

**EFFECTS OF PRENATAL
ALCOHOL EXPOSURE ON THE
FETAL AND PUPS PANCREAS OF
ALBINO RATS**

By

Joseph Kariuki Kweri

**A thesis submitted in fulfillment of the requirement for the degree of Doctor
of Philosophy in the University of Nairobi.**

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DECLARATION


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DEDICATION

To my wife Jane and my children Brian and Linet, for being there for me and inspiring me to reach high academic heights

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LIST OF ABBREVIATIONS USED

APM:	Apical plasma membrane
ATJ:	Apical tight junction
BaPM:	Basal plasma membrane
BV:	Blood vessels
CoE:	Coefficient error
CT:	Connective tissue
ECI:	Endocrine cells interstitial space
EM:	Electron microscopy
EnCN:	Endocrine cell nucleus
ER:	Endoplasmic reticulum
GA:	Golgi apparatus
HAG:	High alcohol group
LAG:	Low alcohol group
LPM:	Lateral plasma membrane
LuPM:	Luminal plasma membrane
M:	Mitochondrion
MAF:	Modified adehyde fuchsin stain
MAG:	Medium alcohol group
SG:	Secretory granules
TM₁:	Trimester one
TM₂:	Trimester two
TM₃:	Trimester three
TEM:	Transmission electron microscope
UCM:	Undifferentiated cell mass
ZG:	Zymogenic granules

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SUMMARY

Background: The pancreas is a mixed exocrine and endocrine accessory gland of the gastrointestinal tract located in the upper abdomen and connected to the duodenum. Its endocrine part produces important hormones such as insulin, glucagon and somatostatin while the exocrine part produces pancreatic juices containing digestive enzymes. Alcohol usage is known to interfere with oxidative dehydrogenate pathway that cause injury to the pancreatic tissues. However, the structural alterations resulting from exposure to alcohol have not been fully elucidated. It is also not known, how alcohol exposure to the pancreas during critical periods of development would affect its growth.

Objective: The objective of this study is to identify and analyze the qualitative and quantitative changes in the fetal and pups pancreas following inutero exposure to alcohol.

Study design: The study was a case control experimental study

Materials and methods: A total of 100 pregnant albino rats were used in this study and they were divided into 10 control and 90 experimental subjects. The 90 rats in the experimental group were divided into three broad study categories of 30 rats each as Low (2.5gms/kg/Bw), Medium (5gms/kg/Bw), and High (6.5gms/kg/Bw) dose treatment groups. The 30 rats in each of the three broad study categories were further subdivided into three sub groups of 10 rats each to march with the three trimester in a gestation period as TM₁ (GD 1 to

20), TM₂ (GD 7 to 20) and TM₃ (GD14-20). Each group of 10 rats was further sub-divided into prenatal and postnatal groups of 5 rats each. Both the control and experimental group received a standard diet containing 72% carbohydrates, 12% lipids, 20% proteins, 54mg/Kg/Bw zinc and water was provided *ad-libitum*.

Pregnant animals were euthanized with an overdose of sodium pentobarbitone and perfused with 5% Zenkers solution for light microscopy and 4% phosphate buffered glutaldehyde for electron microscopy. The pancreas of fetuses and pups were dissected out and processed for light and electron microscopy for both morphological and morphometric analysis.

Data analysis: Data were analyzed using the Statistical Package for Social Sciences (SPSS) for Windows Version 11.5 Chicago Illinois, and statistically tested using one way analysis of variance (ANOVA). Group means with a significance F-value ($p < 0.01$ or 0.05) were further tested by Sheffes' multiple comparison procedure. Comparison on morphometric parameters between the control and the experimental groups were done by Mann-whitney T-test. The Scheffe' Family Confidence Coefficient (SFCC) was applied as an expression of the confidence that all comparisons made among the sets of groups means were correct.

Results: The effects of prenatal alcohol exposure depicted a wide range of teratogenic outcomes that were seen to be sustained from prenatal to postnatal

life. Morphologically, the acini and islets depicted irregularly arranged clusters with reduced cellular counts per islet and per acini cluster. The sizes, shapes and cell compositions also depicted mixed population of “*mature*” and “*immature*” cells in a cluster with the latter type predominating in the alcohol treated groups. In the exocrine pancreas there was also delayed acini canalization, increased basophilia, reduced zymogen granules and increased fibrosis in the inter-acinar spaces. There was disaggregation of the islet clusters and reduction in the mean cellular counts per islet. The ducts were also disorganized and depicted plug formation. Ultrastructurally, the acinar cells, and the islet cells including the Beta, Alpha, PP-cells and D-cells revealed mixed populations of immature cells with reduced cytoplasmic granules, increased cytoplasmic lipid droplets, degeneration of the membrane bound organelles, and cytoplasmic vacuolations.

Quantitatively, the mean volume density of acini (V_{va}), the mean volume density of islets (V_{vis}) and the mean volume density of blood vessels and ducts (V_{vBvD}) in the alcohol treated groups significantly ($P < 0.05$) reduced while the volume density of fibrous connective tissue stroma (V_{ICTs}) significantly increased when compared with the control. At moderate alcohol doses (MAG) and with exposure in the first trimester (TM_1), alcohol caused 43.1% increase in stromal tissue deposition, 41% reduction in acini mass, 27% reduction of islet mass, and 38% reduction of beta-cell mass. It also caused 23% reduction in

total islet volume, 45% reduction in total beta-cell volume, and 32% reduction in the volume-weighted mean islet volume in the fetal pancreas.

Conclusion: The findings of this study show that prenatal alcohol exposure has both qualitative and quantitative teratogenic effects on the fetal pancreas that are sustained postnatally. These prejudicial effects of inutero alcohol exposure are also dose and time dependant. The high alcohol dose of 6.5mgs/Kg/Bw at first and second trimester presents the best "*window of opportunity*" for expression of alcohol teratogenesis on the pancreas of albino rats. Such an effect of alcohol to the pancreas of children born to alcoholic mothers therefore suggest a predisposition to pancreatic disorders in postnatal life.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.0.0 Introduction

The pancreas, a mixed exocrine and endocrine gland, is an accessory gland of the gastro-intestinal tract (GIT) whose ducts open into the duodenum (Young and Heath, 2006). The exocrine part comprises a compound tubulo-acinar gland that produces digestive enzymes. The endocrine part on the other hand, is made of islets of Langerhans consisting of Alpha (α), Beta (β), delta (D) and PP cells that produce various peptidases like insulin and glucagon for regulation of blood glucose (2005; Ackermann and Maureen, 2007).

The normal cellular histogenesis of fetal pancreas starts with proliferation of progenitor pancreatic endodermal epithelium forming a ventral and dorsal diverticulae on day 9.5 in rat, equivalent to 20 days in humans. The two buds fuse approximately at day 12.5 (Pictet *et al*, 1972; Slack, 1995; Kim and Macdonald, 2002). Perturbations to normal pancreas morphogenesis by prenatal alcohol exposure is thought to be as a result of interference with this early pattern of differentiation of pancreatic mesenchymal cells (Polka *et al*, 2000).

Perturbations from prenatal exposure to alcohol can alter the normal pancreas morphology in the fetus. This alteration to the fetal pancreas can be a predisposition risk factor that is associated to increased vulnerability to diabetes mellitus in the offspring and later in adult life (Kotch *et al*, 1995; Kao *et al*, 2001). The histomorphology and morphometric alteration on the fetal pancreas following the prenatal alcohol exposure is not well elucidated nor is the critical dose and the most vulnerable periods.

The prenatal effects of alcohol could be targeting specific developmental activities in branching morphogenesis, tubulodifferentiation or histogenesis of the mesenchymal cells that determine the structural composition and organization of the pancreas morphology (Montanya *et al*, 2002; Kao 2001; Edlund, 2002). Further, the histomorphometric changes of fetal and pups pancreas, the prenatal critical doses, and vulnerable periods of alcohol teratogenesis to the fetal pancreas remain unclear. This study aims to elucidate the prenatal effects of alcohol exposure on the morphology and morphometry of fetal and pups pancreas in albino rats.

1.2.0: Literature Review

The pancreas is a compound glandular organ of the digestive and endocrine system (Longnecker 1996; Edlund *et al*, 2002; Bock *et al*, 2005). Its complex developmental process can be perturbed by alcohol, altering its structural composition and organization of its cellular components (Abel *et al*, 1992; Apte and Wilson, 2003). The quantitative and histomorphological alterations of pancreas due to teratogenic agents like alcohol have been observed to be dose and time dependant, as well as on the genetic predisposition of the organism (Bock *et al*, 2005). Understanding the histomorphometric alterations, critical prenatal doses, vulnerable periods and postnatal effects of alcohol on the pancreas are important in interpreting the teratogenicity of alcohol on the fetal and pups pancreas. However, this information is generally scarce.

1.2.1 Effects of prenatal alcohol exposure on the fetal pancreas

Prenatal alcohol exposure is associated with dismorphogenesis of fetal pancreas (Apte and Wilson, 2003). The perturbations to the development of normal pancreatic structures targets specific cellular components of the fetal pancreas as has been observed through its causal association to neonatal and adult

pancreatic related disorders like diabetes mellitus (Xi-Pie *et al*, 2003; Marriann *et al*, 2000; Edlund 2002; Li Chem *et al*, 2003).

Maternal alcohol intake interferes with fetal intrauterine environment resulting in direct fetal alcohol intoxication to the pancreas (Abel and Greizestein, 1979; Kotch *et al*, 1995; Kaufman and Woolam, 1991; Apte and Wilson, 2003). Once the alcohol has accumulated in the fetus, the effects on the developing fetal pancreas is thought to have multi-factorial dimensions including altered signal programming, intrauterine nutritional effects, fetal hypoxia, alteration in enzyme activities, alteration of cell functions important in cell division and membrane integrity, and alterations in cellular metabolic pathways (Apte *et al* 1997; Werner *et al*, 2002; Kim and Macdornald, 2002; Apte and Wilson, 2003; Wang *et al*, 2006).

The resultant parenchymal defects to the organs like the pancreas following maternal alcohol intake are linked to direct or indirect effect on the functional or morphological organization, as well as normal development of the pancreatic cells during prenatal life (Persaud, 1985; Apte *et al*, 1998; Georgia and Bhushan, 2004; Johnson and Imrie, 2005). Specific effects on pancreas result from interference with the early pattern of cellular differentiation of the pancreatic parenchymal cells (Polka *et al*, 2000).

The pancreas is thought to be highly susceptible to alcoholic metabolic alterations due to its close biochemical and morphological features with the liver (Haber *et al*, 1998; Apte *et al*, 1998; Ammann, 2001). A study by Majumdar *et al*, (1986) reported similar morphological and biochemical changes in the liver and pancreas of rats when treated with acetyl aldehyde. Minoti *et al*, (2000); Apte *et al*, (2000); Apte and Wilson (2003), reported that alcohol directly stimulates activation of stellate cell cascade of activities, leading to pancreatic fibrogenesis and pancreatitis. A study by Chao *et al*, (1997) observed that the genetic polymorphism of the alcohol metabolizing enzymes coupled with alcoholism in Chinese patients led to pancreatic organ damage. Criddle *et al*, (2006) reported that fatty ethyl esters cause calcium toxicity via inositol triphosphate receptors and hence loss of ATP synthesis in pancreatic cells exposed to alcohol.

Prenatal alcohol exposure is associated with mal-developed pancreas, endocrine abnormalities like glucose intolerance (diabetes mellitus), beta cell dysfunction, and autoimmune pancreatic disorders (Ruhland *et al*, 1991; Simmons *et al*, 2001; Colombat *et al*, 2007; Green *et al*, 2010). Chen and Nyomba, (2003); Beijing and Bao, (2005) reported that prenatal growth retardation resulting from maternal alcohol intake perturbs the B-cell development with consequent

inherent persistent defects associated with B-cell dysfunction, glucose intolerance and diabetes mellitus type 1 and II in rats. Simmons *et al*, (2000), observed that abnormal intrauterine milieu on pancreas morphogenesis can induce permanent changes in glucose homeostasis after birth, and diabetes type II in adulthood.

A study by Ruhland *et al* (1991) reported that ethanol alters Major Histocompatibility Complex (MHC) class I and class II products on a variety of cell types including human fetal islet clusters influencing development of diabetes in genetically predisposed individuals. These findings are in tandem with the hypothesis that the rising incidence of diabetes type 1 and II are seen in areas of the world where per capita consumption of alcohol is also increasing, and may be a consequence of the immunological effects of alcohol intake (WHO, 2006).

