would help experts on the histostereological changes that occurred on the fetal liver following alcohol consumption during pregnancy. In absence of such data, there would be increasing cases of liver related mortality and morbidity in both fetal and adult life. Therefore there was need to carry a histostereological study that would show the anatomical changes on the fetal liver following *in-utero* exposure to varying doses of alcohol

1.4 Significance of study

This data on the intrauterine perturbations from alcohol contributed to the repository of scientific data on effects of alcohol consumption during pregnancy and its teratogenic effects on the developing fetal liver. The data helped in elucidating some of the baseline causal of adult liver related diseases like liver cirrhosis and liver cancer among others that were among the leading causes of deaths of economically productive people globally and whose causes were yet to be elucidated. The study findings were also useful in showing the actual histoquantitative teratogenic effects of alcohol when used in different gestational periods at varying doses. Such data was also helpful to the country in the development of policies on prenatal alcohol use and helped come up with health messages related with alcohol consumption to the expectant mothers and the community at large.

1.5 The research question and objectives

1.5.1 Study questions

1) What are the maternal and fetal pregnancy outcomes following prenatal exposure to varying doses of alcohol?
2) What are the histo- morphological changes that occur in fetal liver histo-cytoarchitecture following maternal exposure to varying doses of alcohol?
3) What are the histo- stereological changes on the fetal liver architecture following prenatal exposure to varying doses of alcohol?
4) Are the histo- stereological changes on the fetal liver cyto-histoarchitecture following *in-utero* exposure to varying doses of ethanol dose and time dependent?

1.5.2 Broad objective

The broad objective of the study was to evaluate the histomorphological and histostereological changes on the fetal liver histo-architecture following *in-utero* exposure to varying doses of alcohol at different gestational periods.

1.5.3 Specific objectives

1) To establish the pregnancy outcomes following prenatal exposure to varying doses of alcohol in albino rats.
2) To establish the histomorphological changes on the fetal liver of albino rat following prenatal exposure to varying doses of alcohol.
3) To determine the histostereological changes on the fetal liver of albino rat following prenatal exposure to varying doses of alcohol.
4) To evaluate whether the histostereological changes on the fetal liver cyto-histoarchitecture following *in-utero* exposure to varying doses of alcohol are dose and time dependent.

1.6 Hypothesis (H_0)

There is no relationship between prenatal alcohol consumption and fetal liver teratogenic effects.

1.7 The study assumptions

In carrying out this study it was assumed that the albino rats (*Rattus norvegicus)* model used in this experimental study would replicate the actual teratogenic induction scenario that would occur to humans. This is due to the known close association of this laboratory rats with human biological and functional outcomes when exposed to different teratogens.

1.8 Study limitations

Some of the study limitation included inadequate data on stereological studies:-This lead to scanty past literature related to the current study although this did not stop the researcher from carrying out the study. This was due to the fact that the available past literature served as a guide in the development of the study protocol.

CHAPTER TWO

LITERATURE REVIEW

2.1 Alcohol

2.1.1 Chemistry of alcohol

Alcohol is a teratogenic molecule which is also known as ethanol (Ethyl alcohol) (Ornoy & Ergaz, 2010). This two terms are often used interchangeably in the clinical context (Zelner *et al*., 2013). Alcohol is composed of a single hydroxyl group and a short, two-carbon aliphatic chain (CH_3CH_2OH) with a low molecular weight of 46.069g/mol (Perry *et al*., 2017; Zelner *et al*., 2013). In addition, it has both polar and non-polar molecules in that the non-polar molecules are proportionally more as compared to the polar molecules therefore making alcohol to be more water soluble. Although alcohol is more water soluble, it is also less soluble in lipids due to the few numbers of polar molecules (Singh *et al*., 2016 ;Zelner *et al*., 2013). This ambiguity of polarity facilitates a rapid diffusion of alcohol through the blood placental barrier (Singh *et al*., 2016).

2.1.2 Route of administration and absorption

The most common route of alcohol administration is through the oral route (Singh *et al*., 2016). On the other hand, 80% of the alcohol volume consumed is absorbed from the small intestines while 20% of the alcohol volume is absorbed from the stomach (Singh *et al*., 2016). Via the hepatic portal vein, the absorbed alcohol gets in to the maternal liver and finally into the systemic circulation (Marek & Kraft, 2014). Due to the low molecular weight, lipid and water solubility properties of alcohol, it finally crosses the blood placental barrier into fetal circulation (Manzo & Saavedra, 2010; Zelner *et al*., 2013). Because of the counter current flow of maternal and fetal blood that exist, the fetal and maternal blood alcohol concentrations attains an equilibrium and in this the fetal organs are highly exposed to alcohol (Manzo & Saavedra, 2010).

Further, the fetal blood alcohol concentration is highly dependent on the maternal blood alcohol concentration and the mode of excretion (Nakhoul *et al*., 2017). For instance,food in the maternal gut as well as consumption of beverages with low alcohol content has been

shown to lower alcohol absorption from the gut therefore promoting its excretion. As a result the alcohol concentration in the fetal circulation is reduced. However,consumption of too much alcohol at a single sitting has been shown to increase the rate of alcohol absorption from the gut (Ornoy & Ergaz, 2010).

2.1.3 Distribution, metabolism and excretion of alcohol in the mother

After absorption, alcohol is distributed throughout body fluids, and the distribution is rapid until the tissue levels approximating the blood concentration. The volume of distribution is approximately 0.4-0.7 l/kg (Nakhoul *et al*., 2017). In addition, the time from the last drink to maximum concentration of alcohol in blood usually ranges from 30 to 90 minutes and following this, alcohol readily crosses the blood placenta barrier into fetal circulation (Singh *et al*., 2016).

Alcohol is primarily metabolized in the liver through three different enzymes namely alcohol dehydrogenase (ADH) which is the key enzyme in alcohol metabolism, cytochrome P450 2E1 (CYP 2E1), and catalase (Weathermon, Pharm, & Crabb, 1999). Although majority of the alcohol is metabolized in the liver, 2-10% of alcohol is excreted unchanged in urine, breath and sweat (Perry *et al*., 2017). During alcohol metabolism, ADH catalyses' alcohol oxidation via a coenzyme known as nicotinamide adenine dinucleotide (NAD) forming acetaldehyde which is injurious to the liver cells. This NAD is reduced to nicotinamide adenine dinucleotide (NADH) (Pal & Ray, 2016). Subsequent oxidation of acetaldehyde by NAD forms acetic acid and more NADH and this reaction is catalyzed by aldehyde dehydrogenase (ALDH) (Kent, 2012). The acetaldehyde produced during alcohol metabolism leads to the formation of reactive oxygen species that are highly reactive and toxic to liver cells (Kent, 2012) (Figure 2.1.1). Excessive NADH levels can inhibit glucose production (gluconeogenesis) and breakdown (oxidation) of fat molecules as well as stimulation of fat synthesis resulting into hypoglycemia, acidosis and fatty liver (Zakhari & Ph, 2006).

On the other hand, CYP 2E1 works in the microsomes, and this enzyme is stimulated only in chronic alcoholics and in this reaction hydrogen is released that binds with oxygen and NADPH to form water and NADP otherwise ADH is the main enzyme

involved in alcohol metabolism (Nykjaer *et al*., 2014). Lastly, catalase enzyme mostly metabolizes alcohol in the brain and has been associated with alcohol addiction (Donohue, 2007).

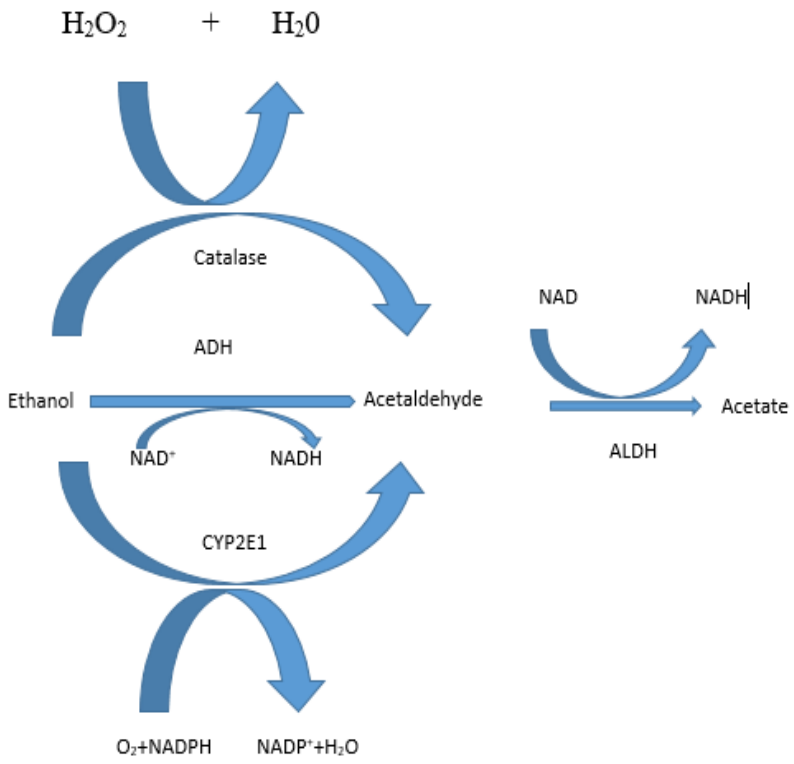


Figure 2.1.1: Alcohol metabolism

2.1.4 Distribution of alcohol in fetus

Alcohol distribution becomes increasingly important during pregnancy, because alcohol tends not to accumulate in the body fat, but rather it is absorbed and carried along the maternal blood circulation and via the placenta it leads in to the fetus (Marek & Kraft, 2014; Zelner *et al*., 2013). Blood alcohol content is undoubtedly of more crucial importance in the pathogenesis of liver defects rather than actual maternal consumption of alcohol (Manzo & Saavedra, 2010). Studies have shown that there is bidirectional placental transfer of alcohol in the maternal-fetal unit such that the alcohol concentration in maternal and fetal blood are similar (Zelner *et al*., 2013). Similarly, alcohol has been shown to be readily transmitted across the

placenta in laboratory rodents (Zelner *et al*., 2013). In the fetus, alcohol is distributed to all body organs including the liver that appear to achieve the highest levels (Marek & Kraft, 2014).

2.2 Effects of alcohol on maternal and fetal pregnancy outcomes

In humans, increased rate of spontaneous abortion, still birth and post natal organs mal-development have been linked to *in-utero* exposure to alcohol (Roberts, 2019). The severity of the malformations following maternal alcohol consumption ranges from FAS, which is evident in 4–6% of infants. This FAS is also associated with minor effects like low birth weight and intra uterine growth retardation (IUGR) (Manzo & Saavedra, 2010). In rodents, *in-utero* exposure to alcohol has been shown to cause embryonic loss, fetal growth retardation and development defects raging from limb anomalies, GIT anomalies, CNS anomalies among others (Downing *et al*., 2010). This clearly indicates that alcohol is teratogenic to both human and rats and its most devastating teratogenic effects occurs on the visceral organs including the liver (Downing *et al*., 2010; Hong & Krauss, 2017).

At the molecular level, alcohol has been shown to interfere with the packing of molecules in the phospholipids bilayer of the cell membrane particularly those cell membranes having low cholesterol levels (Mukherjee, 1999 ; Steiner & Lang, 2017). Therefore, alcohol causes this teratogenic pregnancy outcomes through different mechanisms including increased cellular oxidative stress, depletion of the normal body antioxidants like glutathione, as well as disturbance in metabolism of glucose, protein and lipid (Ghimire *et al*., 2008; Goumard *et al*., 2014). Through this mechanisms, alcohol further leads to increased cellular apoptosis and gene variations (Shankar et al., 2007).

Maternal weight gain during pregnancy and fetal development have been related in that the fetal birth weight may be dependent on the maternal weight during pregnancy. For instance, consumption of light-to-moderate volume of alcohol has been associated with less maternal weight gain and this has been reported to be a risk factor to IUGR which is characterized by a reduction in the anthropometric parameters (Wang *et al*., 2011). In another study, normal fetal anthropometric

parameters namely, the birth weight, head circumference and crown rump length were observed irrespective of maternal alcohol consumption. Further there were no meaningful changes in maternal weight gain following maternal alcohol consumption during pregnancy and this was associated with good nutritional status of the mother (Carter *et al*., 2017). On the other hand, poor maternal nutritional status following alcohol consumption has been associated with poor development of the fetus during intrauterine life (Shanker *et al.,* 2007). This has been associated with nutritional deficits which occurs following prolonged periods of maternal alcohol consumption.

Acetaldehyde which is a toxic metabolite produced during alcohol metabolism has also been shown to interfere with the proliferation of trophoblastic cells as well as the normal placental villus development (Lui *et al.,* 2014). This interference has been shown to occur during the first trimester, the period essential for normal fetal organogenesis (Lui *et al.,* 2014). As a result intrauterine fetal death or abnormal fetal development ensues (Lui *et al.,* 2014; Carter *et al*., 2017).

2.3 Comparative prenatal development and the normal morphology of the liver in fetuses of both humans and rat

2.3.1 Embryology of the liver

The early development of fetal liver is manifested by the formation of primitive streak in the rat embryo that begins around gestational day (GD) 6.5 (Gruppuso & Sanders, 2016). This time period from day 6.5 to 11.5 is developmentally important and has been called the period of organogenesis when vital organs including the liver begin to form (Gruppuso & Sanders, 2016). Embryologically, both human and rats liver primodia correlate in that they both originates from the embryonic endoderm of the distal primitive foregut (Gordillo *et al.*, 2015). Their stage of development too corresponds to the period when exposure to teratogens interferes with the normal hepatogenesis (Gordillo *et al*., 2015; Lobo *et al*, 2012). Their developmental stages also correlate and the fetal liver developmental stages includes:- i) Endodermal proliferation – this occurs on day 6.5 and day 23-26 in rats and human respectively (Gordillo *et al*., 2015). A monolayer of cells happens on the ventral aspect of the developing embryo forming liver cells (Gordillo *et al*., 2015;

Lippincott & Wilkins, 2011). This is followed by: - ii) the formation of liver diverticulum –this occurs due to the continuous proliferation of the endodermal cells neighboring the cardiac mesoderm and septum transversum. This liver diverticulum further proliferates to form hepatoblasts which delaminates, proliferates and invades the surrounding septum transversum, consequently developing into a liver (hepatic) bud. iii) The hepatic bud elongates cranially and divides forming two buds; a small accessory bud on its right side (pars cystica) and a larger bud (pas hepatica).

Further development of the pars cystica forms the cystic duct and the gallbladder in human. However, this division does not happen in rats hence for this reason the rats lack the gall bladder (Gordillo *et al*., 2015; Lippincott & Wilkins, 2011). The pars hepatica in human is synonymous to liver bud in rats and forms the liver, while further differentiation forms different liver cells namely hepatocytes, cholangiocytes, stem cells and kupffer cells. (Gordillo *et al*., 2015). iv) hepatocyte differentiation – this begins on day 15 and 26 in rats and human respectively and continuous differentiation of the hepatocytes takes place even post-delivery (Gordillo *et al*., 2015; Gruppuso & Sanders, 2016). It has been established that full maturation of the hepatic architecture in rat is achieved by postnatal day 28 in contrast to human which is achieved by 2 years post-delivery (Gordillo *et al*., 2015).

2.3.2 Gross and histological morphology of the liver

The liver is the largest internal organ, as well as the largest accessory gland of the gastro-intestinal tract (GIT) (Lobo *et al.*, 2012). In terms of its location, the liver is found located in the right hypochondriac region of the abdomen, extending to the epigastric and left hypochondriac regions both in humans and rats (Gordillo *et al*., 2015). The gross morphological comparison between the rat and human liver shows a lot of similarities in structure and appearance. For instance, the liver of human and rat consist of four lobes namely: right, left, caudate, and quadrate lobe. However, unlike in humans, the right and caudate lobes of the rat further splits into two sub-lobes although not functionally important (Gruppuso & Sanders, 2016). Similarly their histomorphology correlates in that the liver parenchyma is contained in a thin capsule of fibro-connective tissue known as the glisson capsule (Vaissi *et al*.,

2017).Thin septas originates from this capsule dividing liver parenchyma in to lobules (Ebrahim *et al*., 2018).

The liver parenchyma consist of plates of hepatocytes (endocrine cells) which are one cell thick separated from each other by hepatic sinusoids while between adjacent hepatocyte runs the bile canaliculi that forms the exocrine cells of the liver (Vaissi *et al*., 2017).These exocrine cells secrete bile via system of duct in to the duodenum essential for digestion. On the other hand, the endocrine cells secrete numerous plasma proteins that includes:-albumin, fibrinogen, prothrobin and lipoproteins among others into the blood stream (Giancotti *et al*., 2019).