Studies and epidemiological data have indicated that the prevalence of diabetes mellitus is on the increase worldwide (Longneker 1996; Wild *et al*, 2004; Roglic *et al*, 2005; Johnson and Imrie, 2005; World Health organization, 2006; World Diabetes Federation, 2006).

In other studies, alcohol induced injuries to the fetal pancreas have been attributed to accumulation of Free Fatty Ethyl Ether (FEE) radicals in blood

when administered early in the first trimester (Ammann and Muellhaupt, 1994; Hunt, 1994; Apte *et al* 2006).

Alcohol also impairs absorption of some nutritional components like zinc, vitamin A and other important nutrients necessary for the maintenance of pancreatic cells (Sonoda *et al*, 2005; Mccarroll *et al*, 2006; Wang *et al*, 2006). A study by Miyasaka *et al* (2005), reported that alcohol affects the carboxyester gene responsible for carbohydrate metabolism in the pancreas. Nutritional impairment during pregnancy as a result of alcohol consumption has also been observed to cause intrauterine growth retardation with direct reduction in cell population in the endocrine pancreas (Garofano *et al*, 1997; Grofano *et al*, 1998; Garofano *et al* 1999).

Several studies conducted in humans and in animals (Georgia and Bhushan 2004; Kushner *et al*, 2005; Georgia and Bhushan, 2006; Ackermann and Maureen, 2007) suggest that abnormal morphogenesis due to teratogenic exposure to alcohol coupled with genetically susceptible individuals, may result in interference with cascade of genes regulating development, leading to either over expression or under expression of some genes, leading to alteration of pancreatic morphology.

1.2.2 The in-utero alcohol doses and effects on the fetal pancreas

It is now becoming clear that adverse effects of prenatal alcohol exposure on pancreas development have a dose response relationship (Chen and Nyomba, 2003). In humans, post natal organs mal-development has been linked to high alcohol doses (above 7.0gms/kg/Bw) in utero (Chernoff, 1977; Wilson *et al* 1990; Korsten *et al* 1992; Heber *et al*, 1996). A study by Kono *et al* (2001), found out that high doses of alcohol above 8gm/kg/BW caused fibrosis, steatosis inflammation, and necrosis of the adult rat pancreas.

Radall and Taylor, (1979) suggested some working hypothesis associated with alcohol teratogenesis and pancreas induced morphological changes. The study suggested that ethanol at high dose, could increase endotoxin levels and this would increase infiltration of leukocytes such as macrophages and neutrophils that trigger production of free radicals in the pancreas with a key factor to the alcohol dose and time of exposure.

Another study by Persaud (1988), investigated the effects of moderate alcohol consumption on early embryonic development in rat using vitamin and mineral enriched modifications. The study found that there was a significant increase in fetal resorptions in ethanol treated groups. Further, the mean fetal weight of the animals exposed to the ethanol was significantly reduced like what was

observed in long term malnutrition by Blackburn and Vinijcharikul, 1969; Blondean *et al* 1999.

Study by Randall and Taylor (1979), had investigated the teratogenic effects of ethanol, on mice, by giving multiple doses of ethanol. The results showed that only the high doses of ethanol (*6gms/kg/ body weight*) produced teratogenic effects on the limbs and viscera. Garofano *et al* (1997) observed that the blood alcohol content is undoubtedly of more importance in the pathogenesis of fetal pancreatic defects than actual maternal consumption of alcohol, while Woollman (1985) and Brien *et al* (1983) reported that the concentration of alcohol in the fetus is roughly equal or even greater than that present in maternal serum, which depends largely upon the amount of maternal alcohol consumption.

1.2.3 Vulnerable periods of pancreas morphogenesis to alcohol

The gestational day 9.5 to day 11.5 is considered developmentally important as it is the period of organogenesis in the rats and it is a period highly susceptible to teratogenic manipulations (Hebel and Stromberg, 1986; Butler and Juurlink, 1987). Selective developmental activities between day 6.5 to day 10.0 leading to histogenesis and cytodifferentiation of pancreatic mesenchymal cells are prone to effects of teratogenic agents like alcohol as they are the ones

responsible in giving rise to the definitive pancreatic organs (Lih *et al*, 1999; Kim and Macdonald, 2002; Ackermann and Maureen, 2007).

Barker (1992) noted that day 6.5 to day 10.5 is a critical stage of organogenesis, as any substance affecting normal organs development at this stage marks the fetal and infant origin of adult diseases and therefore needs to be guarded from teratogenic agents like alcohol and others. Studies have also investigated the teratogenic effects of alcohol on the mouse using a dose of 0.03 mls/g of freshly prepared 25% (v/v) solution of absolute alcohol (ethanol-‘Analar’) administered through gastric gavage from gestational day one (GD₁) through gestational day twelve (GD₁₂). Though these studies suggest alcohol teratogenicity to be severe during proliferative stage of cell development at first trimester (TM₁), the most vulnerable period of pancreatic dysmorphology is unclear due to dynamic changes in cell differentiation that occur throughout the gestation period.

The pancreas dysmorphogenesis occurs at an exquisite critical period of the organs growth and development during which at a certain “*window period of opportunity*” referred to as the vulnerable period in the histogenesis and cytodifferentiation of the organ is perturbed (Goldman, 1980; Ralier *et al*, 1981; Amart and Saunder, 1996). Lack of adequate data on the histomorphometric

effects of alcohol on the cyto-differentiation of fetal pancreas is a key research area that requires to be explored to shed more light on the vulnerable periods of alcohol teratogenesis (Elliot, 1998).

1.3.0 Research questions, objectives and hypothesis

1.3.1 Research questions

- (1) What are the effects of prenatal alcohol exposure on the morphology and morphometry of fetal and pups pancreas?
- (2) Do the histomorphometric changes on fetal and pups pancreas depend on the dose of the alcohol administered?
- (3) Are the histomorphometric effects of prenatal alcohol exposure to the fetal pancreas sustained post-natally?

1.3.2 Broad objective

The aim of this study was to determine the histomorphometric effects of prenatal alcohol exposure on the fetal pancreas, whether the induced structural defects are dose and time dependant and whether the fetal pancreatic histomorphometric defects are sustained postnatally.

1.3.3 Specific objectives

- (1) To determine the effects of prenatal alcohol exposure on the morphology and morphometry of the fetal and pups pancreas.
- (2) To establish the vulnerable periods of alcohol teratogenesis on fetal pancreas histomorphogenesis.
- (3) To evaluate the effects of various prenatal alcohol doses on morphology and morphometry of the fetus and pups pancreas
- (4) To establish whether effects of prenatal alcohol intake on fetal pancreas are sustained postnatally

1.4.4 Hypothesis (H₀)

Prenatal ethanol exposure has no effects on morphology and morphometry of the fetal, and pups pancreas nor a relation with the alcohol doses and the time of exposure, and that induced pancreatic structural defects in the fetal pancreas are not sustained at postnatal life.

CHAPTER TWO

MATERIALS AND METHODS

2.1.0 Experimental animals

In this study, albino rats (*Rattus norvegicus*) were used due to their low incidence of spontaneously occurring congenital defects, a relatively short gestational span, a large litter size, low cost of maintaining the animals and the availability of considerable amount of the reproductive data (Chernoff 1977). A total of 100 female albino rats weighing between 150-250 grams were purchased from the School of Biological Sciences in the University of Nairobi. The albino rats were fed on a standard diet as determined by *American institute of nutrition* (1977), and left at the animal house for a minimum period of seven days to allow them to acclimatize to the new environment (room temperatures 23°C and natural lighting system). All animals received food (rodent pellets) and water *ad libitum*.

2.2.0 Breeding and fetal harvesting

All these rats were put in cages each containing two rats each. A male albino rat was introduced into each of the cages with two female rats at 2:30 P.M. (+/- 30 minutes). At 09:00 A.M. (+/- 30 minutes) the following morning, the males were returned to their separate cages. The animals were handled only by

the investigator or a trained research assistant for the purpose of obtaining daily weights at 8.00 am, feeding and alcohol administration.

Vaginal smears were taken from the females the next morning and successful mating was determined by the presence of spermatozoa in the smears followed by vaginal wash 24 hours later to assess estrous changes denoted by changes in epithelial cells from squamous to polyhedral marking the start of pregnancy i.e. the first day of gestation (GD1) (Hubscher *et al.* 2005)

Once pregnancy was confirmed, animals were randomly assigned to either the pre or post natal study categories of 50 rats each. The 50 rats in each of these two categories were further randomly assigned into either control or experimental group of 5 and 45 rats respectively. The 45 rats in the experimental category were further assigned into three broad study groups of 15 rats each as low (LAG), Medium (MAG) and High Alcohol group (HAG) of 15 rats each. The 15 rats in each of the broad subgroups of the LAG, MAG and HAG were further subdivided into three categories according to trimester as first (TM₁), second (TM₂) and third (TM₃) trimesters comprising of 5 rats each.

2.3.0 Alcohol Administration

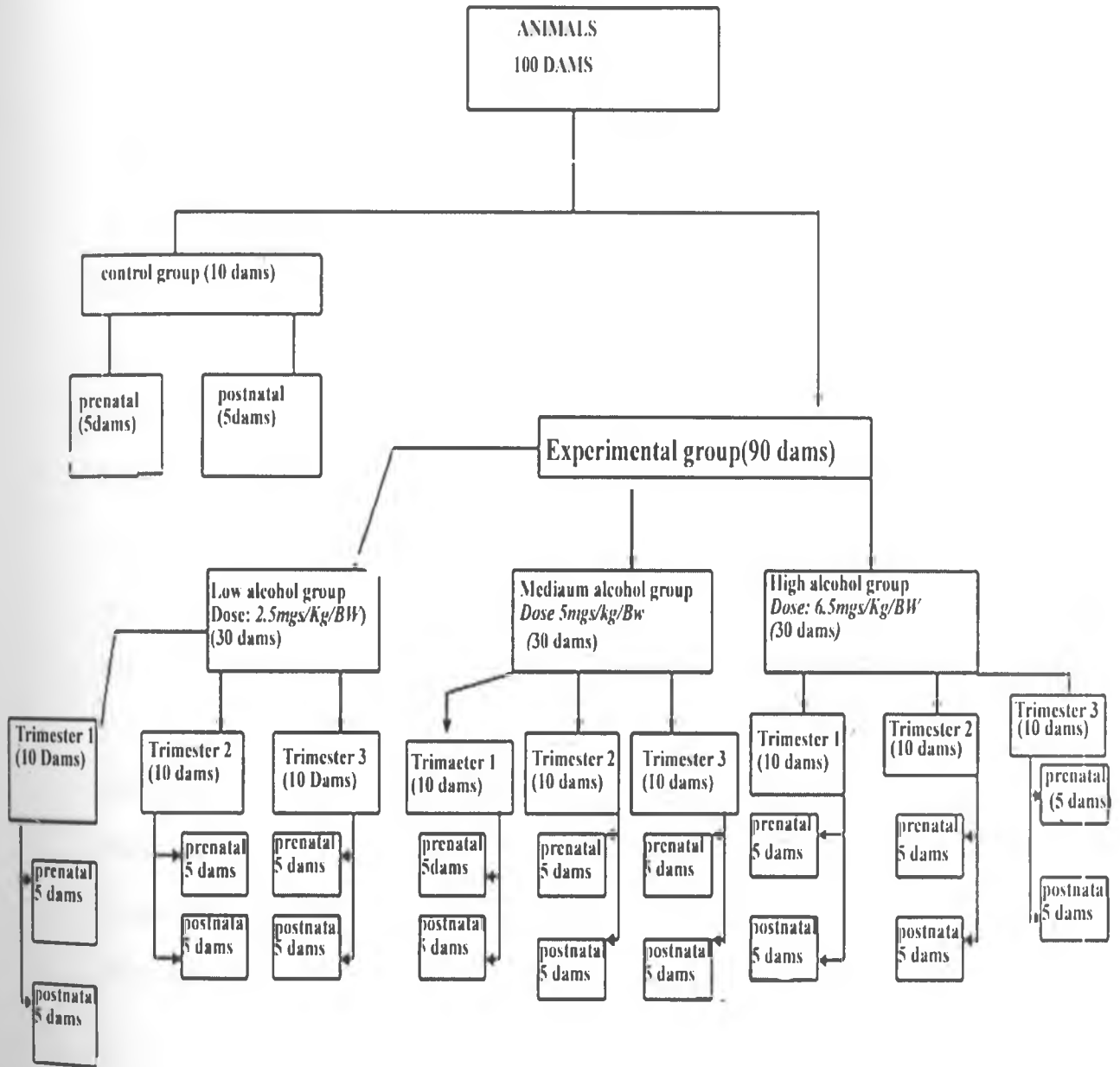
2.3.1 The Control sub group (C)

All the 10 rats in the control category received standard diet of rodent pellets from Unga feeds Kenya limited company containing: 68% starch, 4% cellulose, 5% lipid (corn oil) and 20% protein) and by calories:- 20% proteins, 72% carbohydrates, 12% lipids, and 54mg/kg zinc and water *ad libitum* for the whole of the gestation period day 1-20. For the the experimental groups, all the animals received the standard diet and water *adlibitum* like in the control. In addition they received varying doses of alcohol via gastric gavage (Gauge 1.8 2R2 needle) at 12.30 pm according to their respective study groups as follows, (a) The *Low alcohol group-(LAG)* received a constant daily dose of alcohol of 2.5gms/Kg/Bw, (b) The *Medium alcohol group-(MAG)* received a constant daily dose of alcohol of 5gms/Kg/Bw and (c) The *High alcohol group-(HAG)* received a constant daily dose of alcohol 6.5gms/Kg/Bw.