Histologically, studies have shown that a normal liver is made of classical lobules of hepatic parenchyma which is hexagonal in shape. Each of this hexagonal lobule consist of centrilobular vein and portal tract at each of the six (6) corners (Lobo, 2014). Further, the hepatic parenchyma consist of hepatocytes separated from each other by sinusoids. This sinusoids are unique liver vascular structures lined by fenestrated endothelial cells (Pal & Ray, 2016). Majority of the liver cells are the hepatocytes and forms 80% of the cells. Other liver cells includes kupffer cells, endothelial cells, stellate cells as well as cholangiocytes (Gruppuso & Sanders, 2016). All these liver cells are very key in alcohol metabolism and alcohol consumption can injure any of this cells (Lobo, 2012).

2.4 Histomorphological effects of alcohol on the liver

Prenatal exposure to alcohol has been shown to interfere with the normal histomorphogenesis of the fetal liver (Corsello & Giuffrè, 2012). Such teratogenic dysmorphogenesis following prenatal exposure to alcohol are an important causal predictor of the post natal liver disorders. These post natal liver disorders includes hepatitis, liver cirrhosis, liver cancer among other whose causes are yet to be established (Wong & Huang, 2018). Such defects have been attributed to many maternal risk factors that are teratogenic to the fetal liver histomorphogenesis (Habib-ur-rehman, 2011; Wong & Huang, 2018). Among the leading cause of congenital anomalies during organogenesis are maternal alcohol intake and chemical

substances among others (Corsello & Giuffrè, 2012). These manifested dysmorphogenesis occurs at different gestational periods of fetal liver development.

Chronic alcohol consumption has been shown to causes steatosis (fatty liver disease) with a centrilobular pattern of injury which leads to perivenular fibrosis and/or pericellular fibrosis (Lanker *et al*., 2019). Studies have shown that progression of liver steatosis usually drives into pericellular fibrosis, linking vascular structures and paving the way for the development of cirrhosis. In addition, the progression of alcoholic cirrhosis has been shown to increase the severity of liver fibrosis due to parenchymal loss and proliferation of fibrous tissues (Lanker *et al*., 2019). In another study, when alcohol was administered in mice, swelling of the hepatocytes with fluid accumulation was observed which was associated with the activation of the liver macrophages (kupffer cells) (Saeed, 2016).

The kupffer cells play a central role in the pathogenesis of alcoholic hepatitis (AH), in that alcohol at high dose has been shown to increase the gut permeability. This increase in gut permeability results in raised levels of serum endotoxins that contains lipopolysaccharides (LPS) (Zakhari & Ph, 2006). The LPS binds to LPS-binding proteins which then activates Kupffer cells. This activated Kupffer cells plays an important role in the inflammatory process resulting in alcoholic hepatitis as well as liver collagen fiber deposition in which the hepatocytes are injured (Suraweera *et al*., 2015). In another study, endotoxin were also shown to increases infiltration of neutrophils other than the kupffer cells in dose and time of dependent manner (Zelner *et al*., 2013). Further, alcohol also has been associated with the formation of focal macro and micro abscess in liver parenchyma that are dependent on the alcohol concentration (Saeed, 2016; Sakhuja, 2014).

Other studies have associated the perturbations of lipid metabolism with chronic alcohol consumption. This is due to the fact that alcohol has been shown to increase adipose tissue lipolysis which leads to ectopic fat deposition within the liver with consequent development of alcoholic fatty liver disease (Stainer and Lang 2017). In addition, alcohol also impacts other functions of adipose tissue and lipid metabolism. Lipid balance in response to chronic alcohol intake favors adipose tissue loss and

fatty acid efflux as lipolysis is up regulated and lipogenesis is either slightly decreased or unchanged. Several regulatory proteins modulated by alcohol contribute to these effects.

In addition, the adipose tissue glucose tolerance is also impaired by chronic alcohol due to decreased glucose transporter- 4 availability at the membrane and this may worsen alcoholic liver disease (stainer and lang 2017). Multiple mechanisms such as oxidative stress, mitochondrial dysfunction, and alteration in gut-liver axis have been proposed for the pathogenesis of alcoholic liver disease (Pal & Ray, 2016). Methanol poisoning during pregnancy has also been associated with severe injurious effects on the developing fetal liver ranging from micro-vesicular steatosis, macro-vesicular steatosis, focal hepatocyte necrosis, mild intrahepatocyte bile stasis and hydropic degenerative changes of the hepatocytes (Akhgari *et al*., 2011).

2.5 Histostereological effects of alcohol

Alcohol related liver damage would be mediated by oxygen free radicals and possibly may also indirectly contribute to fetal liver dysmorphology (Zakhari & Ph, 2006). The injurious effects from alcohol would be observed in the liver hepatocytes and liver macrophages among other cells (Zakhari & Ph, 2006; Shankar *et al*., 2007). Studies have associated alcohol consumption with increase in hepatocytes sizes and cellular volume following its injurious effects (Suraweera *et al*., 2015). An increase in kupffer cells volume has also been reported and this is mostly due to the elevated levels of endotoxins (Suraweera *et al*., 2015). In alcoholic cirrhosis, which range from simple steatosis to hepatocellular carcinoma, some studies have shown an increase in the liver volume while other studies have reported a reduction in liver volume (Pal & Ray, 2016; Rosa *et al*., 2015). In another study the total liver volume, mean numerical density and total number of kupffer cells, were increased in non-alcoholic fatty liver which was induced by administration of fat diet concurrently with alcohol (kiki *et al.,* 2007).

2.6 The patterns of fetal liver teratogenicity on dose and time of exposure

Alcohol can interfere with the activities of growth hormone and insulin-like growth factors, which promote body growth and activity (Gabriela *et al*., 1998). Some of the effects of maternal alcohol consumption on fetal hormone systems may contribute to the adverse effects observed in children with fetal alcohol syndrome and related disorders in time and dose dependent manner (Gabriela *et al*., 1998; Goc *et al.,* 2019). Alcohol exhibits teratogenic effects resulting in growth delays, facial anomalies, neurological defects including intellectual disabilities and behavioral problems whose severity depends on the alcohol dose (Fiorentino *et al*., 2017 ; Beier & McClain, 2010). This has been associated with alcohol ability to directly cross the placental barrier. Further, aspilia africana leaf extract have been shown to cause dilatation of the central veins, cyto-architectural distortions of the hepatocytes, centrilobular hemorrhagic necrosis, atrophic and degenerative changes in rats upon in time and dose dependent manner (Eweka, 2006).

In another study which was done by Gao & Bataller, (2011), they associated the teratogenic effects of alcohol to its metabolism due to the following:-oxidative stress to the liver cells, glutathione depletion, abnormal methionine metabolism, malnutrition, and production of endotoxins that activate Kupffer cells (Gao & Bataller, 2011; Parri & Eriksson, 2006). The injurious effects of alcohol were found to be both time and dose dependent. Similarly, another study shown methanol being teratogenic to the developing fetal liver when exposed *in-utero* in time and dose dependent manner (Akhgari *et al*., 2011). Other studies have associated the inhibition of the rates of protein synthesis to maternal alcohol consumption in both fetal and neonatal livers and this inhibition is responsible for some of the changes observed in fetal alcohol syndrome (Rawat, 1976; Eberhart, 2014). A study done by Lovely & Eberhart, (2014) also found a dose dependent effects of alcohol when administered in laboratory rats.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area/Site

All animal experiments including breeding, handling weighing and alcohol administration were done at the small animal facility for research and innovation (SAFARI) of Jomo Kenyatta University of Agriculture and Technology (JKUAT) main campus which is situated in Juja, Kiambu County, Kenya. Processing of specimens for light and stereological analysis were done in the Histology laboratory Department of Human Anatomy, College of Health Sciences (COHES), JKUAT.

3.2 Study design

The current study was a static-group controlled-experimental study design where female albino rats weighing between 200-230gms were used. Before starting the experiments, female albino rats were fed on a standard diet (Ricci & Ulman, 2005), and left at the SAFARI animal house for a minimum period of seven days to allow them acclimatize to the new environment. All rats were kept in standard animal cages in the animal house and received food (rodent pellet) from Thika Unga feeds. They also received water *ad libitum.*

3.3 Study subject

The albino rats (*Rattus novegicus*) which were used in the current study are laboratory rats which have white fur and pink eyes due to their inability to produce melanin (Modlinska & Pisula, 2020). They originates from Asia, belong to the genus "*Rattus*" and the group "*novegicus*" and they were the first mammalian species to be domesticated for scientific research. This rats were preferred due to the following existing facts: (a) They have low incidence of spontaneously occurring congenital defects, (b) a relatively short gestational span, (c) a large litter size, (d) low cost of maintaining the rats, (e) and considerable amount of the reproductive data on this laboratory rats is already available (Orlu & Ogbalu, 2012).

Albino rats which were used in the current study depicted the following physical characteristic: i) they had white fur that covered their body except the nose, ears and the feet, ii) had pinkish eyes, iii) they were about 9 inches (23cm) from the nose to base of the tail when fully grown. iv) they typically had 16 teeth, the most prominent of which were their ever growing incisors which they use to gnaw in addition to their powerful jaw muscles and v) long tail that range between 8-12 inches (20-33cm) (Modlinska & Pisula., 2020; Schweinfurth, 2020).

The young female rats usually attain their sexual maturity at around 40 - 60 days of age (approximately 5 - 6 weeks). The female albino rats used in the current study were colony from the third series pure breeds, aged between 5-6 weeks. This was considered in order to avoid genetic differences **(**Orlu & Ogbalu 2012)**.** In addition, the female albino rat has bicornuate uterus and is a polyestrous spontaneous ovulatory cycles every 4 - 5 days. Their gestation period is 21 - 23 days and they usually gives birth to an average of 6-8 pups. This pups weigh 4.5 - 6 grams at birth, and are born blind, deaf, and hairless. Their ears open at approximately 12 days and their eyes between days 10 - 12. This pups are also dependent on their mother for their nutritional needs until they are approximately 14 days old. Once they are mobile, they may explore food present in the bottom of their cage, but they still receive most of their calories from milk. Most rats are weaned at 21 days of age. Weaning is usually performed by removing the rats from the mother's cage and separating the rats into single-sex groups **(**Orlu & Ogbalu 2012)**.**

On the other hand, male rats attain sexual maturity at 40 - 60 days of age. The descent of the testes usually occurs between postnatal days 30 - 60. In addition the male rat has an os penis, no nipples but the sperm counts vary by strain. The adult male rat has a prominent scrotum which is directly related to age and a longer ano-genital distance than the female rat. In the current study a colony of healthy males from second series breeds were used in mating in order to get pure breed and they were between postnatal days 45-50. (Pritchett & Corning, 2004; Schweinfurth, 2020).

3.4 Selection criteria

3.4.1 Inclusion criteria

- All rats that were healthy and conceived in the first day after being introduced to a male overnight
- All fetuses that were alive at point of sacrificing the rats

3.4.2 Exclusion criteria

- All rats that tested negative for pregnancy test following the introduction of a male were excluded
- All rats that later shown signs of sickness following treatment with alcohol
- All fetuses in which mother had an underlying disease state were also excluded

3.5 Sample size determination, sampling technique and animal grouping

3.5.1 Sample Size determination

The sample size of the female albino dams was determined through the resource equation method), because the standard deviation from previous studies was not available and the effect size as well. In this method, the value 'E' is measured which is the degree of freedom of analysis of variance (ANOVA) based on a decided sample size. The value ('E') should lie between 10 and 20 rats. A value less than 10 necessitates adding more rats which increases the chance of getting significant results. A value more than 20 has been shown to increase the cost of the study without increasing the significance of the result (Charan & Kantharia, 2013 ; Llyas *et al*., 2017).

Total number of rats (TA) = E (degree of freedom) + Total number of groups (TG)

TA (30) = E (20) +TG (10) (30 albino dams were the desired total number of rats for the study)

E=20 which fell between 10 and 20, therefore 30 was an adequate and representative sample size for this study. The total number of groups in this study was ten (10).

Since, every adult female albino rat was assumed to have a minimum average of six (6)- fetuses per pregnancy (Orlu & Ogbalu, 2012), then the expected number of fetuses was 6x30=180 fetuses

In order to determine the fetal sample size, all fetuses from each mother were weighed and their weights recorded in ascending order. Three fetuses one with the lowest, median and highest weights per rat were objectively selected for light and stereological studies making a total of 90 fetuses. However the remaining fetuses were preserved in zenker solution for future use.

3.5.2 Sampling technique

The colonies of pure breeds from second and third series for male (15) and female (30) albino rats respectively were assigned to the ten (10) animal groups. The male rats were aged between 40-50 days and this was considered because at this age they were sexually mature. The female rats were aged between 5-6 weeks, the period when they are sexually mature. This male and the female albino rats were of same breed in order to avoid genetic variations.

3.5.3 Animal grouping

The 30 rats used in this study were assigned into either control of 3 rat or experimental group of 27 rats (Figure 3.5.1). The 27 rats in the experimental category were non-randomly further divided into three broad study groups of 9 rats each assigned: - low (LAG), Medium (MAG) and High alcohol group (HAG). The broad study groups were further subdivided into first (TM_1), second (TM_2) and third (TM_3) trimesters subgroups comprising of 3 rats each.

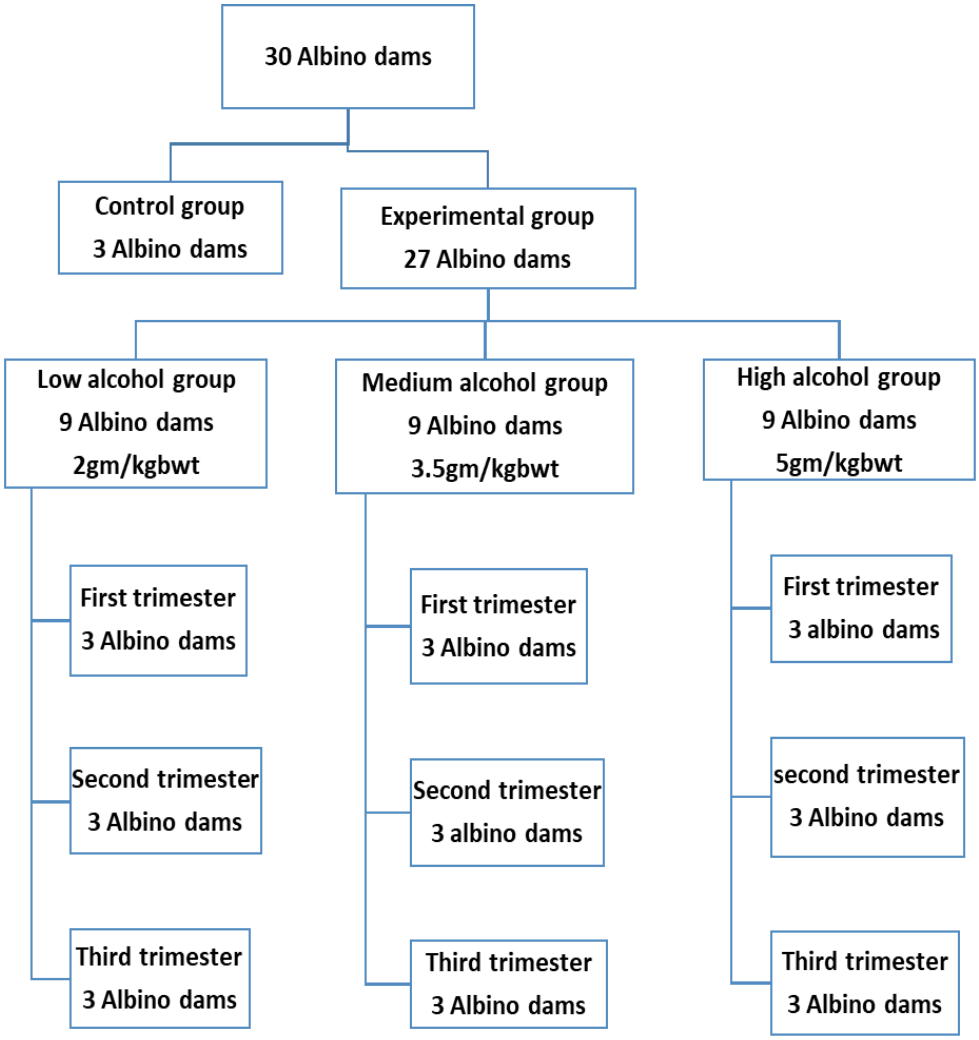


Figure 3.5.1: Animal grouping in the control, Low alcohol group (LAG), Medium alcohol group (MAG), and the High alcohol group (HAG)

3.5.4 Animal feeding

i). The Control sub group(C) -3 rats

All the 3 rats in the control category received standard diet as determined (Ricci & Ulman,2005), containing 20% proteins, 72% carbohydrates and 12% lipids, for the whole of the gestation period, that is day 1-20 (Ricci & Ulman, 2005). They also received water *ad libitum.* The rats were sacrificed on the 20th day of gestation

which were counted from the day pregnancy was confirmed and the fetuses were harvested.

ii) The experimental groups (27 rats)

These were the animal subgroups subjected to varying doses of alcohol. They were organized as follows:-

(a) **The Low alcohol group-(LAG)-**9 rats

Received constant daily dose of alcohol (2gm/kg/bwt) administered as a single bolus through gastric gavage (Guage 16 needle) at 0930hrs for a period of 7, 14 and 20 days for the third, second and third trimester sub groups respectively.