The mothers in the prenatal category were euthanized with an overdose of sodium pentobarbitone on 20th day of gestation while the ones for postnatal category, were allowed to deliver the pups which were followed and sacrificed four weeks after birth. The weight was recorded daily at 8:00 AM and feeds were provided at 9:30 am while alcohol administration was done at 12.30 PM. The timing for the administration of the alcohol was based on the dosage per

the study group and on trimester basis. Since the gestation period of a rat is 21 days, therefore those in TM_1 category received alcohol from gestational day GD1 to GD20, those in TM_2 category between GD7-GD20 and those in third trimester (TM_3) GD14-20.

Diagram 1: The animal grouping in the control and the experimental groups (LAG, MAG, HAG).



2.4.0. Harvesting of fetal and pups pancreases

(a) *The prenatal category (fetuses):*

In all cases, the pregnant rats were euthanized by an overdose of 20% sodium pentobarbitone, between 09:00 and 11:00 A.M. on gestational day 20, to prevent the devouring of any damaged offspring. After anesthesia, the anterior abdominal wall of the mother was opened and the full extent of both uterine horns exposed. The uterine horns were excised along the antimesometrial border to reveal the fetuses, embryonic membranes and placentas. The embryo and the placenta were gently removed in totality from the uterus utilizing the blunt end of a pair of forceps. An incision along the dorsal surface of the membranes revealed the fetuses, then each fetus and its placenta was removed and weighed by use of Ohaus scout pro-digital weighing scale^o model sp 642 and the general fetal morphology examined and recorded immediately.

Fetal sizes were determined by measuring the anal-nasal length to calculate Lee index (Morley *et al*, 1989). External examination was done before and after fixation with Zenker's solution. Pancreases from the dam and the fetuses were dissected out and processed for light and electron microscopy.

(b) *The postnatal category (pups).*

The pups were followed for four weeks (28th day) after which they were euthanized with Sodium pentobarbital, abdominal wall opened and pancreases removed, fixed with Zenker's solution and processed for light and electron microscopy.

2.5.0 Processing the pancreas for light microscopy

After dissecting out the pancreases from the fetuses and the pups, they were immersed in Zenkers' solution for 24 hours to allow for complete fixation to be attained, then the adipose tissue trimmed and processed routinely for paraffin embedding by passing them through ascending concentrations of isopropyl alcohol, (50%, 60%, 70%, 80%, 90%, 95% and 100%) each for one hour and cleared with cedar wood oil for 12 hours. The pancreases were then infiltrated with molten paraplast wax for 12 hours and embedded in paraffin wax. They were then blocked in 1.2 cm cubes using molten wax at 56^oC, and mounted on wooden blocks of 2 cm square. *Leitz wetzlar*^o sledge microtome was used to cut longitudinal thin sections of 7µm thick from head to tail regions. The sections were floated in water bath at 37^oC then mounted onto glass slides coated with egg albumin. Slides were dried in an oven at 37^oc for 24 hours, and then stained with haematoxyline and eosin, haematoxylin phloxine stain or Modified aldehyde Fucshin stain for demostraion of exocrine parenchyma, stromal and

endocrine cellular compositions. Slides in each subgroup selected at sequential order (.i.e always the tenth section) were stained with haematoxyline and eosin to demonstrate the general features of acini, connective tissues, blood vessel and ducts. The other set of slides from each pancreas were stained with Haematoxyline Phloxine stain to demonstrate cellular acini components. Some more slides from each pancreas were stained with Modified aldehyde fuchsin stain (MAF) to demonstrate specific islet cells (*Hamali, 1952*). The slides were then examined at various magnifications, using a *Leica BM^o* microscope.

2.6.0 Processing the pancreas for electron microscopy

After dissecting out the pancreases from the fetuses and the pups, pancreatic tissues were rinsed in distilled water and fixed in 2% phosphate buffered glutaldehyde solution. The pancreases were then cut into small sections of 1 millimeter square cubes and were post-fixed in 1% osmium tetroxide for 12 hours. They were then dehydrated by passing through increasing concentrations of ethanol (50%, 60%, 70%, 80%, 90%, 95% and 100%) for 30 minutes each and twice for one hour in absolute ethanol. The solutions were then cleared in propylene oxide for 30 minutes. Subsequently, the sections were infiltrated in catalyst free durcupan mixture 1 as follows: propylene oxide: 3:1-30minutes; propylene oxide: durcupan 1:1-30 minutes; propylene oxide: durcupan 1:3-30 minutes and then absolute durcupan at 60°C in oven for one

hour. The sections were then embedded in 100% durcupan with catalyst, and polymerized in an oven at 60°C, for 48 hours. The blocks were trimmed and 1µ thick (semithin) sections cut using *Reichert ultramicrotome*[®]. The sections were stained by applying a drop of toluidine blue, mounted in DPX, examined with *Leica BM*[®] light microscope at magnification of x1000 and photographed using *14 Mega pictuxil's*[®] camera on *orthox*[®] light microscope.

For electron microscopy, the blocks were trimmed and ultrathin sections made with *Richerts Ultramicrotome*©. sections were harvested on 200 mesh copper grids stained with uranyl acetate, counterstained with lead citrate (Glauert, 1965), and examined by transmission electron microscope (*EM 201 phillips*©).

2.7.0 MORPHOMETRIC ANALYSIS

2.7.1 Preparation of the pancreatic tissues for morphometry

The pancreases of the fetus and the pups were dissected out and placed in cold saline solution, adipose tissue trimmed off, weighed, and immersed in Zenkers solution for 24 hours at room temperature (23°C) to allow for proper fixation. After the routine tissue processing for light microscopy (*described earlier*) the tissues were allowed to settle randomly in palaplast (the embedding media) to allow for physical randomization of tissue orientation and to ensure that dispositions and orientations of islets attained random location and isotropy for unbiased estimates to be met (Mayhew, 1999; Inuwa, 2005; Inuwa and El Mardi,

2005). Each pancreas was exhaustively sectioned into 7 μ m thick sections. The sections were stained with modified aldehyde fuchsin histochemical method (Bancroft and Gamble, 2002). For this purpose sections were rehydrated, oxidized in 1% potassium permanganate plus 0.5ml concentrated sulfuric acid. Bleaching was done by 1% oxalic acid solution, stained with aldehyde fuchsin stain for 1 hour and counterstaining with orange G and light green solution for purposes of demonstrating stromal tissues (*Appendix 2*).

2.7.2 Sampling pancreas for morphometry

Two sets of ten sections marked as primary and reference sections were sampled from each pancreas by systematic uniform random sampling (SURS) (Howard and Reed, 1998, Partida-Hernandez *et al.*, 2006; Barbera *et al.*, 1997). With a random start between the first 50 sections, every 50th section was sampled and marked as the primary sections. In addition, for every sampled primary section (sections numbered 10, 60, 110, 160, 210, 260 . . .), the lookup section, two sections after it (sections numbered 12, 62, 112, 162, 212, 262 . . .) were sampled and marked as the reference section. All primary and reference sections (40–48 paired sections) from each pancreas were then examined (investigated) using a projecting microscope with an attached TV screen fitted with the transparent counting grid (*Appendix 2*). After investigating both the primary and secondary sections through the systematic uniform random sampling, Applying a two

stage mechanical dissector method, the results of pancreatic parenchyma between the primary sections were compared with reference sections after projecting all images for the whole pancreas on a TV screen using the BH2-Olympus[®] light microscope in the Department of Human Anatomy.

2.7.3 Determination of pancreas volume

After the routine tissue processing for light microscopy (*described earlier*) the tissues were allowed to settle randomly in palaplast (the embedding media) to allow for physical randomization of tissue orientation and to ensure that dispositions and orientations of islets attained random location and isotropy for unbiased estimates to be met (Mayhew, 1999; Inuwa, 2005; Inuwa and El Mardi, 2005). This was also to allow for unbiased random isotropic points to be generated during sectioning with the *leitz sledge* rotary microtome. Each pancreas was exhaustively sectioned into 7 μ m thick sections. The sections were stained with modified aldehyde fuchsin histochemical method (Bancroft and Gamble, 2002). After sampling (*as described above*) the sections were projected onto a TV screen that was superimposed with a transparent grid with marked random points. Using a two stage mechanical dissector method (Gundersen *et al* 1988) Cavalieri principle was employed to estimate the volume of pancreas using the following formula as applied by Mayhew, (1999); Inuwa, (2005); and Mahmoudzadesh *et al* (2006).

$${}_{est}V = \frac{\sum P \cdot a / p \cdot t}{M^2}$$

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

$\sum P$ = was the sum of the number of points landing within the pancreas profiles,

a/p = was the area associated with each point,

t = was the distance between sections and

M = was the magnification (Howard and Reed, 1998; Mandarim-de-Lacerda, 2003).

2.7.4 Determination of pancreas volume densities, total volume, total number of islets

On each sampled section, five fields were selected in a systematic random manner by movement of the microscope's stage in X and Y directions with the aid of mounted transparent grid scale superimposed on the images on the TV screen (*appendix 2*), and points hitting the various components of the pancreas section counted at a final magnification of x40.

Then estimates of the volume density (V_v) of islet components in the reference space were obtained using the formula:

$${}_{est}V_v = P(\text{part})/P(\text{ref}),$$

where **P(part)** and **P(ref)** were the number of test points falling on the samples of the selected structure profiles and in the reference space respectively (Gundersen and Jensen, 1985; Gundersen *et al*, 1988; Howard and Reed, 1998).

In order to estimate the absolute volume, the volume density of that component was multiplied by the reference volume (Howard and Reed, 1998). The total number of islets were determined by the physical fractionators method (Gundersen *et al*, 1988; Bock *et al*, 2005). Briefly, all primary and reference sections (40–48 paired sections) from each pancreas were investigated using a projecting microscope (Barbera *et al*, 1997; Partida-Hernandez *et al*, 2006). An unbiased sampling frame was attached to the TV screen. All primary sections were investigated systematically according to systematic uniform random sampling, and for each islet sampled in the sampling frame were determined whether the islet also appeared in the reference section. The definition of an islet was chosen as a cluster of cells with minimum typical characteristics of islet cells. The total numbers of islets were finally estimated by applying the following formula (Howard and Reed, 1998; Bock *et al*, 2003; Madrin-de-lacerda, 2003; Inuwa, 2005)

$$est N_{isl} = \frac{N_{sect(p-p)}}{N_{sect(p-r)}} \times \frac{\Delta X \cdot \Delta Y}{A_{frame}} \times \Sigma Q_{isl}$$

where $N_{\text{sect}}(\mathbf{p-p})$ = was the number of sections between the primary sections,
 $N_{\text{sect}}(\mathbf{p-r})$ = was the number of sections between a primary section and the corresponding reference section,
 $\Delta\mathbf{X}$ = was the step length in the x direction,
 $\Delta\mathbf{Y}$ = was the step length in the y direction,
 A_{frame} = was the area of the sampling frame corrected for magnification,
 ΣQ_{isl} = was the total number of islets counted (sampled in the primary section but absent in the reference section) from one pancreas (Bock *et al*, 2003).