(b) **The Medium alcohol group-(MAG)-9 rats**

Received a constant daily dose of alcohol (3.5gm/Kg/bwt) administered as a single bolus through gastric gavage (Guage 16 needle) at 0930hrs for a period of 7, 14 and 20 days for the first, second and third trimester sub groups respectively.

(c) **The High alcohol group-(HAG)-9 rats**

Received a constant daily dose of alcohol (5gm/Kg/bwt) administered as a single bolus through gastric gavage (Guage 16 needle) at 0930hrs for a period of 7,14 and 20 days for the first, second and third trimester sub groups respectively. In addition all the rats in the treatment groups received standard diet and water *ad-libitum.*

3.6 Animal handling, breeding and pregnancy determination

3.6.1 Animal Handling

The rats were handled only by the investigator and a trained research assistant for the purpose of obtaining daily weights and feeding between 0900hrs to 0930hrs. All procedures were performed according to the guidelines for care of laboratory rats.

3.6.2 Breeding of the rats

One male albino rat was introduced into a standard animal cage with two female rats (Orlu & Ogbalu, 2012) at 0230hrs (+/- 30 minutes). At 0900hrs (+/- 30 minutes) the following morning, the males were returned to their separate cages.

3.6.3 Determination of Pregnancy

Vaginal smears were taken from the 30 mated females the next morning and pregnancy was determined by vaginal wash 24 hours later to determine the estrous changes that denoted the first day of gestation (GD_1) (Ahmed, 2014). Those rats that tested negative for pregnancy in the first instance were returned in the cage and the males were re-introduced to allow mating to take over for another 24 hours, after which pregnancy was determined.

a) **Materials used in the determination of pregnancy**

i. Cotton tipped swab
ii. 0.85% phosphate buffered saline
iii. Microscope slides
iv. Absolute alcohol (95%)
v. 10 mls blunt tipped disposable pipettes
vi. Gemsa stain

b) The Procedure followed in the determination of pregnancy

1. The animal was restrained using a table cloth against the researchers anterior abdominal wall
2. 1ml of saline was introduced into the vaginal cavity using a blunt tipped disposable pipette (vaginal wash)
3. The cotton tipped swab was moistened with phosphate buffered saline, and gently inserted into the vaginal cavity
4. The swab was then slightly rolled before withdrawing
5. The moist swab was gently withdrawn and rolled onto a clean glass microscope slide

6. The specimen was spray fixed using 95% alcohol
7. The slide was allowed to air dry
8. The slide was then stained with Geimsa stain
9. The slide was viewed under the Optika microscope (from Japan) and observation of large cornfield cells, many neutrophils on the smear and scattered epithelial cells confirmed pregnancy. In addition, presence of sperm cells on the slides confirmed that mating took place.

3.7 Determination and administering of alcohol doses

3.7.1 Materials used and procedure followed in alcohol administration

The administration of the different doses of alcohol was done between 0930hrs daily

a) Materials

i. Alcohol
ii. Gavage needle gauge 16
iii. 5ml syringes
iv. 20 ml beaker for dilution
v. Syringes
vi. Deionized water (500mls)
vii. Pregnant dams
viii. A table cloth

b) Procedure followed in administering various doses of alcohol through gastric gavage

1) The animal was held carefully from the neck region using the left hand
2) The animal was then wrapped with the table cloth to avoid the animal from soiling the investigators clothing and at the same time they were examined for any disease state.
3) The animal was then rested against the investigators anterior abdominal wall with the animal mouth facing the investigator
4) The gavage needle connected to a syringe containing the right alcohol dose was gently inserted into the mouth of the animal turning it gently to pass the esophageal constrictions and the cardiac sphincter

5) The alcohol bolus was then dropped in the stomach of the animal
6) The gavage needle was then gently removed

3.7.2 Determination of the alcohol doses for the experiment

a) Determination of the alcohol doses:

The weight in grams of alcohol for lethal dose (LD_{50}) was 7.1gm/kgbwt in rats. This LD_{50} was already determined in the previous toxic studies in rats. Therefore, a slightly lower dose to this was adopted as the high dose (5g/kgbwt), while the low dose (2g/kgbwt) used in this case was adopted based on the fact that alcohol dose below this has not been associated with teratogenic outcomes (Patten *et al*., 2014). A dose of 3.5g/kgbwt was used as the medium dose.

The alcohol dose for an average weight dam of 200g was calculated as follows:-

Weight of a rat= 200g

Lethal dose LD50= 7.1g/kgbwt

A single rat received=$\frac{200g \times 7.1g}{1000g}$ = 1.42g alcohol

Alcohol used in this study was purchased from Chemo-quip Pharmaceutical Company (Nairobi), with butch number 1614105017, a concentration of 98 % and a molecular weight of 46.07g/mol.

How the alcohol doses in g/kgbwt were determined

(i) The calculation of low alcohol dose group (LAG) that received 2g/kg/bwt (1.3ml)

$\frac{200 \times 2g}{1000}$ = 0.4g/kg of 98 % alcohol

Since alcohol concentration more than 30% has been shown to causes gastric ulcerations in rats, a concentration of 30% was used**.**

Procedure followed in dilution of absolute alcohol into 30% alcohol

Since the alcohol concentration was 98%, this meant that every 100mls of solvent had 98g of alcohol. Therefore, 30mls of alcohol was withdrawn and diluted with 70 mls of distilled water (diluent) to make 100mls of 30% ethanol that is 30g in 100mls of solvent as was in the material safety data sheet.

How the volume of alcohol administered in LAG were determined

(i) The determination of alcohol volume in the low alcohol dose group (LAG) that received 2g/kg/bwt

$$\frac{200 \times 2g}{1000} = 0.4 \text{ g/kg of 98 \% alcohol}$$

$$\frac{0.4g \times 100mls}{30gms} = 1.3mls \text{ of 30\% alcohol}$$

(ii) The determination of alcohol volume in the medium alcohol dose group (MAG) that received 3.5g/kgbwt

$$\frac{200 \times 3.5g}{1000} = 0.7g/kg \text{ of 98 \% alcohol}$$

$$\frac{0.7 \times 100mls}{30gms} = 2.3mls \text{ of 30\% alcohol}$$

(iii) The determination of alcohol volume in the high alcohol dose group (HAG) that received 5g/kgbwt (3mls)

$$\frac{200 \times 5g}{1000} = 1 \text{ g/kg of 98 \% alcohol}$$

1 x100mls = 3.3mls of 30% alcohol

30gms

(iv) The Controls**:** All controls received 3mls of deionized water through gastric gavage.

All the volumes were administered in a standard volume of 3-4mls (the standard allowable daily oral volume of a rat per day) and by following this set up, the critical dose of the alcohol teratogenesis on the fetal liver histomorphogenesis was determined as follows:-

b) Determination of the critical period for alcohol to fetal liver teratogenesis.

In reference to Fig. 3.5.1 on animal groupings, the administration of alcohol in each of the groups was done as follows:

1) In each of the groups (LAG, MAG,HAG), the 9 dams were randomly sub divided into three sub-groups: the Trimester 1(TM_1)= 3 dams, Trimester 2 (TM_2)= 3dams and trimester 3 (TM_3)=3 dams

   The gestation period of a rat is 21 days, therefore trimester one was taken as the period between gestational day GD1 to GD7, while trimester 2 is between GD7-GD14 and third trimester GD14-20.

2) Alcohol was administered as follows:-

   - All trimester ones (TM_1) rats:- (LAG,MAG,HAG) categories received alcohol from gestation day GD_1-GD_{20}
   - All trimester two's (TM_2) rats:- (LAG,MAG,HAG) categories received alcohol doses from gestation day GD_7-GD_{20}
   - All trimester three (TM_3) rats:- (LAG,MAG,HAG) categories received alcohol doses from gestation day GD_{14}-GD_{20}

Sacrificing the rats

- All rats were sacrificed on day 20 before delivery.

c) Determining vulnerable periods of alcohol teratogenesis on the fetal liver

To determine the vulnerable periods of alcohol teratogenesis, alcohol was administered daily throughout the gestation period starting on day 1(GD_1) for the trimester 1 groups, day 7 (GD_7) for the trimester II (TM_2) and day 14 (GD_{14}) for trimester III (TM_3) subgroup.

3.8 Harvesting of fetuses

3.8.1 Humane sacrificing of the rats and harvesting of the liver

Materials

i. The rats (pregnant rat GD20,
ii. Carbon dioxide cylinder
iii. Cotton gauze or cotton wool
iv. Bell or dissector jar
v. Mounting board
vi. Mounting pins
vii. Pair of scissors
viii. A pair of forceps(toothed)
ix. Scalpel blade
x. Scalpel blade handle
xi. Fixatives- formaldehyde
xii. Hypodermic needle guage 20
xiii. Gloves (clean)
xiv. Magnifying glass
xv. Ruler
xvi. Electronic weighing machine
xvii. Specimen collection bottles

3.8.2 Procedure for anaesthetizing and perfusing the animal

1. An empty heavy bell jar with a tight fitting lid was opened and cotton wool was introduced.
2. The expectant rat was then introduced into the heavy bell jar and the tight fitting lid was used to close the jar.
3. While in the bell jar, the tight fitting lid was slightly opened and the cotton wool was soaked in carbon dioxide, the bell jar was tightly closed again to allow the animal to anaesthetize.
4. The animal was allowed to be anaesthetized for 2-5 minutes
5. The animal was then removed from the bell jar and mounted onto the board using mounting pins with dorsal side on the board (Figure 3.8.1A).
6. Using a pair of scissors and forceps, an incision was made in the ventral medial side extending from the xiphoid process to the symphysis pubis (Figure 3.8.1B).
7. The fetuses were then harvested, body weight, crown rump length, and head circumference among other parameters were measured.

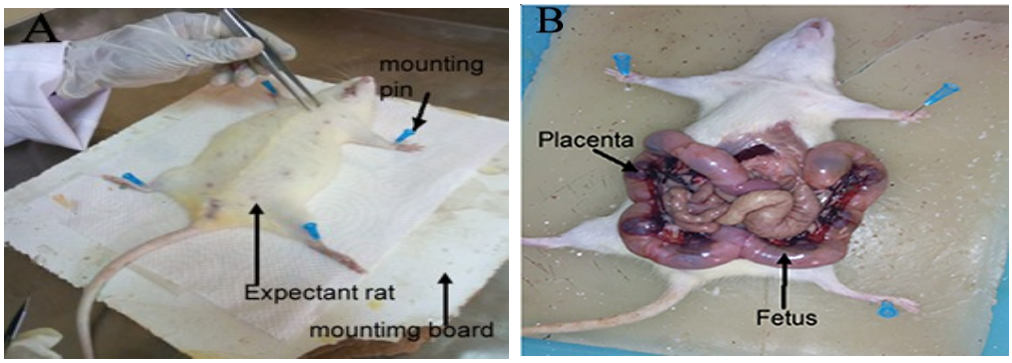


Figure 3.8.1: Image of (A) expectant rat mounted on a dissection board, (B) Fetuses and placenta in the uterine horns after opening the abdomen.

3.8.3 The procedure followed in harvesting of fetuses

a. The uterine horns were excised along the anti-mesometrial borders using a pair of scissors.
b. The live fetuses were determines by using a probe to touch each fetus. Movement of the fetus confirmed that they were alive while lack of movement confirmed the dead fetuses. All live and dead fetuses were counted and recorded
c. The resorbed endometrial sites were counted and recorded (Figure 3.8.2).
d. The fetuses were then harvested by cutting the umbilical cord using a pair of scissors to detach each fetus from the mother. The fetal body weight, crown rump length and head circumference were measured using an electronic weighing balance and tape measure respectively.
e. Congenital anomalies were assessed

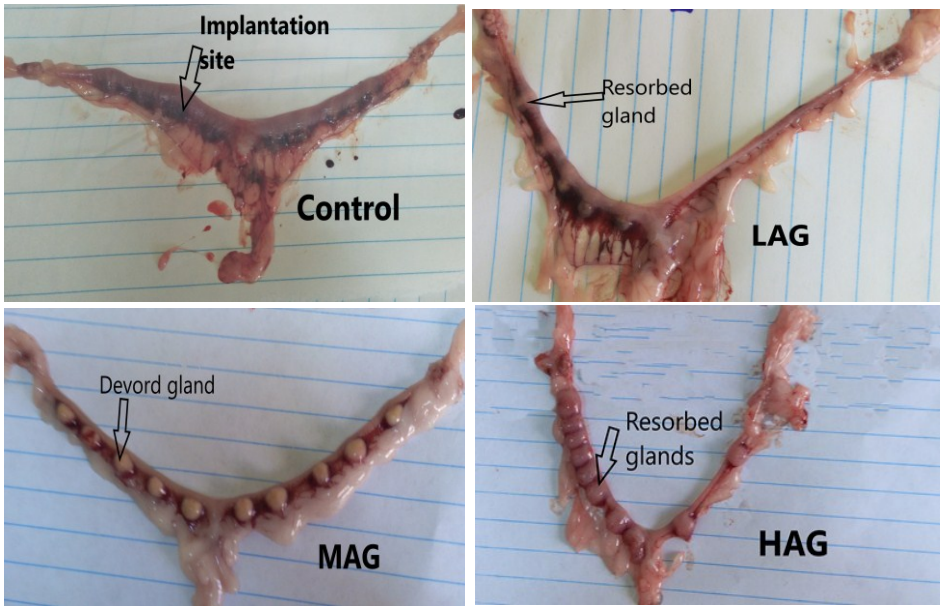


Figure 3.8.2: Image of resorbed endometrial glands/Devoured fetuses in Low alcohol group (LAG), Medium alcohol group (MAG) high alcohol group (HAG) and the Control group (CG)

3.8.4 The procedure followed in harvesting of the fetal liver

a. The fetuses were mounted onto the board using mounting pins(dorsal side facing the board)
b. Using a pair of scissors and forceps, the ventral medial side-up was opened from the xiphoid process to symphysis pubis.
c. The diaphragm was retract up carefully to avoid damaging the liver that lies in the right hypochondriac region.
d. The entire liver was excised at its hilum and examined for any gross malformations
e. The fetal liver was weighed using digital weighing scale
f. The liver was immersed in formaldehyde solution for fixating prior to processing for light microscopy and stereological analysis for 24 hours later.

3.9 Tissue processing for light and stereological microscopy

After fixing the liver in formaldehyde solution for 24 hours, the nine fetal livers per sub group were each placed in a tissue cassette, dehydrated in an ascending concentration of alcohol (50%, 70%, 80%, 90%, 95% and 100%(absolute) each for one hour and cleared with xylene for 30 minutes. The sections were then infiltrated with paraplast wax for 12 hours and embedded in paraffin wax. Leitz sledge microtome was used to cut longitudinal thin liver sections which were floated in water at 37^0 then stuck onto glass slides using egg albumin, applied as thin film with a micro-dropper. From each subgroup, one whole liver was fully sectioned and all sections were stuck in microscope glass slide to determine the total number of sections as well as the different histological changes in the treatment groups against the control.

In each subgroup 20-25 slides were selected through systematic uniform random sampling, dried in an oven at 37^0 for 24 hours then stained with haematoxylin and eosin to demonstrate the general features of fetal liver components(Palipoch & Punsawad, 2013). The same 20-25 selected slides stained with H&E stain were used for histo-stereology. Photographs were taken to show the different histological

changes at different magnifications of x4, x10, x40, and x100 in four systematically selected random fields. The total liver volume for each subgroup was determined using cavalieri principal of point counting and the mean of the four fields recorded as the count for each region**.**

3.9.1 Materials used for staining

i. The specimens (fetal liver)
ii. Zenkers solution (1 litre)
iii. Distilled water
iv. DPX moutant
v. Glass slides and cover slips
vi. Haematoxylin and Eosin
vii. Glass staining square jars
viii. Paraffin wax
ix. Microtome knives
x. Rotary microtome(American optical CO)
xi. Heater and water bath container
xii. Specimen bottles
xiii. Slide holders
xiv. Distilled water
xv. Formaldehyde-10% concentration
xvi. Xylene
xvii. Isopropyl alcohol
xviii. Glass jar for preparing dilutions
xix. Plastic tissue cassettes
xx. Beakers
xxi. Egg albumin
xxii. Dropper

3.9.2 Procedure used for processing the fetal liver specimens for light and stereological microscopy

1. Fetal liver tissue were fixed in formalin solution for 24 hours

2. The liver, were dehydrated in an ascending concentration of alcohol (50%, 70%, 80%, 90%, 95% and 100% (absolute) each for one hour.

3. This was followed by clearing alcohol from the liver tissue by immersing in xylene for 30 minutes.

4. After clearing with xylene, the liver were infiltrated with paraplast© wax for 12 hours at 56^0c

5. Liver tissue were orientated in the longitudinal axis and embedded in paraffin wax on the plastic tissue cassettes

7. Excess wax was trimmed-off till the entire length of the liver tissue was exposed

8. 5µm thick longitudinal sections from right lobe to left lobe were cut with Leitz© sledge rotary microtome

9. The sections were floated in water at 37 degrees to spread the tissue

10. The sections were stuck onto glass slides using egg albumin, applied as thin film with a micro-dropper and 20-25 slides in each subgroup were selected through SURS.

11. The slides were dried in an oven at 37 degrees for 24 hours

12. The slides were stained with eosin and haematoxylin stain for viewing under light microscopy.

3.9.3 The procedure for staining liver specimens for light microscopy and stereology

The liver sections were stained with Hematoxylin and Eosin (Palipoch & Punsawad, 2013), through the following procedure:-

1. The liver tissues were immersed in xylene for the removal of wax because wax impairs staining of the liver cells
2. The liver tissue slides were further immersed in descending grades of alcohol (100%, 90%, 80%, 70% and 50%) to remove xylene and also hydrate the tissue.
3. The tissues were immersed in Haematoxylin stain for 10 mins to stain the liver cell nuclear. This was followed by blueing for 30mins which is done by washing the tissue slides in running tap water.
4. After blueing the tissues were again immersed in Eosin stain for 10 mins to stain the cells cytoplasm, followed by washing away the excess eosin stain.
5. The tissue were dehydrated by taking them through ascending grades of alcohol (50%, 70%, 80%, 90% and 100%).
6. The alcohol was cleared with xylene (xylene I and II).
7. Mounting or cover slipping was done using DPX mountant, then the tissues were allowed to dry for 3 days.
8. The tissues were examine under the light microscope and digital images were taken using a 32mp digital camera for light as well as stereological analysis.

3.10 Stereological analysis

3.10.1 Procedure followed in estimation of the total liver volume and volume densities

The water immersion method was used to determine the initial liver volumes while cavalieri method was used to determine the terminal liver volume after tissue processing.

a) Estimation of the liver volume using Archimedes principle (Water emersion method)

The anterior abdominal walls of fetuses from control and experimental groups were opened, the liver were harvested. The Archimedes volume was estimated by immersing the whole fetal liver tissue into graduated beakers containing normal saline, and the displacement was measured (Altunkaynak, 2009 ; Mohazzab, 2017). The normal saline displaced by the liver represented the actual liver volume which were used as the reference volumes (Altunkaynak, 2009). This was followed by immersing the liver tissue in 10% formalin for fixation over 24 hours. This method was compared to the cavalieri method where the mean and standard error of mean (Mean ± SEM) of the measurements were determined.

b) Estimation of the liver volume using cavalieri principal method

To estimate the total liver volume for each fetus, between 20-25 sections of 5um were sampled from each longitudinal liver section, through systematic uniform random sampling (Asuzu *et al* ,2015). The stained liver tissue (on glass slides) were mounted on the BH2-Olympus light microscope in the Department of Human Anatomy. Digital images were taken for all fields of view in the sampled sections at a magnification of x4. The liver volume was determined by combining the Cavalieri method of segmentation with point-counting on evenly spaced organ slices (Figure 3.10.1) (Altunkaynak, 2009; Asuzu *et al.*, 2015). The following steps were followed: i) Preparation of liver Cavalieri sections ii) Selection of the spacing for the point probe iii) The point probe was tossed randomly onto each section iv) The points that hit the region of interest were counted using STEPnizer stereology tool v) All sections were processed keeping a tally of counts per section vi) The total liver volume was finally determined by applying the Cavalieri formula below:-

$$_{est}V = \frac{\sum_{i-1}^{m} P.\ a/\ p.\ ts}{M^2}$$

Where: $_{est}V$= was the estimation of the volume of the liver,

$\sum$**P**= was the sum of the number of points landing within the various components of the fetal liver profiles,

a/p= the area associated with each point,

t = the distance between sections and

M= represented the magnification (x40)

$\mathbf{m}_{i-1}$ = all points in the fetal liver sections from the first to the last

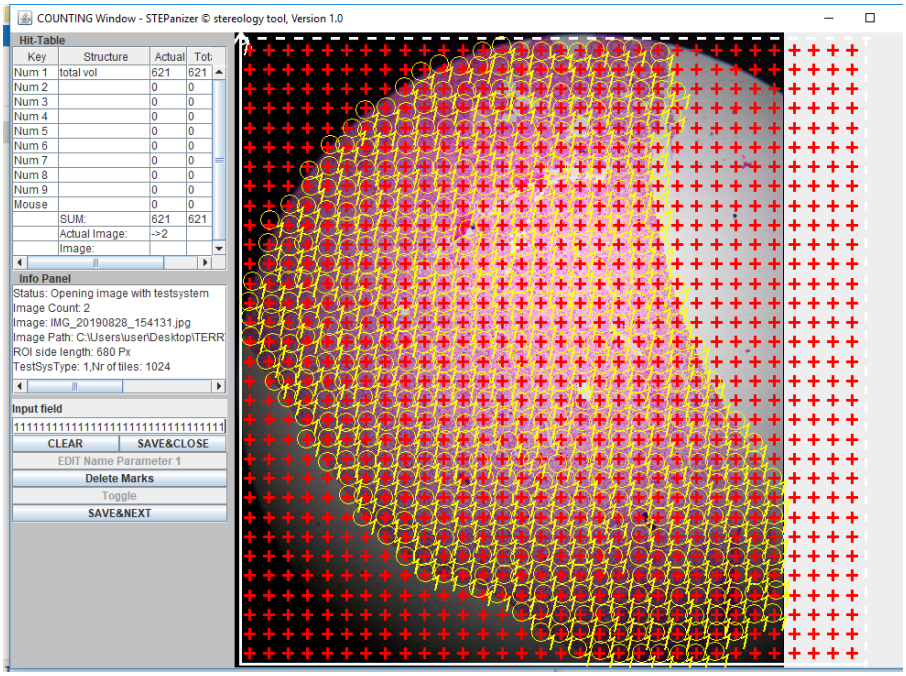


Figure 3.10.1: Photomicrograph of Light microscopic liver slice image with superimposed equidistant point grid.

c) Statistical correction of liver tissue Shrinkage

The following method was applied to quantify shrinkage caused by fixation and histological procedures because shrinkage affects all variables involved in estimating stereological size. The volume of the harvested fetal liver was determined using the water displacement method. After tissue processing and tissue sectioning, the liver

volume was estimated using thereby the Cavalieri method of point counting (Altunkaynak, 2009; Sol & Tips, 2017)

The liver volume shrinkage was then calculated using the formula below(Altunkaynak, 2009):-

$$\text{Volume shrinkage} = \frac{\text{(Volume before)} - \text{(Volume after)}}{\text{(Volume before)}}$$

Volume before were the liver volume which were determined through the water immersion method, while volume after were the volumes determined through the cavalieri method.

3.10.2 Estimation of liver volume densities

To estimate the mean volume density (Vv), of the hepatocytes, sinusoids, portal triad and central vein on each sampled section, the STEPnizer tool was used. Using the STEPnizer tool, a transparent grid was then superimposed on the digital images projected on the computer screen on the liver hepatocytes, central vein among other structures. The points hitting these areas of interest were counted at a magnification of x10 in all fields of view and the volume determined by using the Cavalieri method. Then estimates of their volume density, (Vv) in the reference space were obtained using the formula:

$$\mathbf{estVv = P\,(part)/P\,(ref)}$$

Where **P (part)** and **P (ref)** were the number of test points falling in all structure profiles and in the reference space, respectively **(**Altunkaynak, 2009)

3.11 Photography (materials and procedures)

3.11.1 Materials used in photography

a) Liver tissue histological slides
b) Digital techno camera
c) Light microscope-BP Olympus microscope

d) Memory card

3.11.2 Procedure followed in taking photomicrograph

1. Histological slide were mounted on the stage of the microscope
2. The focus was adjusted until the image to be photographed was in focus
3. The field was magnified as deemed proper
4. A 32 megapixel digital camera was used to take photomicrograph
5. Photographs of the regions were taken as viewed best under the focus of the microscope
6. The photomicrographs were uploaded in a laptop.

3.12 Statistical data analysis

Data was analyzed using the Statistical Package for Social Sciences (SPSS) for Windows Version 23 Chicago Illinois. Differences among groups were analyzed using one way analysis of variance (ANOVA). Group means showing significance difference (p-value <0.05) were further analyzed by the Tukey on post-hoc t-tests. The Tukey test was applied as an expression of the confidence that all comparisons made among the sets of group means were correct. Turkey test was also used to compare the means of the various stereological parameters for the various ethanol treated groups with the control. The results were expressed as mean ± standard error of the mean (SEM) for all values. All results whose $P<0.05$ were considered to be statistically significant.

3.13 Ethical consideration

Ethical approval was sought from animal ethical and research committee of JKUAT with reference number: JKU/2/4/896A (see appendix IV) and all procedures were performed according to the guidelines for the care of laboratory animals (Bayne, 1996).

CHAPTER FOUR

RESULTS

4.1 The maternal and fetal pregnancy outcomes.

4.1.1 The mean maternal weight gain throughout the gestational period

The control group recorded the highest maternal weight gain throughout the gestational period of 93.67±3.53gm while the HAG recorded the lowest at 35.67±2.03gm when treated at TM_1, followed by MAG at 42.0±1.0gm and lastly 52.7±1.45gm for the LAG and a statistically significant as observed (P=0.001). This was also the case in TM_2 where the mean maternal weight gain for the HAG was 65.33±6.69gm, the MAG at 64.33±3.55gm and LAG at 76.33±7.13gm and was found to be statistically significant (P=0.019) while the control group had 93.67±3.53gm (Table 4.1.1). At TM_3 the mean maternal weight gain did not depict any statistical significant difference (p=0.226) when the alcohol treated groups were compared with the control group.

Table 4.1.1: The TM_1, TM_2 and TM_3 mean maternal weight gain in the LAG, MAG, HAG and the control group

Parameter	Control group	Low alcohol group(LAG)	Medium alcohol group (MAG)	High alcohol group(HAG)
TM_1 Mean maternal weight gain (gms)	93.67±3.53[a]	52.7±1.45[b]	42.0±1.0[c]	35.67±2.03[c]
TM_2 Mean maternal weight gain (gms)	93.67±3.53[a]	76.33±7.31[b]	64.33±3.53[b]	65.33±6.69[b]
TM_3 Mean maternal weight gain (gms)	93.67±3.53[a]	79.33±7.31[a]	77.7±3.8[a]	79.0±4.9a

*KEY: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA, with Tukey test on post-hoc t-tests. * indicates that differences are statistically significant (p<0.05).*

4.1.2 The resorbed endometrial glands and placental weights.

At TM_1 and TM_2 and TM_3 the endometrial glands resorptions were observed to have direct correlation with the dose and time of alcohol exposure in that with increasing alcohol doses and duration of exposure, there was a marked increase in endometrial gland resorptions although there was no statistical significant difference observed (p=0.160) except in the HAG TM_1 (P=0.025) when they were compared with the control group (Table 4.1.2).

The mean placental weight is a key indicator of the nutritional exchange between the mother and the fetus. The mean placental weights in the alcohol treated group at TM_1, TM_2 and TM_3, show that alcohol interfered with development of the placenta. A significant reduction (P=0.001) in the mean placental weights was observed when alcohol was administered at TM_1 with the lowest weights in the HAG followed by the MAG and LAG. Similarly, this was also the case when alcohol was administered in trimester two (TM_2) in dose and time dependent manner. However no significant difference (P= 0.059) was observed between the LAG and MAG at TM_3 (Table 4.1.2).

Table 4.1.2: The TM_1, TM_2 and TM_3 means resorbed endometrial glands and placenta weight in the control and alcohol treated groups (LAG, MAG and HAG).

Animal group	ETH treatment period	Resorbed glands (gm)	Placental wts (gm)
control	none	0.00±0.00^{a}	0.54±0.02^{a}
LAG	TM1	1.00±0.58^{ab}	0.39±0.015^{b}
	TM2	1.67±0.33^{a}	0.48±0.012^{ab}
	TM3	0.33±0.33^{a}	0.58±0.01a^{b}
MAG	TM1	3.67±2.03^{ab}	0.39±0.007^{b}
	TM2	2.67±1.20^{a}	0.45±0.03^{b}
	TM3	0.33±0.33^{a}	0.52±0.01^{ab}
HAG	TM1	6.67±1.45^{b}	0.30±0.00^{c}
	TM2	3.67±2.03^{a}	0.42±0.007^{b}
	TM3	0.33±0.33^{a}	0.50±0.00^{b}

Key: The means, followed by the same letter in a column are not statistically different at (P<0.05) using one way ANOVA, with Tukey test on post-hoc t-tests

4.1.3 Influence of alcohol on mean fetal weight, CRL and head circumference

It was observed that the mean fetal weights, the mean crown rump lengths and the head circumference depicted an inverse dose response relationship while at the same time depicting a direct dose response relationship with the time of exposure. A statistical significant difference (p=0.001) was particularly observed when the treatment was done in TM_1 and TM_2 across all the alcohol treated groups (LAG, MAG and HAG). The treatment at TM_3 did not show statistical significant difference (P>0.05) in the LAG and MAG when compared with the control group in the three fetal parameters (Table 4.1.3)

Table 4.1.3: The TM_1, TM_2 and TM_3 means fetal birth weight, crown rump length and head circumference in the control and alcohol treated groups (LAG, MAG and HAG)

Animal group	ETH treatment period	Fetal bwt in gm	CRL in cm	Head circumference in cm
control	none	6.27±0.033[a]	4.62±0.099[a]	3.73±0.1453[a]
LAG	TM1	4.18±0.117[b]	3.20±0.058[b]	2.37±0.033[b]
	TM2	5.17±0.067[b]	3.933±0.088[b]	3±0.00[b]
	TM3	6.10±0.058[a]	4.533±0.0333[a]	3.5±0.00[ab]
MAG	TM1	4.07±0.056[b]	3.083±0.0441[b]	2.03±0.033[bc]
	TM2	4.097±0.033[b]	3.6±0.0577[c]	2.67±0.088[bc]
	TM3	6.13±0.03[ab]	4.27±.0145[ab]	3.23±0.088[b]
HAG	TM1	3.63±0.088[c]	2.93±0.067[b]	1.633±0.133[c]
	TM2	4.87±0.088[c]	3.33±0.067[c]	2.33±0.033[c]
	TM3	5.8±0.12[c]	4.03±0.033[b]	3.2±.0100[b]

The means, followed by the same letter in a column are not statistically different at (P<0.05) using one way ANOVA, with Tukey test on post-hoc t-tests

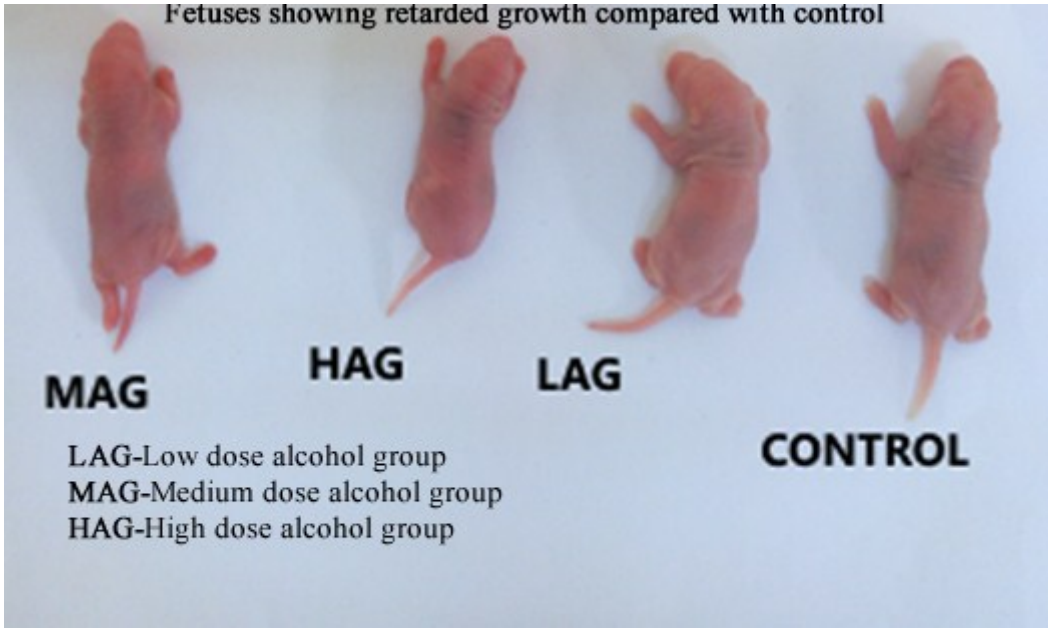


Figure 4.1.1: Image showing fetuses with retarded growth in the LAG, MAG and HAG against the control.