2.7.5 Determination of Islets volume-weighted mean volume

To determine the islet volume-weighted mean volume, five systematic random fields were sampled from each primary section. At a final magnification of x40, a grid of standard points and a set of parallel lines of random orientation were superimposed randomly onto the image on the TV screen and the volume-weighted mean volume of the islets estimated using the point sampled intercepts method (Gundersen and Jensen, 1985; Gundersen *et al*, 1988). Measurements were performed using a 15-glass ruler with a total length of 35 mm (Howard and Reed, 1998; Gundersen and Jensen, 1985; Skau *et al*, 2001; Mahmoudzadeh *et al*, 2006). In each animal, 100–200 islets were sampled

(Gundersen *et al*, 1988). The Coefficient of error (CoE) for point counting in each measurement was less than 0.05. Data was entered into a result sheet and the volume-weighted mean volume of the islets estimated by this formula:

$${}_{est}Vv = \frac{\pi}{3} \ell_0^3 \cdot f$$

where, Vv = was the volume-weighted mean volume of the islets,

ℓ_0^3 = the mean measured intercepts lengths cubed

f is $= (1/\text{Magnification}^3)$ (Skau *et al*, 2001; Mahmoudzadeh *et al*, 2006).

2.7.6 Determination of the total mass of beta cells, islets, and pancreas

Total beta cell and islet mass was determined as previously described by the works of Montanya *et al*, (2002). Briefly, using the measurement of total beta-cell mass as an example, the sections were investigated using a projecting microscope equipped with a projecting arm to project the image onto the TV screen. All stained sections from each pancreas were then systematically investigated according to systematic uniform random sampling (Bock *et al*, 2003; Bock *et al*, 2005) starting at a random position outside the sections and then moving the stage with fixed step lengths in the X and Y directions through all sampled sections from each pancreas. A point-counting grid with 99 points,

one (1) of them encircled (the unit point), attached to the grid on the TV screen, and for each pancreas the total number of grid points that hit beta cells and the total number of unit points that hit the pancreas and islets counted. The total beta-cell mass for each pancreas were then estimated by:

$$\text{est}M_{\beta} = \frac{P_{\beta} \times M_{tis}}{99 \times (P_{tis})}$$

Where: M_{β} = was the total beta-cell mass,

P_{β} = was the total number of grid points that hit cells β -cell in all investigated sections from one pancreas,

P_{tis} = was the number of unit points that hits the sampled tissue, and

M_{tis} = was the wet weight of it.

The **mass of pancreas** was then estimated from this formula:-

$$\text{est}M_{pan} = \frac{P_{pan} \times M_{tis}}{P_{tis}}$$

Where: P_{pan} was the number of unit points that hits the pancreatic tissue.

2.8.0 STATISTICAL ANALYSIS

Data was analyzed using statistical package for social sciences (SPSS) for Windows Version 11.5 Chicago Illinois, and statistically tested using one way analysis of variance (ANOVA). Group means with a significance F-value ($p < 0.01$ or 0.05) were further tested by Sheffes multiple comparison procedure (Mood *et al* 1974, Conover 1980; Hollander and Wolfe 1999). The Scheffe' Family Confidence Coefficient (SFCC) was applied as an expression of the confidence that all comparisons made among the sets of group's means were correct (Gibbons *et al*, 2003; Wasserman and Larry, 2007; Corder and Foreman, 2009). Mann whitny U-values were applied to compare the means of the various stereological parameters for the various alcohol groups with the control (Mann and Whitney, 1947; Lehmann, 1975).

CHAPTER THREE

RESULTS

3.1.0 General observations

The pancreas of albino rat fetus (20 days old) consists of a head, a narrow neck, broad body and a branched tapering tail (*Figure 1A*). Microscopically it is contained in a thin fibrous capsule that sends in septa dividing it into lobules (*Figure 1B*). Each lobule comprises of numerous evenly distributed acini that are clustered in the head and neck region and relatively scattered in the body and the tail regions (*Figure 1C*). The acini clusters showed regional variation with head and neck having 25-35 clusters per field (*Mag X10*) while the body has between 20-30, and the tail recording between 15-20 clusters per field (*Table 1*). On average, each acinus is made of 15-25 acinar cells arranged around a lumen (*Figure 1D*). The acinar cell contains conspicuous cytoplasmic zymogen granules with high affinity to acidic dyes making the cytoplasm appear acidophilic (*Figure 1A*). Ultrastructurally, the acinar cell displays typical features of protein synthesizing cell with numerous golgi complex apparatus, numerous mitochondria, lysosomes and conspicuous electron dense granules in the cytoplasm (*Figure 2*).

The duct system starts with inter-cellular secretory canaliculi that extend from the central lumina at the centre of the acini and approaches the basement membrane

of the acini (*Figure 3a*). Several of these secretory canaliculi unite to form the intercalated ducts between groups of acini within the same lobule (*Figure 3A*). The cells that line the intercalated ducts are cuboidal and extend up to the lumen of the acini appearing in the lumen as the intra-acinar cells (*Figure 3A*). Several of these from the same lobule join to form a single intralobular duct that consist of a simple cuboidal epithelium surrounded by a layer of connective tissue (*Figure 3B*). The intralobular duct leaves the lobule and combines to form interlobular ducts (*Figure 3c*). The later eventually merge to form the main pancreatic duct made up of low lying columnar epithelium on their luminal surface (*Figure 3D*).

The stroma comprises connective tissue rich in blood vessels, nerves, fibroblastic cells, loose areolar tissue and other extracellular matrix that maintain the frame work of the gland. The blood vessels are concentrated near the head and the body along the midline (*Figure 4A&B*).

Scattered between the acini are clusters of purplish staining cells that form the so called Islets of Langerhans. In each of the islets of langerhans, all the endocrine cells were seen closely associated to the blood vessels with no particular order in the alignment of the specific cells along the blood vessels. The cell are arranged in cords and clusters between which are blood vessels (*Figure 5A*). With Haematoxyline and eosin staining the B-cells are seen forming the mantle and constitutes the largest population of cells (*Figure 5B*). The quantitative analysis of the B-cell counts showed regional variations with the body recording the highest

number of B-cells per cluster of between 80-120 cells per islet, the tail recording 50-80 cells while the neck and head regions recorded an average of between 55-75 cells per islet cluster (*Table 1*). Based on the cell composition, two types of islets were distinguishable; the pp-rich islets that were predominantly seen in the head and neck regions and alpha (glucagon rich islets) that were predominantly seen in the body and the tail region (*Figure 6A-D*).

The normal body weight of a fetus at term (Gestational day 22) ranges between 5 to 6.4gms with a mean weight of 5.57 ± 0.12 gms. The weight of its pancreas ranges between 10.5 mgs to 12.0 mgs with a mean weight of 11.14 ± 0.02 mgs while its volume calculated in cubic millimeters ranges between 9.00-12.05 mm³ with a mean of 10.02 ± 0.001 mm³ (*Table 2*)

The exocrine acini contribute the greatest part of pancreatic tissue with a volume density of between 65-70%, followed by the connective tissue stroma with volume density of between 25-29%, then blood vessels and duct contributing 2-5.5%, and lastly the islets with a volume density of 2.5-3% (*Table 2*). The percentage proportion of the fetal endocrine pancreas was between 1.5 - 1.7% to the total pancreas mass while the volume ranges between 1.10 and 1.15mm³ to the total pancreas volume. This percentage proportion of the islets translates to a total mass of between 2.00 to 2.5mgs with a mean of 2.01 ± 0.002 mgs.

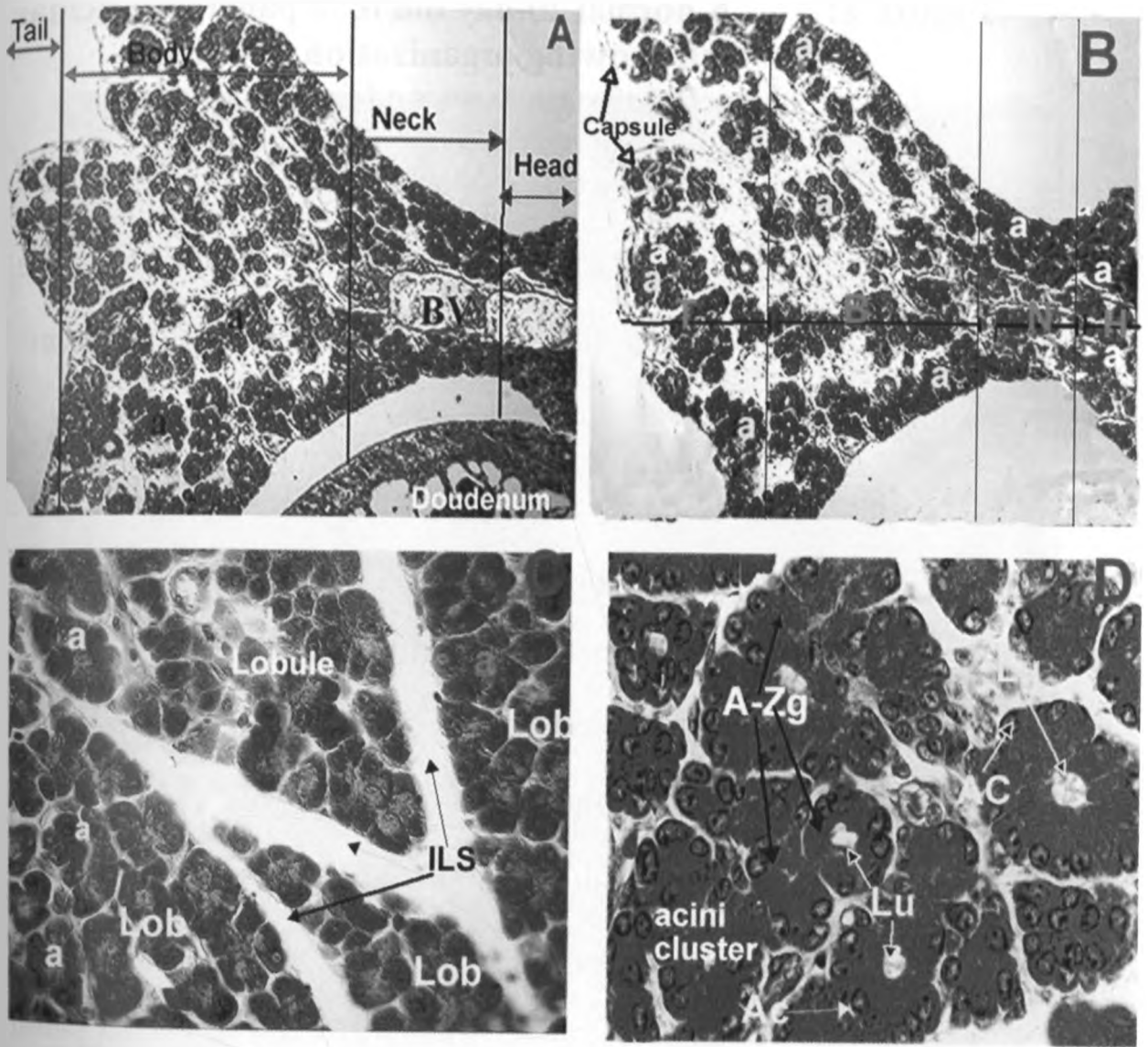
A zonal arrangement in the islet cells was observed. Alpha cells (α) were arranged in clusters scattered at the periphery of the islets (*Figure 5A&B*) and the β -cells were located in the central portion of the islets forming the core and the mantle of the islet are while the rest of the cells including, delta, Pancreatic Polypeptide (PP)-cells and C-cells were found peripherally located (*Figure 5A&B*). Though most of the D cells were situated peripherally, a few cells were found to occupy an intermediate position while the PP cells were seen to be arranged singly or in clusters at the periphery of the islet (*Figure 5A&B*). On the cellular composition of the islet, the beta cells contributes the highest cellular counts of between 67 to 75% followed by alpha cells with 20-25%, then delta cell ranging between 2-5% (*Table 3*). It was however observed that, though the percentage proportion of the cell types in an islet did not vary much between one islet to the other, the numerical counts of the different cells type in an islet cluster varied with the size of the islets that also varied from one islet to the other. In the rat pancreas, 61-64% of the beta cells were seen lying side by side in a cord like structures of between 8-16 beta cells (*Figure 5B*). The volume density of beta cells per islet is 73.4% of the total cellularity while the logarithmic totals of beta cells calculated into tens exponential three ($\times 10^3$) ranges between 2.5-2.9 (*Table 4*).

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Figure 1 A-D: Sections of a normal (20-day old) fetal pancreas showing the parts and distribution of acini.

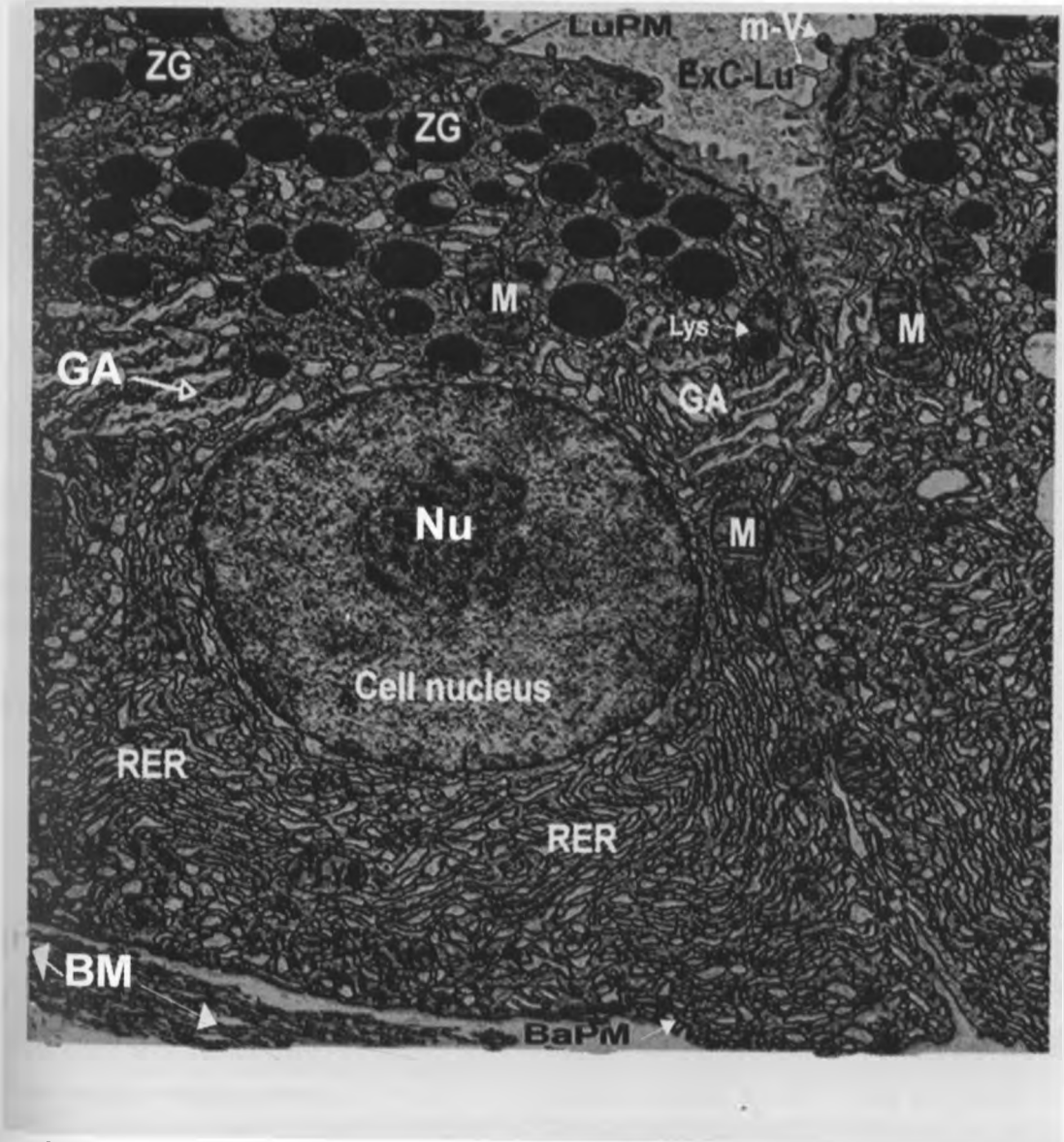
- 1A:** Longitudinal section through the middle of the fetal pancreas. Note the expanded head (H) as it joins the second part of duodenum, the narrow neck (N) joining the head (B) and body, and the broad body (B) and the branched tail (T) that look like it is branched. Note the distribution of the acini clusters (a) and the blood vessels (BV) along the midline. *H&E Mag x10*
- 1B:** Longitudinal section of a fetal pancreas showing the regional distribution of acinar clusters (a). Note the high clustering of the acini in the head (H) and the neck (N) region the relative scattering of the acini clusters in the body (B) and the tail (T) regions. *H&E Mag x10*
- 1C:** A section of fetal pancreas showing the organization of the acinus clusters into lobules (Lob). Note the Interlobular septa (ILS) that is an extension of the outer fibrous covering capsule. Note the organization of the acini (a) in the lobule (Lob). *H&E Mag x 40*
- 1D:** A section of fetal pancreas showing the organization of cinus clusters (a). Note each acini cluster is made up of 15-20 columnar acinar cell (AC) that surround a lumen of the acinus (Lu). Note the acidophilic appearance of the zymogenic (A-Zg) in the cytoplasm. Note the canalization of the acini (CA). *H&E Mag x40*

Figure 1 A-D:



NB> all are sections of a 20 days old fetal pancreas

Figure 2:



NB> a normal 20 day old fetal acinar cell (TEM)

Table 1: The Normal ranges and the mean number of β -cells per islet as well as numerical counts of the acini clusters per field.

Parameters	Acini distribution according to parts of the pancreas							
	Head		neck		body		tail	
	Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE
No. of acini per field	25-35	28.7 \pm 0.3	25-29	26.1 \pm 0.7	20-30	22.6 \pm 0.2	15-20	17.8 \pm 0.9
No. of beta cell per islet cluster	55-75	69.2 \pm 0.5	82-97	88.7 \pm 0.8	80-120	96.2 \pm 0.7	50-80	59.7 \pm 0.8

NB> The mean values on the regional distribution of acini clusters per field as well as the numerical counts of beta cells per islet across the parts of the pancreas were statistically significant ($P < 0.05$) by Chi-square test at (95%) confidence intervals when compared across the parts of the pancreas.

Table 2: The normal ranges and the means of the fetal body weights, pancreas weight, the exocrine and connective tissues morphometric parameters

Parameters	The normal ranges of the Fetal morphometric parameters	
	Ranges	Means \pm SE
Fetal Body weight (gms)	4.90 - 6.40	5.57 \pm 0.12
Pancreas wet mass (mg)	10.50 - 12.00	11.14 \pm 0.02
Pancreatic volume (mm ³)	9.00 - 12.05	12.02 \pm 0.03
Volume density of acini (%)	65.00 - 70.0	67.35 \pm 0.97
Volume density of Connective tissues (%)	25.00 - 29.0	27.90 \pm 0.09
Volume density of Blood vessels and ducts (%)	4.00 - 7.00	5.49 \pm 0.003

Table 3: The normal ranges, means and percentages of fetal endocrine cells in an islet cluster.

Cell type	The ranges of cell counts per islets	Mean numerical counts \pm SE	The percentage (%) range of cellular compositions
Beta cells(β)	63 - 131	98.45 \pm 2.53	67 -75%
Alpha cells (α)	15 - 39	22.47 \pm 1.29	20-25%
Delta cells (δ)	4 - 13	5.61 \pm 0.08	2 - 5%
Others (PP,Cells)	1 - 5	1.79 \pm 0.01	0.1- 0.9%

NB> percentages among the endocrine cells only

Table 4: The normal fetal endocrine morphometric parameters

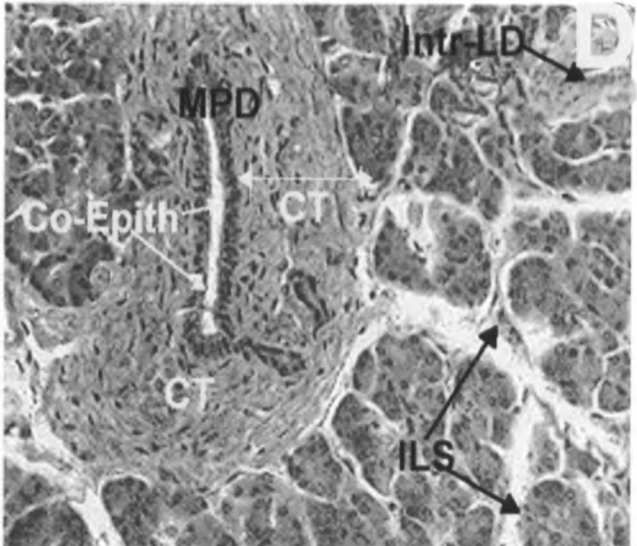
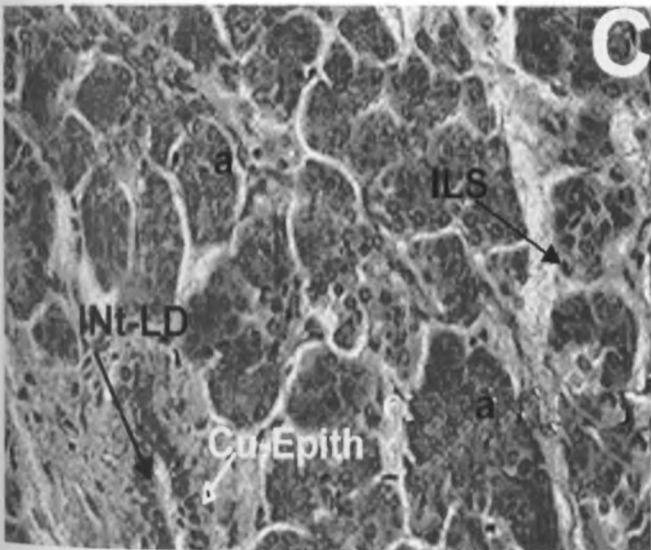
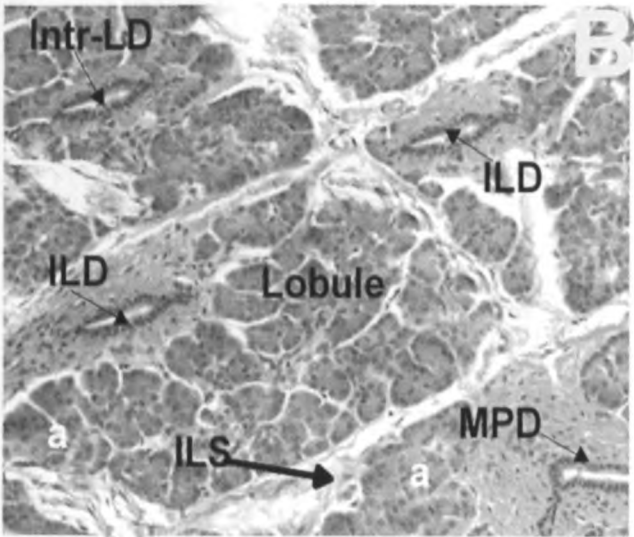
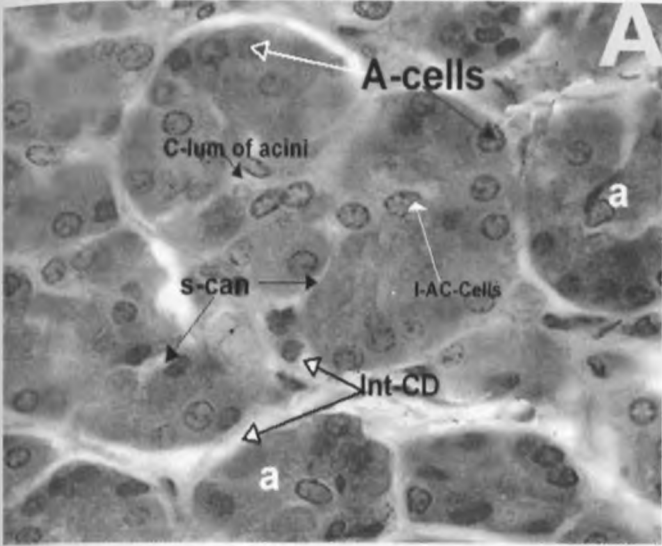
Parameters	The ranges and the means of the endocrine stereological parameters	
	Ranges	Means \pm SE
Islet mass (mg)	2.00 - 2.50	2.2 \pm 0.001
Beta cells mass (mg)	1.50 - 1.75	1.6 \pm 0.002
Volume density: β -cell /islet (%)	65.01 - 70.00	73.4 \pm 1.5
Total β -cell volume (mm ³)	0.10 - 0.15	0.12 \pm 0.003
Volume density: Islet/pancreas (%)	2.50 - 3.01	2.87 \pm 0.08
Total islet volume (mm ³)	2.50 - 2.9	0.93 \pm 0.002
Volume -weighted mean islet volume (mm ³)	0.85 - 1.00	0.95 \pm 0.006
Total islet number ($\times 10^3$)	0.10 - 0.15	2.67 \pm 0.012

NB> In this study positive pearson correlations were observed between the islet mass and the beta cell mass($r=0.77$, $p<0.0005$); volume density of the beta cell per islet and the volume density of islets per pancreas ($r=0.69$, $p<0.0035$); the total islet volume and the total islet numbers ($r=0.64$; $p<0.005$). However, the volume-weighted mean islet volume did not show statistical significance difference ($P>0.05$) with the group. Additionally, the pancreatic endocrine tissue per unit body weight and the pancreatic tissue per unit body volume was also statistically insignificant ($p>0.05$) when fetal endocrine histomorphometric values were compared within the group.