4.1.4 Influence of alcohol on congenital defects

When the number of congenital defects in the alcohol treated groups were compared against the control, a total of 36 fetuses were found to have congenital anomalies (Table 4.5). The external abnormalities observed were seen in the low, medium and high dose alcohol treated groups. There was observed defects of the vertebral column, limb and cranio-facial gross features. The commonest congenital defect that was observed was limb amelia that occurred in dose dependent manner.

Table 4.1.4: The congenital anomalies observed and their distribution across the study groups that is LAG, MAG & HAG against the control

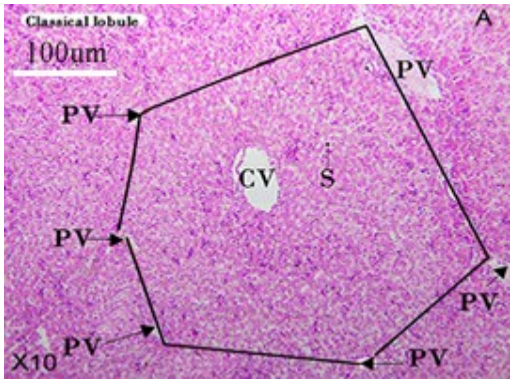
Type of congenital Anomaly observed	Number of fetuses	Distribution of fetuses with a anomalies across the study groups				% out of the total number of fetuses (90)
		HAG (%)	MAG (%)	LAG (%)	Control (%)	
Limb amelia	14	7(50%)	4(28.6%)	2(14.3%)	1(7.1%)	15.56%
Posterior limb hypoplasia	5	3(60%)	1(20%)	1(20%)	0(0%)	5.56%
Anterior limb hypoplasia	9	5(55.6%)	3(33.3%)	1(11.1%)	0(0%)	10%
Failure of closure of the vertebral column & neurotube	1	1(100)	0(0%)	0(0%)	0(0%)	1.1%
kyphosis	1	1(100%)	0(0%)	0(0%)	0(0%)	1.1%
Spina Bifida	2	1(50%)	1(50%)	0(0%)	0(0%)	2.2%
Cranio facial deformities	4	2(50%)	1(25%)	1(25%)	0(0%)	4.44%
Total	**36**	**20(55.6%)**	**10(27.8%)**	**5(13.9%)**	**1(2.8%)**	**40%**

4.2 Histomorphological findings

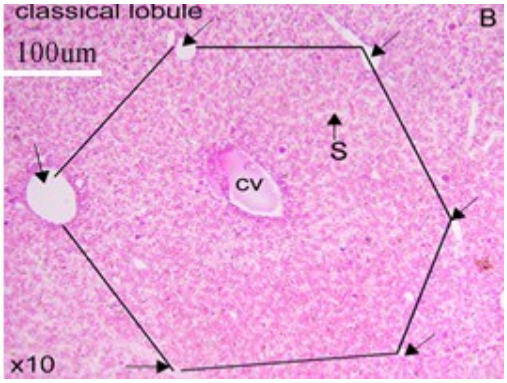
In decribing the histomorphological findings on the effects of prenatal exposure to varied doses of alcohol, paramentres including the general organization of the classical liver lobules, portal triad, haemopoetic tissue, the cellular organization and stromal tissue from the three alcohol groups (LAG, MAG and HAG) were compared against the control and reported per the trimesters of exposure as follows:-

4.2.1 The TM1, TM2 and TM3 comparative histomorphological features of the classical liver lobule between LAG, MAG and HAG against the control

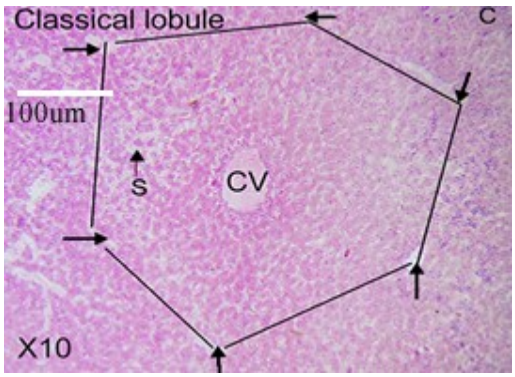
When alcohol was administered at TM_1, TM_2 and TM_3, it was observed that the classical liver lobule (hexagonal area enclosed in black in photomicrographs A-D), (were well developed in the control group (Photomicrograph A) and they had an even distribution of the hepatocytes. On the other hand, the classical liver lobules in the alcohol treated groups as per the photomicrographs B for the LAG, photomicrograph C for the MAG, and photomicrograph D for the HAG, shown sparse distribution of hepatocytes across the three trimesters in dose dependent manner. In addition varying degrees of sinusoidal dilation directly correlated to dose and time of alcohol exposure across the three trimesters. The central vein **(CV)** in the classical liver lobule of the alcohol treated groups (Photomicrograph B, C and D) were constricted in TM1 and TM3 except in TM_2 HAG and MAG which were constricted. In addition, there were aggregation of cells around the CV depicting presences of inflammation. (Figure 4.2.1, 4.2.2 and 4.2.3).



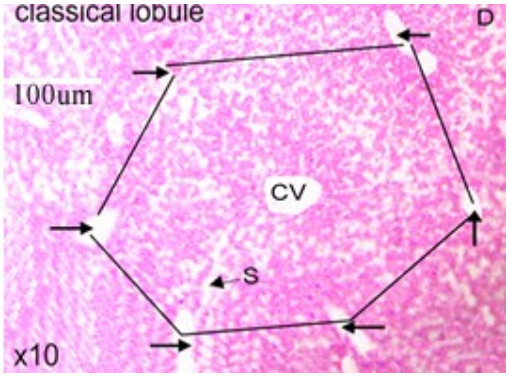
A: CONTROL- showing hexagonal classical liver lobule of the fetal liver (outlined in black continuous lines) stained with H & E with well distributed cells and normal sinusoids (**S),** central vein (C**V)** and portal vein **(PV)**.



B: LAG TM_1- showing large hexagonal classical liver lobule of the fetal liver stained with H & E with sparse distribution of cells, sinusoidal (**S**) dilatation and small central vein **(CV)** surrounded by inflammatory cells

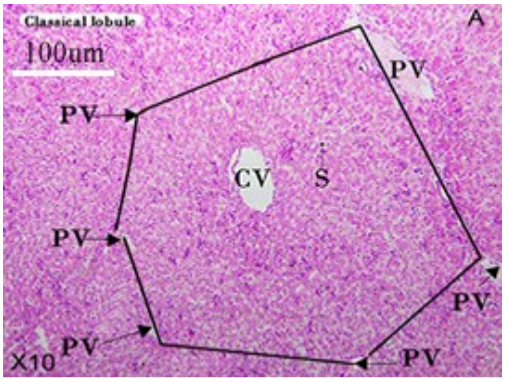


C: MAG TM_1- showing larger hexagonal classical liver lobule of the fetal liver stained with H & E with more sparse distribution of cells, further sinusoidal (**S**) dilatation, smaller central vein **(CV)** surrounded by more inflammatory cells).

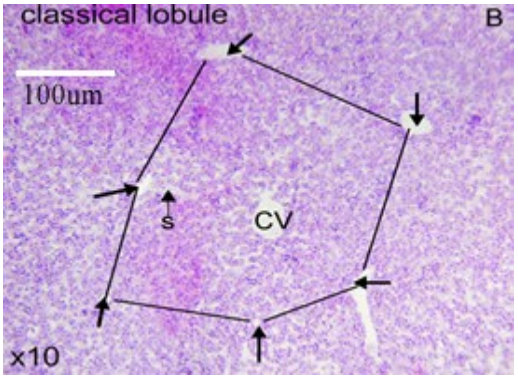


D: HAG TM_1- showing the largest hexagonal classical liver lobule of the fetal liver stained with H & E with the most sparsely distribution of cells, severely dilated sinusoids (**S**) and the smallest central vein (**CV)**

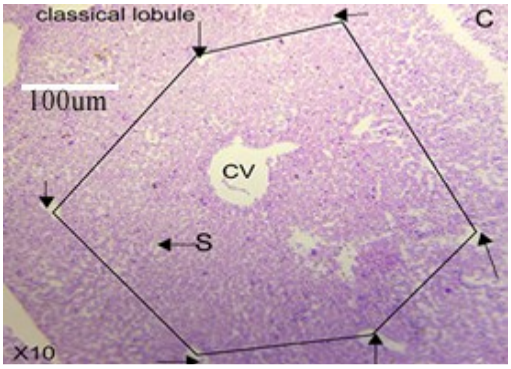
Figure 4.2.1: The TM_1 Photomicrograph of comparative histomorphological features of the classical liver lobule in:- (A) control; (B) LAG; (C) MAG; and (D) HAG



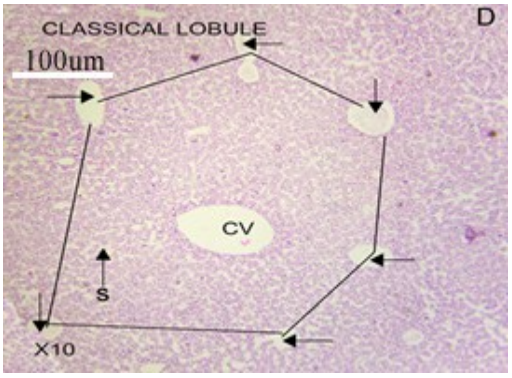
A: CONTROL- showing hexagonal classical liver lobule of the fetal liver (outlined in black continuous lines) stained with H & E with well distributed cells and normal sinusoids (**S),** central vein (C**V)** and portal vein **(PV)**.



B: LAG TM_2- showing hexagonal classical liver lobule of the fetal liver stained with H & E with sparse distribution of cells, sinusoidal (**S**) dilatation and small central vein **(CV)**

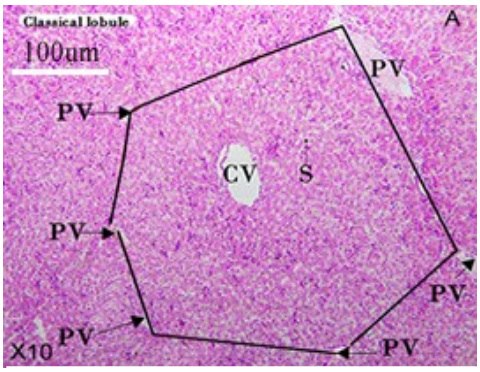


C: MAG TM_2- showing larger hexagonal classical liver lobule of the fetal liver stained with H & E with further sparse distribution of cells, further sinusoidal (**S**) dilatation, larger central vein **(CV)** surrounded by more inflammatory cells).

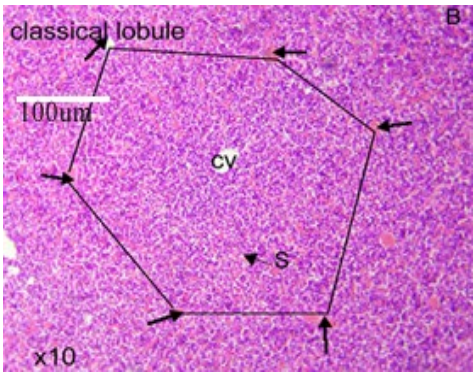


D: HAG TM_2- showing the largest hexagonal classical liver lobule of the fetal liver stained with H & E with the most sparsely distributed cells, severely dilated sinusoids (**S**) and the largest central vein (**CV)**

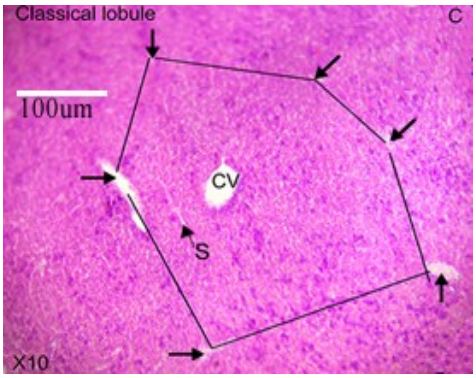
Figure 4.2.2: The TM_2 Photomicrograph of comparative histomorphological features of the classical liver lobule in:- (A) control; (B) LAG; (C) MAG; and (D) HAG



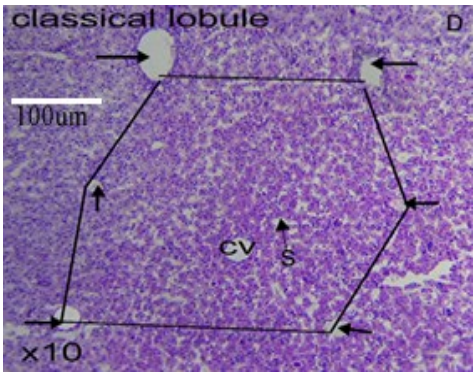
A: CONTROL- showing hexagonal classical liver lobule of the fetal liver (outlined in black continuous lines) stained with H & E with well distributed cells and normal sinusoids (**S),** central vein (C**V)** and portal vein **(PV)**.



B: LAG TM3- showing large hexagonal classical liver lobule of the fetal liver stained with H & E with normal cells, normal sinusoidal (**S**) and small central vein **(CV)**.



C: MAG TM_3- showing larger hexagonal classical liver lobule of the fetal liver stained with H & E with poor cellular distribution, mild sinusoidal (**S**) dilatation, smaller central vein **(CV)**.

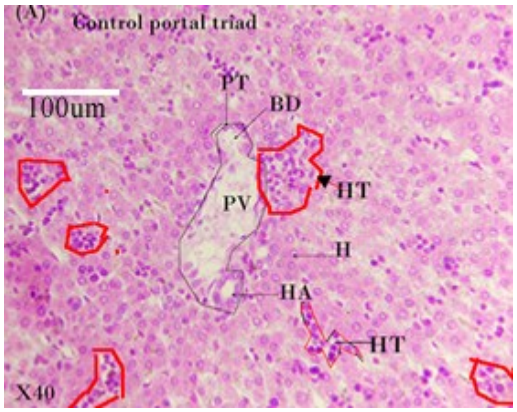


D: HAG TM_3- showing the largest hexagonal classical liver lobule of the fetal liver stained with H & E with poorer cellular distribution, severely dilated sinusoids (**S**) and the smallest central vein (**CV)**.

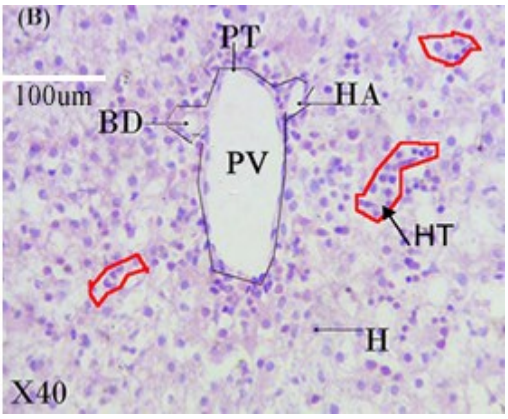
Figure 4.2.3: The TM_3 Photomicrograph of comparative histomorphological features of the classical liver lobule in:- (A) control; (B) LAG; (C) MAG; and (D) HAG

4.2.2 The TM1, TM2 and TM3 comparative histomorphological features of the hematopoietic tissue and portal triad between LAG, MAG and HAG against the control

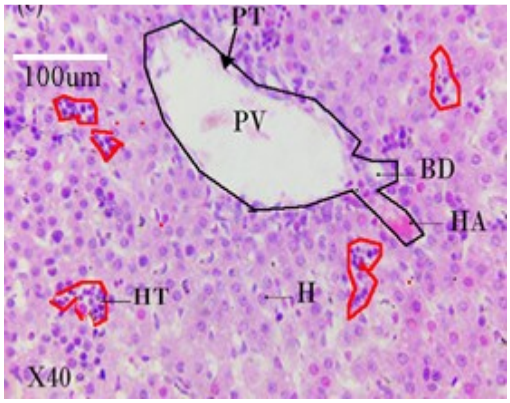
When alcohol was administered in TM_1, TM_2 and TM_3 across the study groups, the hematopoietic tissue around the portal triad (**PT**) aggregated in clusters and this aggregation varied according to the doses in that in the HAG (photomicrograph D), the hematopoietic tissues (**HT**) were less aggregated and sparsely distributed when compared to the control (photomicrograph A). The hematopoietic cells in the control group were densely aggregated and uniformly distributed (Figure 4.2.4, 4.2.5 and 4.2.6). The hematopoietic cells around the portal triad were sparsely distributed across the alcohol treated groups with the HAG depicting the lowest distribution followed by the MAG and the LAG possiblely due to the diminished haematopoietic fuction of the fetal liver following toxic effects of alcohol on the hepatocytes. Further it can be observed that the portal triads in the control group was well developed with its clear structure including the hepatic artery (**HA**), the bile duct (**BD**) and the portal vein (**PV**) as seen in Photomicrograph A when compared with photomicrographs B for the LAG, photomicrograph C for the MAG, and photomicrograph D for the HAG across the three trimesters. However the portal vein (**PV**) was massively dilated in the HAG, followed by the MAG and least in the LAG across the three trimesters. On the other hand the hepatic artery (**HA**) appears slightly larger in size with thicker walls as compared to the bile duct (**BD**) whose walls are thinner. Both hepatic artery and the bile duct were not shown to have any difference from the control group across the trimesters. Fatty changes were observed in TM_1 HAG unlike other alcohol treated groups (Figure 4.2.4, 4.2.5 and 4.2.6)



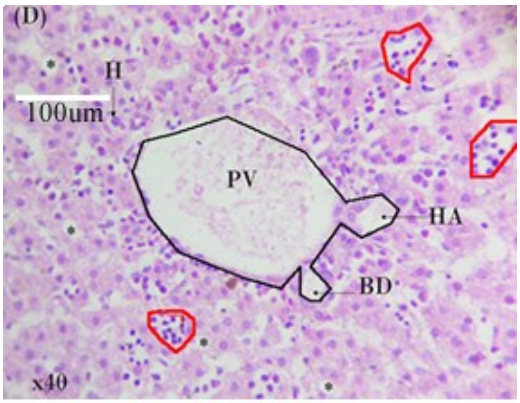
A: control- showing many aggregates of fetal hematopoietic tissue (**HT)** as outlined in red around the portal triad (**PT)** stained with H & E ,normal thick walled hepatic artery (**HA**), thin walled portal vein (**PV**) and thin walled bile duct (**BD**).



B: LAG TM_1. showing few aggregates of hematopoietic tissue (**HT)** stained with H & E, thin walled dilated portal vein **(PV**), normal thick walled hepatic artery (**HA**) and thin walled bile duct (**BD**).

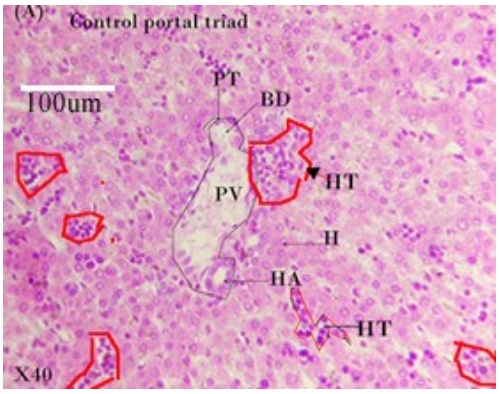


C: MAG TM_1- showing fewer aggregated hematopoietic tissue (**HT)** as outlined in red and further dilatation of the portal vein (**PV**).

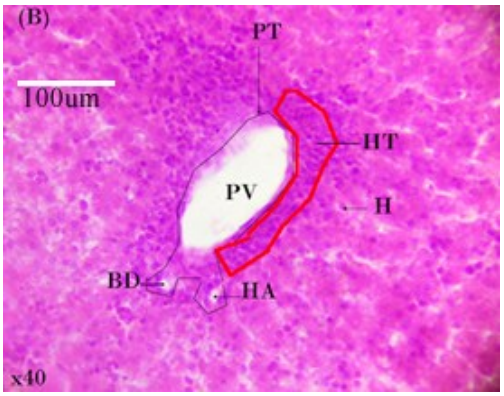


D: HAG TM_1. showing fewest aggregates of hematopoietic tissue (**HT)** as outlined in red and the most dilated portal vein (**PV),** asterisk(*) indicates fatty change.

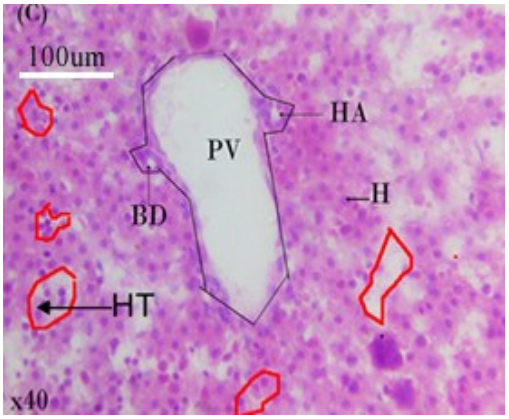
Figure 4.2.4: The TM1 photomicrograph of comparative histomorphological features of the hematopoietic tissue around the portal triad in:- (A) control; (B) LAG; (C) MAG; and (D) HAG



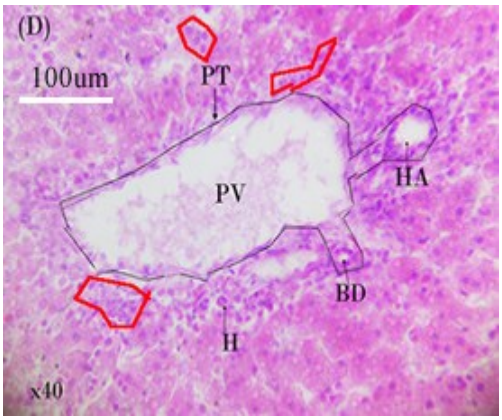
A: control- showing many aggregates of fetal hematopoietic tissue (**HT)** as outlined in red around the portal triad (**PT)** stained with H & E ,normal thick walled hepatic artery (**HA**), thin walled portal vein (**PV**) and thin walled bile duct (**BD**).



B: LAG TM_2 - showing few aggregated fetal hematopoietic tissue (**HT)** as outlined in red, around the portal triad (**PT)** stained with H & E, dilated thin walled dilated portal vein **(PV**), normal thick walled hepatic artery (**HA**) and thin walled bile duct (**BD**).

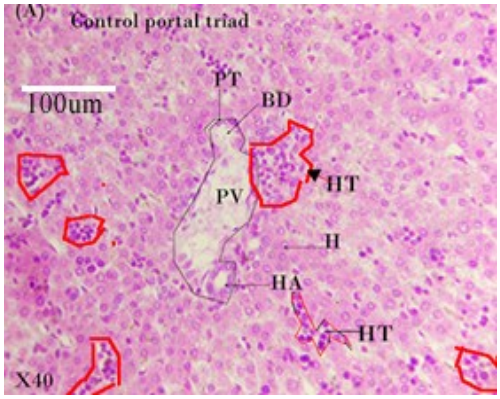


C: MAG TM2- showing fewer aggregated fetal hematopoietic tissue (**HT)** as outlined in red, around the portal triad (**PT)** and further dilatation of the portal vein (**PV**).

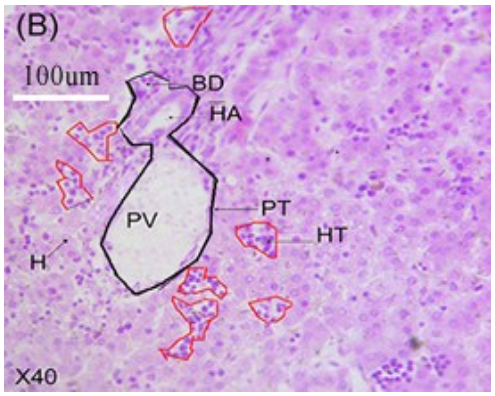


D: HAG TM_2- showing fewest aggregate of fetal hematopoietic tissue (**HT)** as outlined in red, around the portal triad (**PT)** and the most dilated portal vein (**PV).**

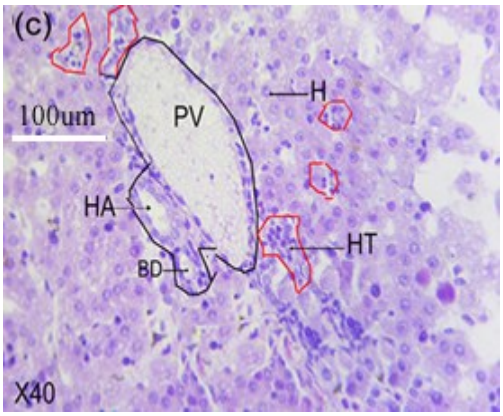
Figure 4.2.5: The TM_2 photomicrograph of comparative histomorphological features of the hematopoietic tissue around the portal triad in:- (A) control; (B) LAG; (C) MAG; and (D) HAG



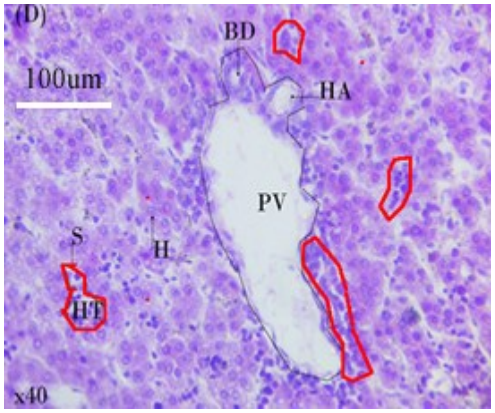
A: control- showing many aggregates of fetal hematopoietic tissue (**HT)** as outlined in red around the portal triad (**PT)** stained with H & E ,normal thick walled hepatic artery (**HA**), thin walled portal vein (**PV**) and thin walled bile duct (**BD**).



B: LAG TM_3 - showing many aggregated fetal hematopoietic tissue (**HT)** when stained with H & E, thin walled dilated portal vein **(PV**), thick walled hepatic artery (**HA**) and thin walled bile duct (**BD**).



C: MAG TM_3- showing few aggregated fetal hematopoietic tissue (**HT)** as outlined in red, around the portal triad (**PT)** and further dilatation of the portal vein (**PV**).

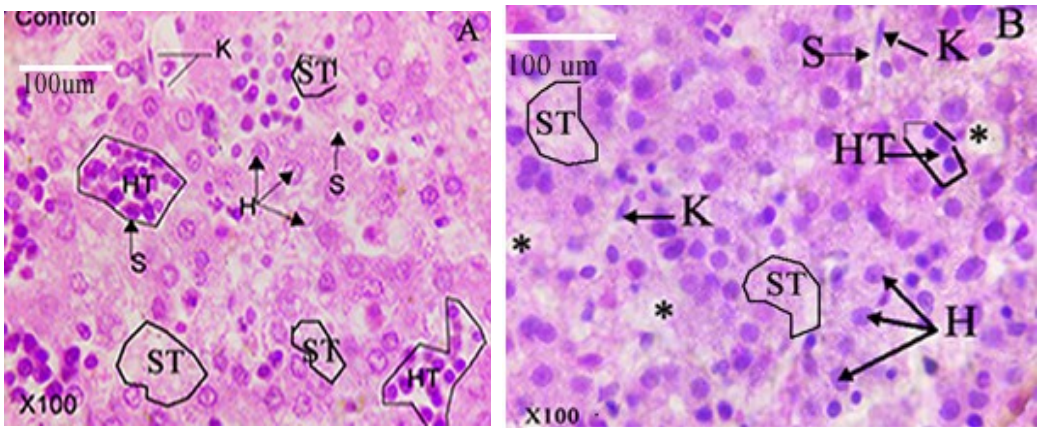


D: HAG TM_3. showing fewer aggregated fetal hematopoietic tissue (**HT)** as outlined in red and the most dilated portal vein (**PV**)**.**

Figure 4.2.6: The TM_3 photomicrograph of comparative histomorphological features of the hematopoietic tissue around the portal triad in:- (A) control; (B) LAG; (C) MAG; and (D) HAG

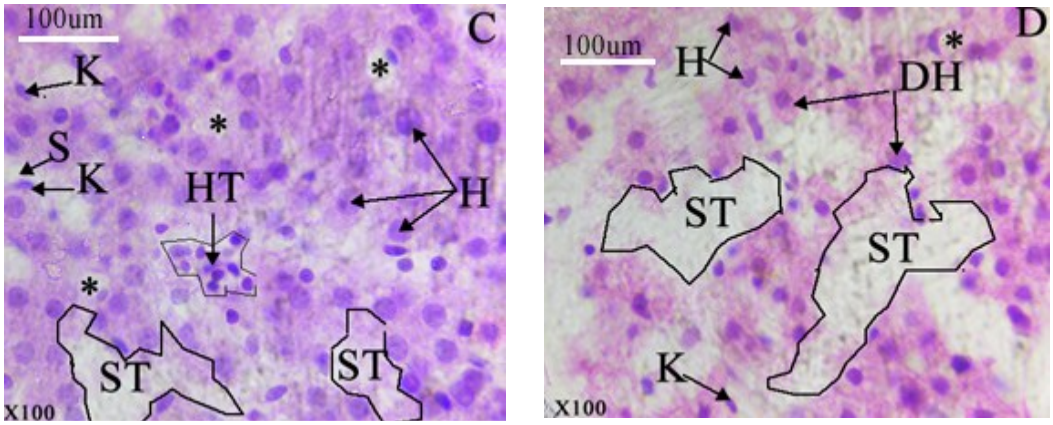
4.2.3 The TM_1, TM_2 and TM_3 comparative histomorphological features of the liver parenchyma of the LAG, MAG and HAG groups against the control

When alcohol was administered in TM_1, TM_2 and TM_3 across the study groups, the liver parenchyma was seen to vary according to the doses and time of alcohol exposure. Further it was observed that the liver parenchyma in the control was well developed with its clear structure including the hepatocytes (**H**), the kupffer cells (**K**), hematopoietic tissue (**HT**) and the stromal tissues (**ST**) as seen in Photomicrograph A when compared to photomicrographs B for the LAG, photomicrograph C for the MAG, and photomicrograph D for the HAG. The stromal tissue was not uniform across the alcohol treated groups in that the HAG shown obvious stromal tissue spaces followed by the MAG and the LAG across the three trimesters. Similarly, severe fatty changes were observed in the HAG, followed by the MAG and LAG across the trimesters. Further the hepatocytes appeared similar in morphology in the control group while in the alcohol treated groups the hepatocytes were varying in morphology in dose and time dependent manner. On the other hand, the kupffer cells were conspicuous in the alcohol treated groups as compared to the control groups (Figure 4.2.7, 4.2.8 and 4.2.9).



A: control-: showing normal cellular component of the fetal liver stained with H & E, uniform Hepatocytes **(H)**, less conspicuous kupffer cells **(K)** within the sinusoids (**S**), uniform stromal tissue **(ST)** and hematopoietic tissue **(HT)**.

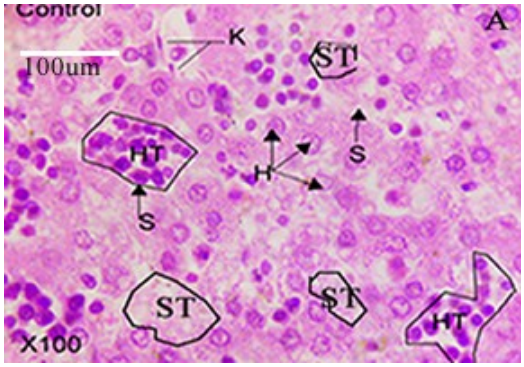
B: LAG TM_1-: showing different sizes of hepatocytes **(H)**, conspicuous kupffer cells **(K)** within the sinusoids (**S**) and disorganized stromal tissue **(ST)** with fatty changes [*(**Asterisk**)]



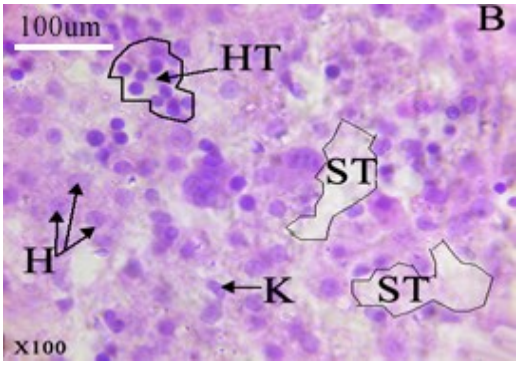
C: MAG TM_1-: showing different sizes of hepatocytes **(H)**, more conspicuous kupffer cells **(K)** within the sinusoids (**S**), further disorganization of stromal tissue **(ST)** with more fatty changes [*(**Asterisk**)] and fewer hematopoietic tissue **(HT)**.

B: HAG TM_1-: showing degenerating hepatocytes **(DH)**, conspicuous kupffer cells **(K)** within the sinusoids (**S**), highly disorganized stromal tissue **(ST)** with fatty changes [*(**Asterisk**)] and fewer hematopoietic tissue **(HT)**.