Figure 3A-D: Sections of normal 20-day old fetal pancreatic ducts system in albino rats.

- 3A:** A section of a fetal pancreas indicating the beginning of the duct system. Note the acinar cell (A-cells) that make up the acinus cluster (a) and the central lumen of the acini (C-lum of acini) that give rise to the secretory canaliculi (S-can) joining up to form the intercalated duct (Int-CD). Note the cuboidal intra acinar cell (I-AC-cells) that line the intercalated duct (Int-CD). *H & E Magx100*
- 3B:** A section of a fetal pancreas indicating the lobulations (Lob) and the intralobular ducts (Intr-LD) that unite to form the interlobular ducts (Int-LD). The interlobular ducts join up into the main pancreatic duct (MPD). Note the interlobular septa (ILS) dividing the lobules and the acini (a) distribution in the lobule. *H&E Magx40*
- 3C:** A section of a fetal pancreas illustrating the acinus (a) and the cuboidal epithelial (Cu-epith) lining of the Inter-lobular duct (Int-LD). Note the lobulations denoted by the Inter-lobular septa (ILS). Note the acinus (a) in each lobule. *H&E Mag x40*
- 3D:** A section of a fetal pancreas showing the structure of the main pancreatic duct (MPD). Note the thick layer of connective tissue (CT) lining the main pancreatic duct. Note the low lying columnar epithelium (Co-Epith) that lines the main duct. Note the lobulations and the inter lobular duct (Int-LD). *H&E Mag x40*

Figure 3 A-D:



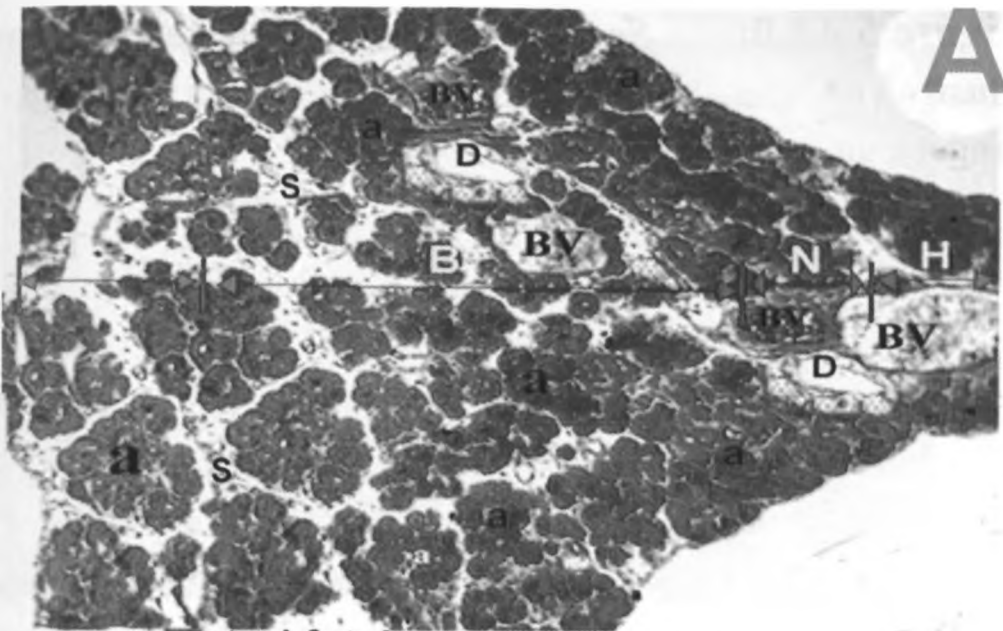
NB> sections from a 20 day old fetuses

Figure 4 A&B: Sections of a-20-day old fetal pancreas showing deposition of stromal tissue in an alcohol treated group compared with the control.

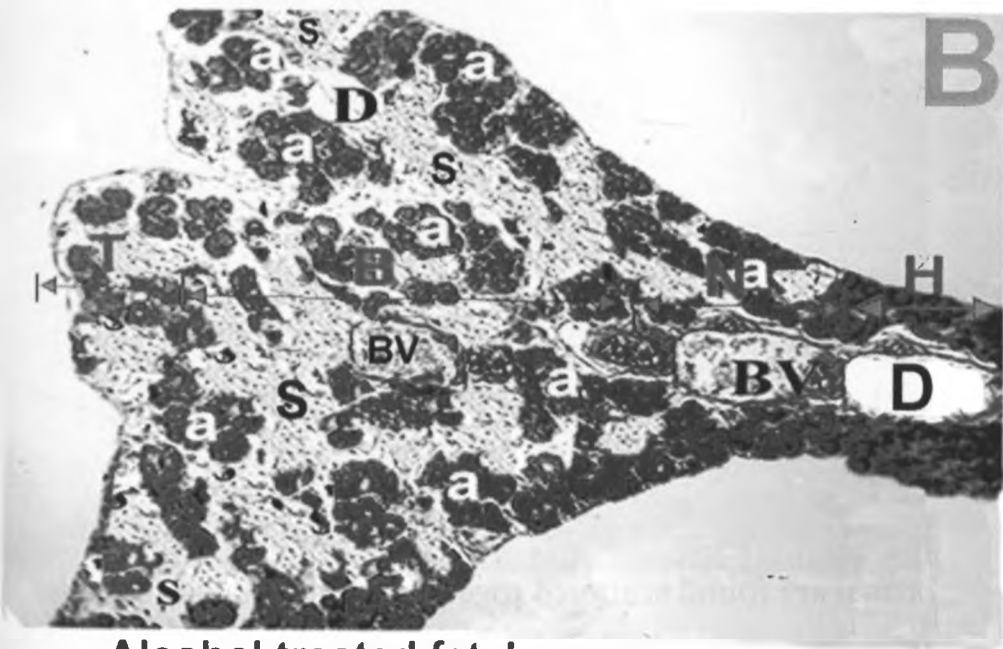
4A: A longitudinal section of a control fetal pancreas showing the regional distribution of stromal tissues (S) in the interacinar spaces, the acini clusters (a) and the central position occupied by the blood vessels (BV), ducts (D) in relation to the parts of the pancreas including the head (H), Body (B), Neck(N) and the tail (T) regions. *H &E Mag x10*

4B: A longitudinal section of an alcohol treated fetal pancreas showing the parts and the regional distribution of the fibrous connective tissue deposits. Note the extensive deposition of fibrocystic stromal tissue (S) in the inter acinar spaces and the position of blood vessels (BV) and ducts (D). Note the relative reduction in acini (a) clusters across the fetal pancreas in the head (H), Neck (N), Body (B) and the Tail (T) regions. *H&EMag x10*

Figure 4 A&B:



control fetal pancreas



Alcohol treated fetal pancreas

Figure 5 A&B: Section of a-20-day old fetal pancreas illustrating the histological structure of the islets of langerhans.

5A: A section of fetal pancreas showing the islets of langerhans in between the acini clusters. Note the distribution of the beta cells in the islet cluster that make up the core and the mantle of the islet cluster. Note the capsule like tissue (*marked with the green arrows*) that surrounds the islet cluster and separating it from the acini. *H&E Mag x10*

5B: Fetal islet of langerhans illustrating the histo-cyto-architecture of the specific islet cells in a cluster. Note that the Beta (β) cells are arranged in cord like structure of two columns with cells lying side by side. The alpha (α) cells that stain chocolate brown are found scattered together with the delta (δ) cells. The PP and the C- cells were mostly seen along the periphery of the islets. *Modified aldehyde fuchsin stain. Mag x1000*

Figure 5A&B:

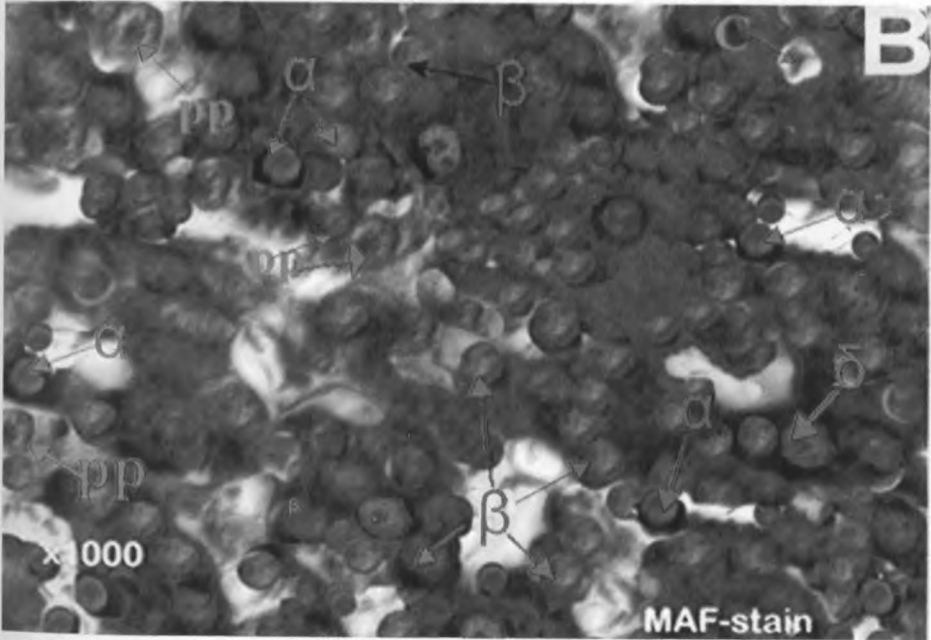
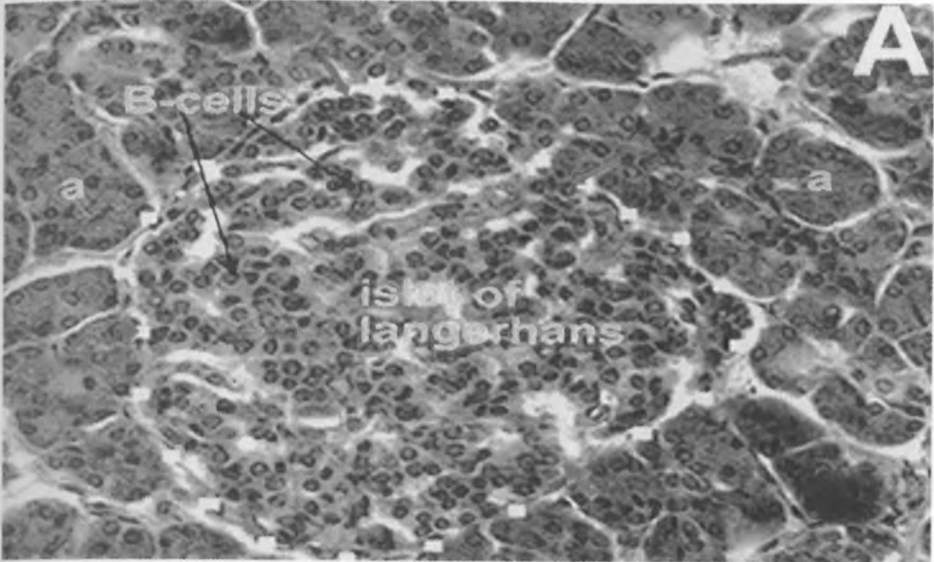
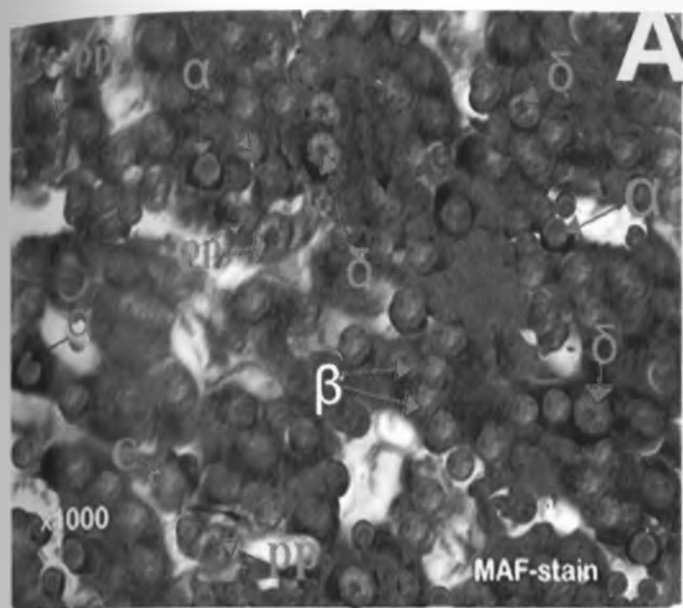