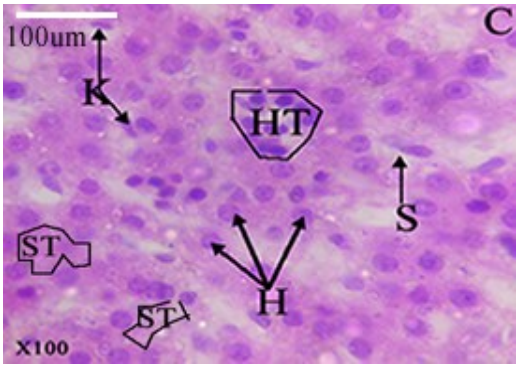
Figure 4.2.7: The TM_1 photomicrograph of histomorphological features of the liver parenchyma in:- (A) control; (B) LAG; (C) MAG; and (D) HAG



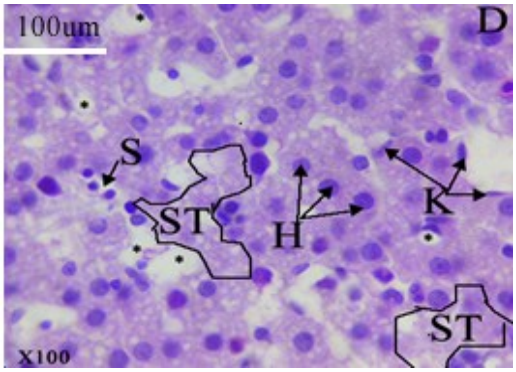
A: control-: showing normal cellular component of the fetal liver stained with H & E, uniform Hepatocytes **(H)**, less conspicuous kupffer cells **(K)** within the sinusoids (**S**), uniform stromal tissue **(ST)** and hematopoietic tissue **(HT)**.



B: LAG TM_2.: showing different sizes of hepatocytes **(H)**, conspicuous kupffer cells **(K)** within the sinusoids (**S**) and less disorganized stromal tissue **(ST).**

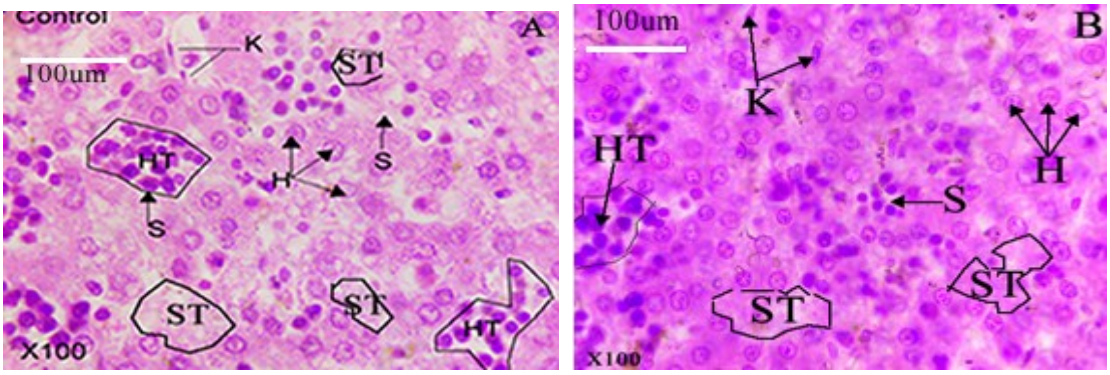


C: MAG TM_2 - showing different sizes of hepatocytes **(H)**, more conspicuous kupffer cells **(K)** within the sinusoids (**S**), and more disorganization of stromal tissue **(ST)**



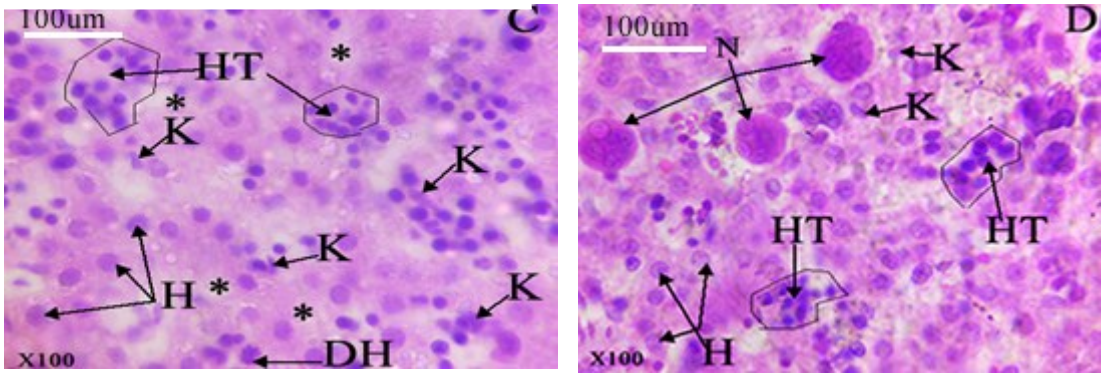
D: HAG TM_2 - showing different sizes of hepatocytes **(DH)**, more conspicuous kupffer cells **(K)** within the sinusoids (**S**), highly disorganized stromal tissue **(ST)** with fatty changes [*(**Asterisk**)]

Figure 4.2.8: TM_2 photomicrograph of histomorphological features of the liver parenchyma in:- (A) control; (B) LAG; (C) MAG; and (D) HAG



A: control-: showing normal cellular component of the fetal liver stained with H & E, uniform Hepatocytes **(H)**, less conspicuous kupffer cells **(K)** within the sinusoids (**S**), uniform stromal tissue **(ST)** and hematopoietic tissue **(HT)**.

B: LAG TM_3.: showing uniform sizes of hepatocytes **(H)**, less conspicuous kupffer cells **(K)** within the sinusoids (**S**), normal stromal tissue **(ST)** with no fatty changes.



C: MAG TM_3 -showing different sizes of hepatocytes **(H)** and degenerating hepatocytes **(DH)**, conspicuous kupffer cells **(K)** within the sinusoids (**S**) and fatty changes (*).

D: HAG TM_3 - showing different sizes of hepatocytes **(H),** conspicuous kupffer cells **(K)** within the sinusoids (**S**) and N-Neutrophils.

Figure 4.2.9: The TM_3 photomicrograph of histomorphological features of the liver parenchyma in:- (A) control; (B) LAG; (C) MAG; and (D) HAG

4.3 The histostereological findings on the fetal liver

4.3.1 The gross appearance and weight of the fetal liver

The gross appearance of the fetal liver from the alcohol treated groups particularly at HAG and MAG appeared larger in size, edematous with brownish color, rough in texture when compared to the control which appeared smaller in size, pink in color with smooth texture. At the same time the mean fetal liver weights for all alcohol treated groups across all trimesters showed significant increase (P=0.001) except for the LAG and MAG at trimester three (LAG -TM_3, MAG –TM_3) (Table 4.3.1).

Table 4.3.1: **The TM_1, TM_2 and TM_3 inter and intra-group mean fetal liver weight in the control and alcohol treated groups (LAG, MAG and HAG).**

		TRIMESTERS			
Parameter	**Treatment group**	**Control**	**TM_1**	**TM_2**	**TM_3**
Mean fetal liver weight in gms	HAG	0.453±0.029[a]	0.897±0.015[d]	0.730±0.015[c]	0.60±0.012[b]
	MAG	0.453±0.029[a]	0.810±0.038[b]	0.690±0.021[b]	0.550±0.029[a]
	LAG	0.453±0.029[a]	0.773±0.015[b]	0.647±0.024[c]	0.450±0.029[a]

*KEY: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA with Tukey test on post-hoc t-tests. * indicates that differences are statistically significant (p<0.05).*

In addition, the mean fetal liver weight was high in the HAG TM1 at 0.897±0.015gm followed by TM2 at 0.730±0.015gm and lastly in TM3 at 0.60±0.012gm. A statistical significant difference (p=0.001) was observed when they were compared with the control which was at 0.453±0.029gm. Similarly, a statistical significant difference (p=0.001) was also observed in TM_1 and TM_2 MAG unlike TM_3 MAG and LAG in the same trimester (Table 4.3.1).

4.3.2 The influence of alcohol on the total fetal liver volume

The reference and calculated mean total fetal liver volume as determined by use of water immersion method (WIM)which is the goal standard method and cavalieri method was found to depict a direct dose response relationship in that when the dose of exposure to alcohol increased, the mean total liver volume had a corresponding increase and *vice versa*, (Table 4.3.2). On the other hand, when the total liver volume was compared with the time of exposure, it depicted a direct response relationship to the time of alcohol exposure in that when alcohol treatment was administered at trimesters one, two and three (TM_1, TM_2 TM_3), the liver volumes increased directly with the time of exposure. For instance when the alcohol treatment was done at TM_1 the highest mean increase in the total liver volume was noticed in TM_1 at 0.766±0.006, followed by TM_2 at 0.666±0.010 mm^3 and lastly TM_3 at 0.526±0.011mm^3. All the intra and intergroup comparisons were also found to be statistically significant ($p<0.05$) when compared with the control group. (Table 4.3.2).

Table 4.3.2: The TM_1, TM_2 and TM_3 comparative reference, calculated total mean fetal liver volume using WIM and cavalieri method in the control and alcohol treated groups (LAG, MAG and HAG).

Animal group	Alcohol treatment period	WIM volume in mm^3	Cavalieri volume in mm^3
control	none	0.548±0.007[a]	0.511±0.007 [a]
Low alcohol group(LAG)	TM1	0.816±0.201[c]	0.766±0.006c
	TM2	0.706±0.0103[b]	0.666±0.010 [b]
	TM3	0.551±0.012 [a]	0.526±0.011 [a]
Medium alcohol group(MAG)	TM1	0.858±0.008[d]	0.796±0.013 [d]
	TM2	0.742±0.009 [c]	0.708±0.011 [c]
	TM3	0.608±0.006 [b]	0.572±0.008 [b]
High alcohol group(HAG)	TM1	0.930±0.010 [d]	0.882±0.008 [d]
	TM2	0.787±0.006 [c]	0.757±0.005 [c]
	TM3	0.652±0.010 [b]	0.612±0.007 [b]

KEY: The means, followed by the same letter in a column are not statistically different at ($P<0.05$) using one way ANOVA with Tukey test on post-hoc t-tests.

4.3.3 The comparative volume densities of the liver hepatocytes, sinusoids, portal vein and central vein in the LAG, MAG and HAG against the control

When the mean volume densities of the fetal liver hepatocytes, sinusoids, portal triad and central vein in the first trimester at varying doses was compared with the control it was observed that the fetal liver volume densities were significantly increased ($p< 0.05$) in dose dependent manner when administered at TM_1 and TM2 except in TM3 which was found not to be statistically significant ($P>0.05$). The highest mean volume density were observed in the HAG followed by MAG and lastly the LAG when compared to the control. Further, the Post hoc results for the mean volume densities also revealed that the MAG was significantly different from HAG, but not significantly different from the LAG (Table 4.3.3).

Table 4.3.3: The comparative mean volume densities of the liver hepatocytes, sinusoids, portal vein and central vein in the LAG, MAG & HAG against the control when administered at TM_1, TM_2 and TM_3.

Animal group	ETH treatment period	Mean vol. density of hepatocytes (mm^3)	Mean vol. density of sinusoids(mm^3)	Mean vol. density of portal triad(mm^3)	Mean vol. density of central vein(mm^3)
control	none	0.2811±0.0039 [a]	0.1278±0.0018[a]	0.7667±0.0010[a]	0.0256±0.0004 [a]
LAG	TM1	0.4210±0.0036 [b]	0.1914±0.0016[b]	0.1148±0.000 [b]	0.0382±0.0003 [b]
	TM2	0.3660±0.0054 [b]	0.1663±0.0025[b]	0.998±0.0015 [b]	0.0331±0.0005 [b]
	TM3	0.2890±0.0062 [a]	0.1314±0.0028[a]	0.7883±0.001 [a]	0.0263 ±0.0006 [a]
MAG	TM1	0.4401±0.0085 [b]	0.2000±0.0038[b]	0.1200±0.002 [b]	0.4000±0.0008 [b]
	TM2	0.3892±0.006[c]	0.1769±0.0028 [c]	0.1061±0.001 [c]	0.353±0.0006 [c]
	TM3	0.3166±0.0053 [b]	0.1439±0.0023[b]	0.863±0.0014 [b]	0.0288±0.0005 [b]
HAG	TM1	0.4852±0.0044 [c]	0.2206±0.0020 [c]	0.1323±0.001 [c]	0.4411±0.0004 [c]
	TM2	0.4161±0.0029 [d]	0.1892±0.0013[d]	0.1135±0.000 [d]	0.378±0.0003[d]
	TM3	0.3367±0.0040 [c]	0.1531±0.0018 [c]	0.918±0.0011 [c]	0.0306 ±0.0004 [c]

KEY: The means, followed by the same letter in a column are not statistically different at ($P<0.05$) using one way ANOVA with Tukey test on post-hoc t-tests.

4.4 The fetal liver teratogenicity based on alcohol dose and time of administration

The current study has established that teratogenic effects of *in-utero* administration of alcohol had a direct correlation with the dose and time of alcohol exposure as was shown when the intergroup and intragroup comparisons were done. It was established that when alcohol was administered in the first, second and third trimester at low (2g/Kgbwt), medium (3.5g/kgbwt) and high (5g/kgbwt) alcohol doses ,both histomorphological as well as histostereological findings were statistically significant ($p<0.05$) as compared to the control. This effects were illustrated in the histomorphological and quantitative results.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMEDATION

5.1 DISCUSSION

5.1.1 The maternal and fetal pregnancy outcomes

In this study, the maternal pregnancy outcomes included devoured endometrial glands, placental weights and mean maternal weights gains throughout the gestational period, while the fetal pregnancy developmental outcomes included fetal weights, CRL, head circumference and the observed gross congenital abnormalities.

The findings of this study shown that there was a significant reduction in mean maternal weight gain ($p<0.05$) in the alcohol treated groups as compared to the control in dose dependent manner (Table 4.1.1). Similar effects of adverse effects were also described upon administration of ethanol to expectant rats (Fiorentino *et al*., 2017). This liver teratogen were found to cause oxidative stress to the maternal liver cells which further interfered with metabolism function of the liver. The interference of the maternal liver metabolic function was shown to cause maternal malnutrition which lead to low maternal body weight gain which could be the case in the current study. In another study, low maternal weight gain was associated with the negative effects of alcohol on the function of different body organs following cellular oxidative stress (Carter *et al*., 2017). This contradicts another study in which moderate alcohol consumption was shown to cause weight gain with subsequent obesity. This maternal weight gain was associated with the high alcohol calories and the time of consumption (Wang *et al*., 2011)

Further, embryonic resorption which is defined as prenatal death followed by subsequent degeneration of the conceptus (Tine *et al*., 2004), was found to be statistically significant in the high alcohol group ($p< 0.05$) when compared to the control group (Table 4.1.2). The increased frequency of resorption in the first trimester indicated the adverse effects of *in-utero* alcohol exposure on fetal viability.

This concurs with a study which was done by Mishra *et al* (2015), who also found time and dose dependent embryo resorption upon alcohol administration to mouse.

The current study was in accordance with a study that showed a reduction in fetal body weight, which was associated with placental oxidative stress that consequently interfered with placental villi formation (Bosco & Diaz, 2012; Flores *et al*., 2014) which could also be the case in the current study. It also concurs with another study in which prenatal maternal under nutrition following alcohol consumption lead to an increase in embryo resorption (Musumeci *et al*, 2015).

In this study, low mean placental weight was recorded in the alcohol treated groups (table 4.1.2) as compared to the control group when alcohol was administered in different trimesters revealing the effects of alcohol on the placenta. This study was in agreement with a study in which ethanol caused oxidative stress that interfered with placental villi formation and placental blood flow through vasoconstriction. Consequently this leads to local placental ischemia, infarction and reduced fetal growth (Bosco & Diaz, 2012). Similar effects were also observed by Bosco *et al* (2012), who shown that intrauterine alcohol exposure reduced placental glucose utilization, lead to vascular abnormalities and reduced trophoblastic proliferation thereby placental weight deficits (Lui *et al*., 2014). Burd *et al* (2014). It was further found that, alcohol induced dose dependent vasoconstriction which affected both placental and fetal weights as in the current study.

On the other hand, the mean fetal birth weight and length at gestational day 20 were found to be significantly reduced ($p=0.001$) in the high, medium and low dose alcohol groups when alcohol was administered in the first, second and third trimester as compared to the control group (Table 4.1.3). The varied fetal body weight and fetal body length observed in the current study concurred with a study which was done by Ramsay (2010) who correlated fetal alcohol spectrum disorder with level and nature of alcohol exposure. He further observed that the clinical severity upon *in-utero* alcohol exposure depended on time and the dose of alcohol. Similar effects were also described by Anulika *et al* (2018) who associated teratogenic effects of alcohol to litter size and fetal birth weight. However, it has been demonstrated that

newborns who are born to alcoholic mothers are of small size (weight and or length) for a given gestational age possibly because alcohol increases the production of prostaglandins which consequently increase cAMP activity thus reducing cell division (Randall *et al*., 1987). In addition, alcohol has been shown to interfere with nutritional supply to the fetal-placental unit consequently disrupting the absorption, transport and storage of iron to the developing fetus (Nakhoul *et al*., 2017; Nykjaer *et al*., 2014; Sebastiani *et al*., 2018) which might have been the case in the current study. Another study which was done by Ertem *et al,* (2006) contradicted the current study where no significant difference in fetal birth weight was observed upon prenatal exposure to alcohol and this may have been due to the methodology used. In their study, the pregnant rats were fed on isocaloric modified liquid diet for a period of one week without alcohol. After a week, they were administered alcohol at different concentrations after which the rats were sacrificed (Ertem *et al.,* 2006.).

The current study also concurred with a study which was done by Georges *et al,* (2017) who observed a reduction in fetal head circumference. This reduction was associated with indirect alcohol injurious effects on fetal placental unit as well as direct fetal toxicity from alcohol and its toxic metabolite (acetaldehyde). Therefore, alcohol directly or indirectly has been shown to compromises the nutritional status to the developing fetus. In addition, it has been shown to alter the gene that are involved in the embryological development of the skeletal system along with the GIT viscera's including the liver (Ramsay, 2010; Sebastiani *et al*., 2018). This confirms that alcohol has detrimental effect on fetal growth and development and could be the case in the current study.

The present study has also demonstrated that chronic alcohol consumption is teratogenic as shown by the congenital defects observed. The defects involved limbs, vertebral column and craniofacial defects among others. They were found to be more in the high and medium alcohol groups when compared to the control group probably due to the prolonged exposure and dose of the alcohol (Table 4.1.4). This results agrees with other studies when alcohol was administered before and during pregnancy (Bosco & Diaz, 2012; Gabriel *et al*., 1998; Lui *et al*., 2014). Alcohol has been shown to cause this defects through several mechanisms including interference

with growth factors, apoptosis of fetal cells, alteration of glucose transport and uptake by the cells as well as alteration of homeobox genes among others (Zhou *et al.*, 2011)

5.1.2 Histomorphology of the fetal liver

Alcohol can cause liver damage in the form of fatty liver, fibrosis and liver cirrhosis (Lackner & Tiniakos, 2019). Results from the present study agree with previous studies that show that alcohol intake exerts harmful effects on the liver. Moreover, the amount of alcohol consumed correlates with severity of the damage of liver. In the current study, it was observed that liver of rats that received high doses (5g/kg/bwt) of alcohol showed severe damage in the liver tissues compared to those that received 3.5g/kg/bwt and 2g/kg/bwt of alcohol (Figure 4.2.1, 4.2.2 & 4.2.3). In addition, administration of alcohol from the first trimester showed more injurious effects as compared to the 2^{nd} and 3^{rd} trimester. The histomorphological changes observed in the current study which includes fatty changes, degeneration of hepatocytes and inflammatory changes among others were prominent in the livers of rats fed on high dose alcohol throughout the gestation. This agrees with a study which was done by Saeed (2016) that showed important changes in hepatic trabecular structure and increased hepatocytes with cytoplasmic vacuoles. This study is in accordance with the previous studies in that administration of low, medium and high doses of alcohol affected rat fetal liver histomorphology represented by cellular disorganization, conspicuous kupffer cells, dilated sinusoids, fatty changes, hepatocyte degeneration among others.

The degeneration of hepatocytes observed in the liver of fetuses exposed to alcohol prenatally could be associated with oxidative stress which induces fat deposition in the hepatocyte cytoplasm (Sakhuja, 2014). A study done by Stainer & Lang (2017) documented that the byproducts of alcohol metabolism (oxygen free radicles) react with unsaturated membrane lipids, initiating self-perpetuated peroxidase process (Steiner & Lang, 2017). This reaction produces loss of membrane function and ultimately cell death (Yang et al., 2019). A study by Chen *et al* 2016 showed that liver cells contains glutathione (antioxidant) which is a natural scavenger involved in

elimination of the oxygen free radicles (Chen *et al*., 2016). Unfortunately, when alcohol depletes this antioxidants, oxidative stress occurs. In this study, alcohol administered at different gestational period could have yielded free oxygen radicle during its metabolism in the maternal liver. This oxygen free radicles may have crossed the maternal placental barrier in to the developing fetal liver and this might have depleted the naturally occurring antioxidants. This consequently might have led to the injury of hepatocytes manifested by degenerating hepatocytes. In addition, alcohol and its metabolites has been shown to cause reduction in the activity of alcohol dehydrogenase and cytochrome p-450 which are hormones involved in liver alcohol metabolism (Mukherjee, 1999; Das & Lucia, 2017). As a result of reduced activity of this enzymes, alcohol accumulates in the liver which subsequently leads to more liver cell death as well as dilatation of the sinusoids due to its toxic effects (Das & Lucia, 2017) which could have been the case in the present study. Alcohol has also been found to cause leaky gut in which endotoxins from the gut end up causing liver injury as well as activating the Kupffer cells (liver resident macrophages), that play crucial roles in the inflammatory responses of liver (Suraweera *et al*., 2015; Zeng *et al*., 2016). This could have been the cause of the conspicuous kupffer cells which were observed in the alcohol treated groups.

Other studies have revealed that alcohol exposure impairs with hematopoietic precursor cell proliferation, more so the hepatic stem cells (Di-Rocco et al., 2019). Therefore this might have been the case in the alcohol treated groups where the hematopoietic tissue was reduced. Alcohol causes this by suppressing the normal hematopoiesis process as well as causing dysfunctional hematopoiesis of the hepatic stem cells (Rennert *et al.,* 2012). This consequently interferes with the key role of the fetal liver which could be the cause of the few clusters of hematopoietic cells among the treatment groups in this study (Shi *et al,* 2017).

5.1.3 Histostereology of the fetal liver

In the current study, alcohol triggered hepatomegaly in the albino fetuses when exposed prenatally at different gestational period and doses. This is because the liver, which is the primary site for alcohol metabolism is more prone to alcohol-related

injury (Haseba & Ohno, 2010). For this reason, alcohol intake puts a great metabolic overload on the hepatocytes (Pal & Ray, 2016), and the response to this overload could lead to increased liver weight. Further, the liver mostly prefers alcohol as an energy source and due to this is stops the use of fats which accumulates in the hepatocytes leading to fatty liver (Yang *et al*., 2019) which have been the cause of hepatomegaly in the current study. In addition, the hydrogen produced as a by-product of alcohol metabolism is converted to more fat for the synthesis of cholesterol and lipoproteins which further accumulates as fat droplets in the liver (Yang *et al*., 2019; Manzo & Saavedra,2010). All this could be responsible for the hepatomegaly observed in the HAG, MAG and LAG when alcohol was administered in the first, second and third trimester.

On the other hand, the rats that were exposed to alcohol in the entire gestational period presented with increased liver weight than those that received alcohol for a short duration (Table 4.3.1). This difference may be associated with the toxic effects of alcohol and its toxic byproduct (acetaldehyde) which have been shown to cause varying degree of cytoarchitectural distortion of the hepatocytes. The current study concurs with a study which was done to establish the effects of alcohol on cytoskeletal of the fetal liver where it was shown that alcohol distorted liver proteins which ended up causing hepatomegaly in rats (Rosa-toledo *et al*., 2015).

Following administration of varying doses of alcohol, there was evident increase in the total liver volume and volume densities among the alcohol treated groups as compared to control (Table 4.3.2 & 4.3.3). The liver volume were significantly increased ($p=0.001$) in the high, medium and low alcohol treatment groups that received alcohol in the first and second trimester as compared to the control group. This concurs with a study in which rats were administered 5% ethanol prenatally and an increase in the fetal liver hepatocyte cytoplasm as well as the nucleus volume were established (Renau-piqueras *et al*., 1985). A dose dependent increase in the liver volume was also reported following administration of fatty diet and alcohol concurrently (Kiki *et al*., 2007). In the present study, the increase in liver volume might have been due to the vascular dilatation, inflammation of the liver following alcohol injurious effects, recruitment of inflammatory cells, and liver fatty change

upon alcohol administration. A study done by Altunkaynak, *et al*, (2017), established an increase in total liver parenchyma upon administration of alcohol and a fat diet to rats which concurs with the current study in which alcohol caused increased liver volume in the albino fetuses. The presumed reason for hepatic volume increase would be the ballooning of hepatocytes along with increased vascular dilatation.

In this study, the fetal mean volume densities of the hepatocytes, sinusoids, portal triad and the central vein were shown to increase in a time and dose dependent manner in the treatment groups when compared to the control group. This agrees with a study which was done by Gabriela et al, (1998) which shown interferences in fetal development following prenatal alcohol consumption. This developmental interferences not only occurred directly through adverse effects exerted by alcohol upon crossing the blood placenta barrier into the fetal circulation, but also indirectly, by disturbing the functions and interactions of maternal and fetal hormones (Gabriela *et al*., 1998). Alcohol exposure was shown to impair the functioning of hypothalamic-pituitary-thyroid axis, which regulates the metabolism of almost all tissues therefore promoting histo-cytoarchitecture distortion of the liver parenchyma involved in metabolic processes (Gabriela *et al*., 1998). This parenchymal distortion may consequently increase the liver weight which could have been the case in the current study. *In-utero* exposure to alcohol increased both liver weight and total protein content in the Golgi complex and this altered its morphological and functional properties (Azorín *et al*., 2004). This was based on the fact that alcohol perturbed the developing fetal liver by inducing retention of proteins in hepatocytes thereby resulting into alteration of the liver histo-cytoarchitare (Azorín *et al*., 2004).

5.1.4 The effects of alcohol in relation to dose and time of exposure

The current study established that alcohol teratogenic effects following *in-utero* exposure were time and dose dependent as shown by the histomorphological and histostereological effects, with the most vulnerable period being the first trimester at a dose of 5g/kgbwt. The current findings concurs with a study which was done by Kiki *et al*, 2007 who administered alcohol to rats. They showed an increase in the liver volume in time and dose dependent manner (Kiki *et al*., 2007). The current

study was also in accordance with a study which established an increase in the fetal liver hepatocyte cytoplasm in addition to the nucleus volume, in dose and time dependent manner (Renau-piqueras *et al*., 1985).

5.2 CONCLUSION

In conclusion, the current study has established that:

i. *In-utero* exposure to alcohol caused intrauterine growth retardation which was manifested by the reduction in the fetal birth weight, CRL and head circumference among others.
ii. Prenatal alcohol consumption lead to the distortion of the normal liver morphology which was characterized by the dilatation of the sinusoids, degenerating hepatocytes, increased liver volume and cellular disorganization among others.
iii. *In-utero* exposure to alcohol lead to hepatomegaly in the alcohol treated groups which was manifested by the increase in the liver volumes and volume densities.
iv. The injurious effects of alcohol to the developing fetal liver were established to be time and dose dependent with the most critical period being the first trimester at a dose of 5g/kgbwt.

5.3 RECOMMEDATION

By virtual that alcohol has been shown to be teratogenic across the three trimesters as was shown by the pregnancy outcomes, histomorphological and histostereological effects in dose and time dependent manner the researcher therefore recommends that:-

i. Expectant mothers should abstain from alcohol consumption anytime during pregnancy.
ii. Further studies on histostereological effects of alcohol need to be carried out in non- human primates.

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APPENDICES

Appendix I: The publication

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Effects of in-utero exposure to varied doses of alcohol on fetal growth and development in albino rats (*rattus norvegicus*)

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Abstract: *In-utero exposure to alcohol has been shown to disturb the normal development of various fetal organs. This is due to the fact that expectant mothers continue to use alcohol as a social drink and this has been shown to have injurious effects on the developing fetal organs. Though this data exist, data on whether this effects are dose or time dependent is lacking .This injurious effects on the developing fetal organs can help elucidate some of the behavioral and functional disorders observed in adulthood that are of unknown cause.The broad objective of this study was to determine the growth and developmental parameters upon administration of varying doses of ethanol, therefore to determine this injurious effects, 30 expectant rats weighing between 200 to 230 g were used. Simple random sampling was used to assign them into four study groups A –D (n=9 and 3 for each treat group and control respectively).Groups A served as expectant control group and received food and water ad libitum only while groups B (low alcohol group), C (medium alcohol group) and D (high alcohol group), were each further sub divided into first, second and third trimester ethanol treatment group (n=3) and received 2g/kg, 3.5g/kg and 5g/kg body weight of alcohol respectively once daily via oral gavage. The expectant rats were sacrificed on the 20th day of gestation upon euthanasia with carbon dioxide and the abdomen was excised to expose the uterine horns. The fetal anthropometric body parameters including birth weights, fetal length and head circumference among others were recorded. The number of implantation sites and resorptions were counted and recorded. Data was then entered in excel sheet, analyzed through SPSS version 23 and statistically tested using one way analysis of variance (ANOVA) and p-values of less than 0.05 were taken to be significant. The findings of the study showed that there was a statistical significant decrease I fetal weight, crown rump length as well as the head circumference. Results also showed a statistically significant dose and time-dependent decrease in fetal birth weight, fetal length and head circumference especially in treatment groups C and D ($p<0.05$). In conclusion, the present study revealed that alcohol consumption during pregnancy has negative effects on growth and development parameters. This effects are time and dose dependent therefore more emphasis on complete abstinence from alcohol consumption anytime during pregnancy should be done on the expectant mothers. Hence forth the above study showed that alcohol causes detrimental effects that are time and dose dependent and range from intrauterine growth retardation manifested by decreased fetal birth weight, head circumference, and fetal length.* ---------- ---------- Date of Submission: 14-06-2019 Date of acceptance: 29-06-2019 ----------

I. Introduction

Alcohol, a social drink that continue to be abused by expectant mothers as well as all age groups, has been shown to disturb the normal morphogenesis of the fetal organs when used during intrauterine life (1–3). Globally, alcohol abuse among expectant mothers continues to be on rise (4), and this is related to a huge health burden including morbidity, mortality and disability (5). Despite that, alcohol effects on growth and development have widely been studied, there is paucity of data on the fetal anthropometric measures and morphogenesis of specific fetal organs following in-utero exposure to alcohol. In addition, data on whether this effects are time or dose dependent has not been well established hence the present study was conducted to observe if the effects are time or dose dependent by using rats as the experimental model. Global prevalence of alcohol consumption during pregnancy

has been estimated to be 10% (6,7), which is associated with a wide range of fetal congenital anomalies broadly known as fetal alcohol syndrome (FAS),characterized by Malformations, intrauterine death, growth deficiency, CNS abnormalities, GIT abnormalities and behavioral deficits among others (1).Some studies have associated alcohol injurious effects on the fetal and maternal genetic makeup , while other studies have associated alcohol teratogenic effects on environmental conditions including maternal malnutrition (4,8).These effects could be used to explain some of the structural, neurodevelopmental and behavioral conditions like autism including attention deficit hyperactivity disorder that exist of unknown causes.

Appendix II: Data capture sheet for expectant albino rats

ALBINO RAT IDENTITY……

DATE	**WEIGHT IN GRAMS**	ALCOHOL DOSE IN DEIONISED WATER(g\kg)	GENERAL CONDITION OF RAT

Appendix III: Data capture sheet for the albino fetuses

ALBINO RAT IDENTITY
(**MOTHER**)……..
DATE OF
HARVESTING…….FIXATIVE……
……..
TOTAL NO. OF FETUSES…….
TOTAL NO. OF
RESORPTIONS……
NO. OF DEAD FETUSES…….

	F1	F2	F3	F4	FT	F6	F7	F8	F9	F10	F11	F12	F13	F14
GROSS APPEARANCE														
Fetal crown lamp length(cm)														
FETAL WT(g)														
Head circumference														
Obvious congenital abnormalities														
LIVER														
Gross appearance														
WT(g)														
Obvious congenital abnormalities														
Displacement Volume														

Cavalieri volume														
Placental wt.														

Appendix IV: Letter of ethical approval

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
P. O. Box 62000-00200 Nairobi, Kenya Tel 0675870225 OR Extn 3209
Institutional Ethics Review Committee

April 19th, 2018 **REF: JKU/2/4/896A**

Teresiah W. Musa
Department of Human Anatomy.

Dear Ms. Musa,

RE: HISTO-STEREOLOGICAL STUDY ON THE EFFECTS OF ALCOHOL ON THE DEVELOPMENT OF FETAL LIVER IN ALBINO RATS

The JKUAT Institutional Ethics Review Committee has reviewed your responses to issues raised regarding your application to conduct the above mentioned study with you as the Principal Investigator.

The is to inform you that the IERC has approved your protocol. The approval period is from April 19th 2018 to April 19th 2019 and is subject to compliance with the following requirements:

a) Only approved documents (informed consent, study instruments, study protocol, etc.) will be used.
b) All changes (amendments, deviations, violations, etc.) must be submitted for review and approval by the JKUAT IERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the IERC immediately.
d) Any changes, anticipated or otherwise that may increase the risks to or affect the welfare of study participants and others or affect the integrity of the study must be reported immediately.
e) Should you require an extension of the approval period, kindly submit a request for extension 60 days prior to the expiry of the current approval period and attach supporting documentation.
f) Clearance for export of data or specimens must be obtained from the JKUAT IERC as well as the relevant government agencies for each consignment for export.
g) The IERC requires a copy of the final report for record to reduce chances for duplication of similar studies.

Should you require clarification, kindly contact the JKUAT IERC Secretariat.

Yours Sincerely,

DR. PATRICK MBINDYO
SECRETARY, IERC

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