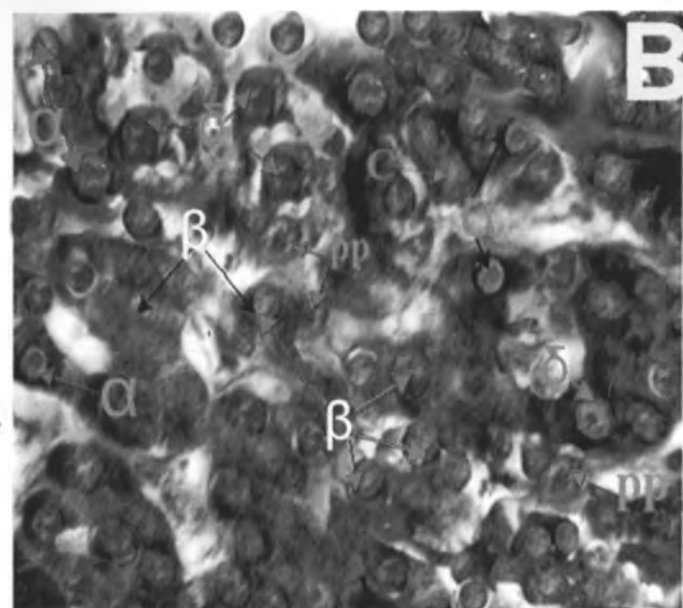
Figure 6 A-D: Sections of normal 20-day old fetal islets showing the PP-rich islets in the head and neck regions and the α -rich islets found in the body and the tail

- 6A: A section of control fetal islet showing the pp- rich islet observed in the head region. Note the numerality of the PP-cells that are mostly seen appearing in pairs. Also note the presence of beta cells (β) that in cord like cytoarchitecture along side alpha (α), delta (δ) and the C-cells (C). *MAFs Mag x1000*
- 6B: A section of a control fetal islets showing the pp-rich islet found in the neck region. Note the numerality of the pp-cells. Also note that the β -cells are more numerous than any other cell type in the islet including the alpha (α), delta (δ),beta (β) and the C-cells. *MAF- Magx1000*
- 6C: A section of control fetal islet showing an alpha rich islet observed in the body region. Note the numerality of alpha cells and paucity of the pp-cells as compared with 6A &6B. Note also the numerality of the other types of cells including beta (β), delta (δ) and the C-cells. *MAF- mag x1000*
- 6D: A section of control fetal islet showing an alpha rich islet observed in the tail region. Note the paucity of the pp-cells and the numerous alpha cells as compared with the 6A &6B. Note presence of the other types of cells including beta (β), delta (δ) and the C-cells. *MAF - mag x1000*

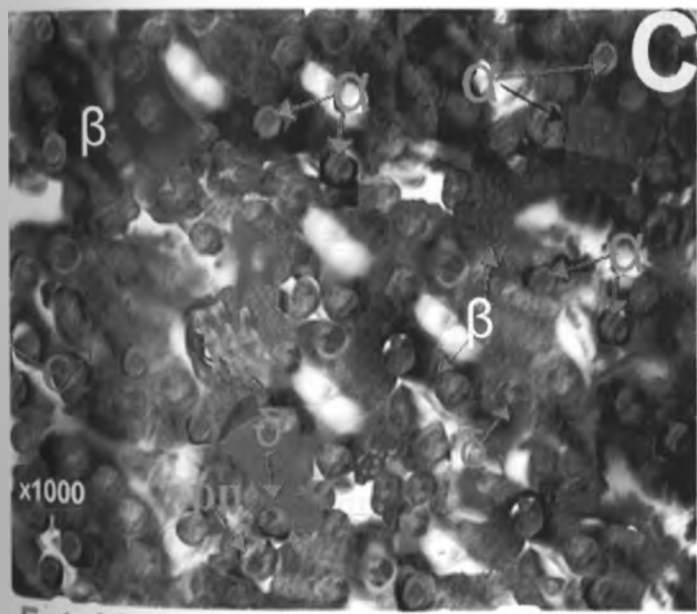
Figure 6 A-D:



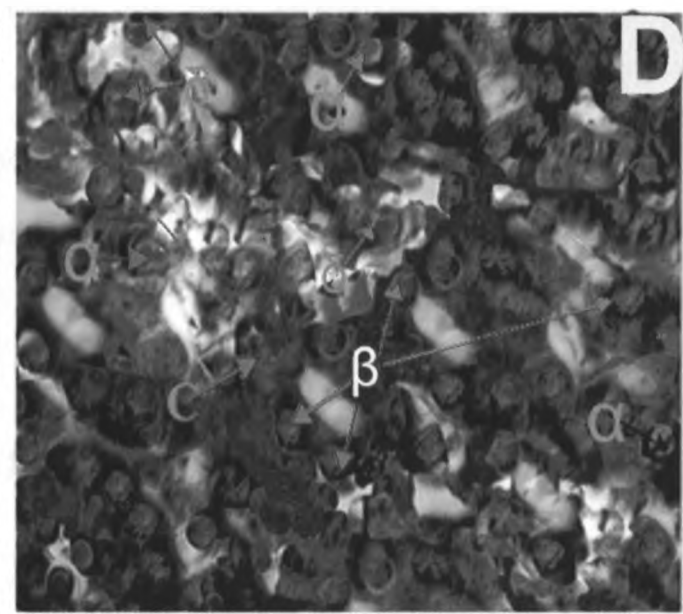
Fetal islet at the head region



Fetal islet at the neck region



Fetal islet at the body region



Fetal islet at the tail region

3.2.0 The prenatal effects of alcohol exposure on the morphology of fetal pancreas

In this study, it was observed that prenatal exposure to alcohol altered the normal patterning of the fetal pancreatic parenchyma with specific structural defects in both the exocrine and endocrine cells. The observed effects were both intra and intercellular alterations including disorganization of intracellular organelles, membrane alterations and secretory impairments. The main effects involve disruptions of acinar clustering, reduced number of acini, acinar cell substructure alterations, organelles and the duct system. On the endocrine pancreas, alcohol reduces the size, number and impairs the cellular composition of the islets of langerhans. These alterations are both qualitative and quantitative varying with time of alcohol exposure and the intragastric doses administered to the mother during pregnancy. Additionally, the fetal pancreatic defects were seen to have an inverse correlation with fetal weights and the alcohol doses.

3.2.1 The effects of alcohol on fetal acini clusters and connective tissues

In the exocrine pancreas, alcohol caused reduction in the numbers of acinar cells per acini cluster as well as reduction of acini clusters per field (*Table 5*). It also caused reduction in size of the cellular components hence the observed reduction

in the volume densities of acini blood vessels and ducts. On the acini, the morphological effects include; delayed maturation of acinar cells, impaired clustering of acini with reduction in numerical counts of acini clusters per field (*Figure 7A-D*). It also caused reduction of acini cells per cluster that was seen to depict regional variation (*Table 5*). The reduction in acini clusters and the delay in canalization of acini were seen to depict regional variations (*Tables 5, 6*). The tail and body suffered highest reduction of acini while the head and neck were not as greatly affected (*Figure 7a-b*). The average counts of acini clusters per field ranged between 15-23 islets cluster per field in the head, 10-15 in the body, 9-15 in the tail for the alcohol treated groups, which was significantly lower ($p < 0.05$) compared with 25-35 in the head and neck, 20-30 in the body and 15-20 in the tail for the control (*Tables 5,6*).

Alcohol delays maturation and canalization of acini (*Figure 7A-D*). The mean numerical counts of canalized acini per field ranged between 5-10 in the head and neck, 10.0-15.0 in the body and 3.5-4.5 in the tail in the control as compared with 3-9 in the head, 5-9.5 in the body and 2-6 in the tail region in the low alcohol treated groups (*Table 7*).

Table 5: The comparative numerical counts and means of the acini clusters per field in the alcohol treated groups compared with the control

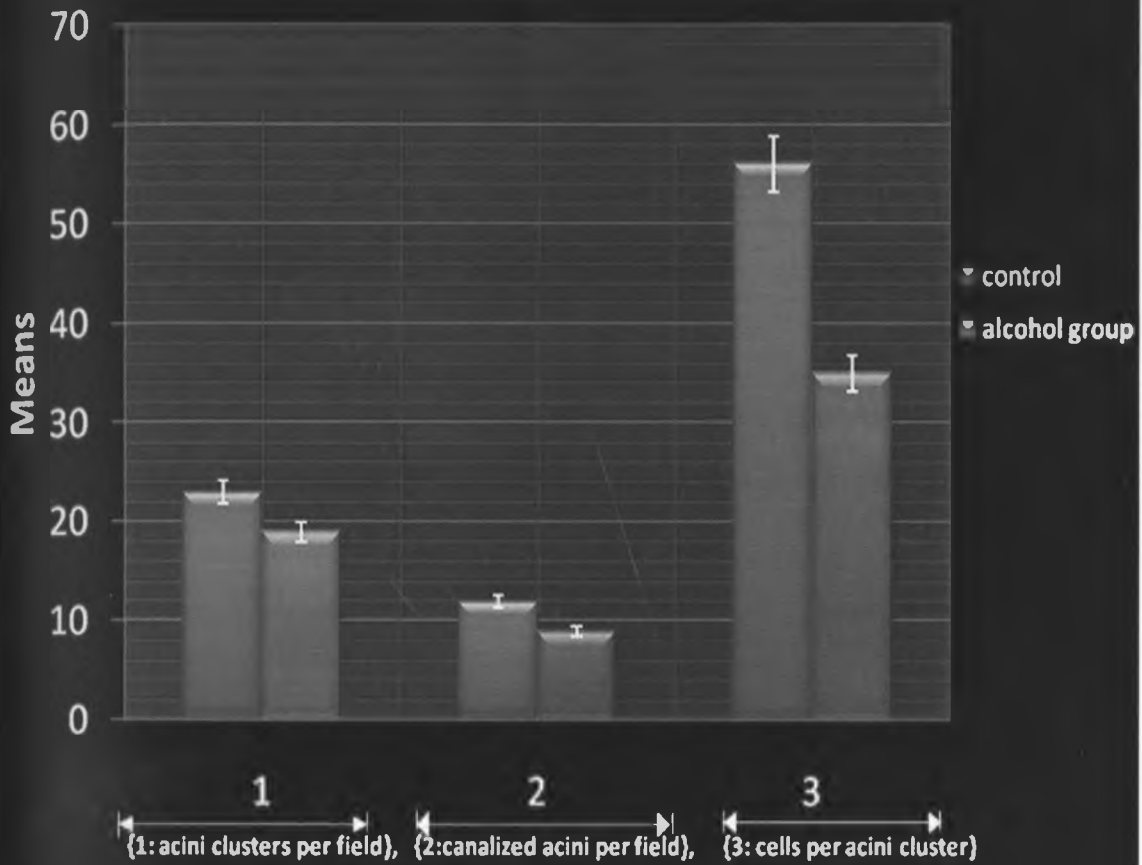
Study group	Parameters	Acini per field with regional distribution							
		Head		neck		body		tail	
		Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Control group	No. of acini per field	25-35	28.7±0.3	25-29	26.1±0.7	20-30	22.6±0.2	15-20	17.8±0.9
Alcohol treated group	No. of acini per field	20-30	27.9±0.7	15-20	25.1±0.9	16-25	19.6±0.2	10-15	13.8±0.6
	P-value		0.049		0.031		0.027		0.038

Table 6: The ranges and the means of canalized acini per field in the head, neck, body and the tail regions

Study group	Parameters	The ranges and means of canalized acini per field							
		Head		neck		body		tail	
		Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Control:	Canalized acini per field	5-10	7.6±0.3	5-12	7.1±0.4	10-16	12.6±0.2	8-15	11.8±0.9
Alcohol Treated groups (5mg/kg/Bw)	Canalized acini per field	4-7	5.2±0.5	4-9	6.7±0.8	8-14	9.6±0.7	2-6	5.7±0.8
	P-values		0.0477		0.013		0.0214		0.029

NB> The distribution of canalized acini and the number of acini per field in the alcohol treated group are statistically significant ($p < 0.05$) when compared with the control by both Chi-square test and with ANOVA.

Graph 1: A bar graph showing the numbers of acinar cells per cluster, acini clusters and canalized acini clusters per field between the control and the alcohol treated groups.



NB> Note the differences in the numbers of canalized acini, the number of acini clusters per field and the numbers of acinar cells per cluster between alcohol treated groups and the control.

The alcohol treated groups exhibited a 12.62% reduction in volume densities of acini and 6.5% in the blood vessels and ducts while the deposition of fibrous connective tissues stroma increased by 17.09%. These quantitative effects on acini, blood vessels and ducts were seen to be statistically significant ($p < 0.005$) by both ANOVA and the Chi square tests when a comparative non parametric Mann whitenny U-test was applied (*Table 7*). The U-values and the probabilities of occurrence of U-value on the median values across the study groups were also statistically significant ($p < 0.05$). An exponential reduction in the volume densities of the acini, connective tissues, blood vessels and duct was noted in the alcohol treated groups when compared with the control (*Graph 2*).

Table 7: The non-parametric Mann Whitennys U-test on the fetal stereological parameters of the acini, connective tissues, pancreas and fetal weights between the experimental and the control groups

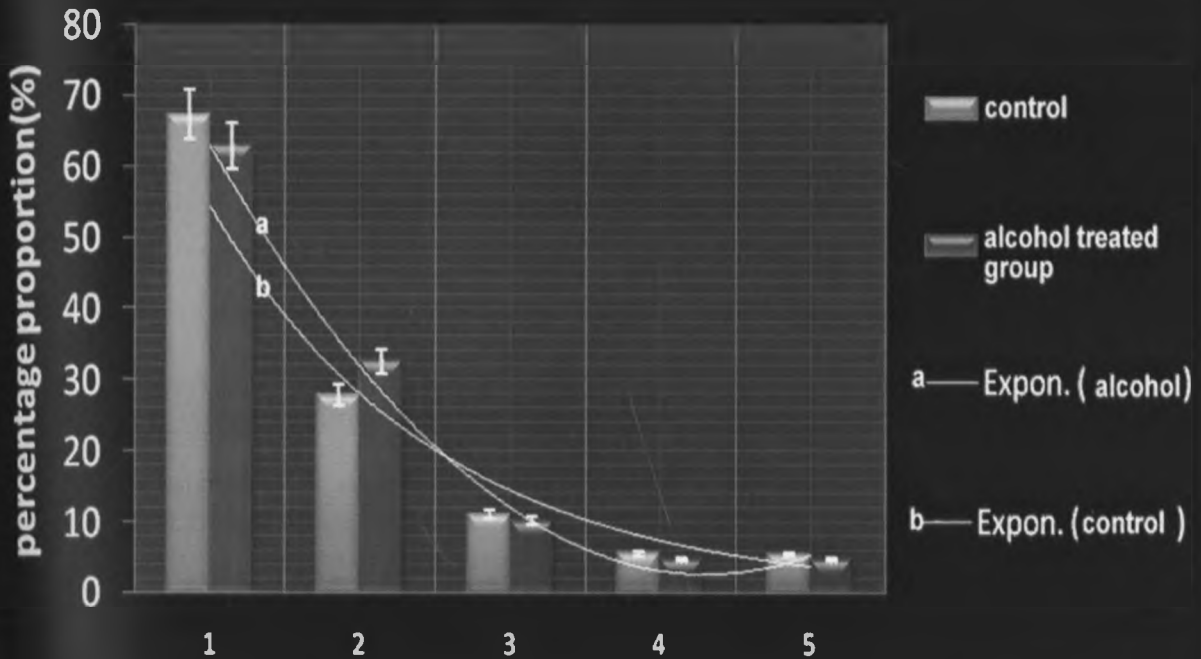
Parameter	Means \pm SE		Mann whitenny U values-Alcohol group (MAG) versus the control groups			
	control	Alcohol group	The median values Used		[U] Calc	P[U]
			control	Alcohol		
Volume density of acini (%)	67.35 \pm 0.92	62.92 \pm 0.61	67.12	63.4	0*	0.004
Volume density BV and ducts (%)	5.49 \pm 0.03	4.83 \pm 0.002	5.33	5.00	0*	0.004
Vol. density fibrous CT (%)	27.90 \pm 0.09	32.67 \pm 0.04	29.27	31.35	2*	0.042
Pancreas wet weight(mgs)	11.14 \pm 0.01	10.26 \pm 0.02	11.01	10.53	2*	0.016
Fetal body weight(gms)	5.67 \pm 0.19	4.93 \pm 0.16	5.63	5.12	0*	0.004

* U-values whose P- value was less than or equal to 0.05

U-calc = calculated Mann-whiteny U-value

P[U]= probability of occurrence of calculated U value

Graph 2: A comparative percentage proportion of the acini, connective tissues, pancreas and fetal body weights between the experimental and the control groups



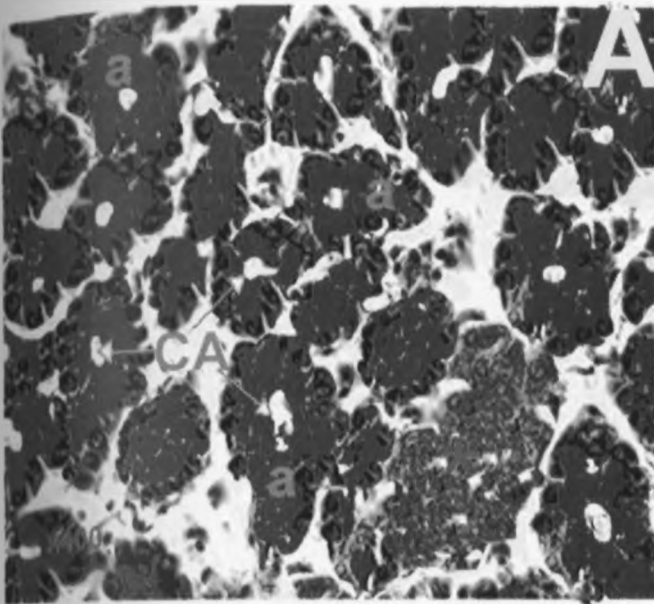
The study parameters: 1=vol. density acini; 2=vol. density Fibrous CT; 3=pancreas wgt; 4=fetal weight; 5=Vol density BV and ducts

NB> Note the exponential differences in the volume densities of acini, fibrous connective tissue deposits, blood vessels and ducts between the alcohol treated group compared with the control.

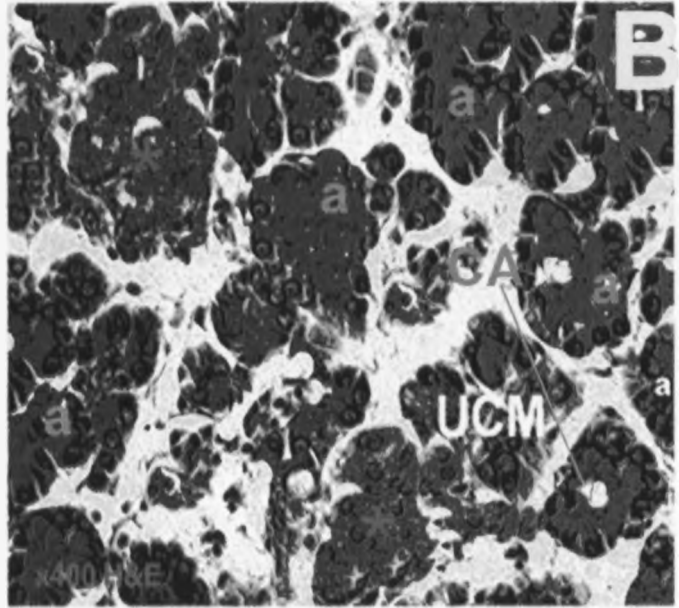
Figure 7 A-D: Sections of a-20-day old fetal pancreas of the alcohol treated groups (MAG) illustrating delayed maturation and canalization of acini compared with the control

- 7A: A section of a control fetal pancreas showing the morphology of acini in the head and neck region. Note an even distribution of mature acini with many canalized acini (CA) per the field. Note the classical aggregation of the islets of langerhans [*as indicated by a star (*)*]. *H&E Mag x40*
- 7B: A section of alcohol treated fetal pancreas showing the morphology of the acini in the head and neck regions. Note the relative reduction in acini clusters (a) as well as the canalized acini (CA). Note the delayed cytodifferentiation of the cells depicted by presence of undifferentiated cell masses (UCM). Note the poorly developed islet clusters [*as indicated by a star (*)*]. *H&E Magx40*
- 7C: A section of a control fetal pancreas showing the morphology of the pancreatic acini at the body and tail region. Note an even distribution of mature acini and presence of canalized acini (CA) in the field. Note the classical aggregation of the islets of langerhans [*as indicated by a star (*)*]. *H&E Magx 40*
- 7D: A section of an alcohol treated fetal pancreas showing the morphology of the acini in the body-tail region. Note the relative reduction in acini clusters (a) as well as reduction in the canalized acini (CA). Note the delayed cytodifferentiation of the cells depicted by presence of undifferentiated cell masses (UCM). Note the poorly developed islet clusters [*as indicated by a star (*)*]. *H&E MagX40*

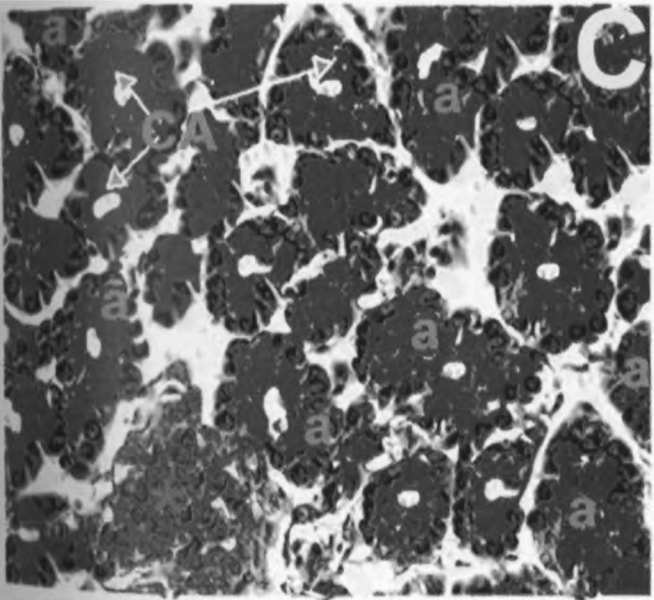
Figure 7 A -D:



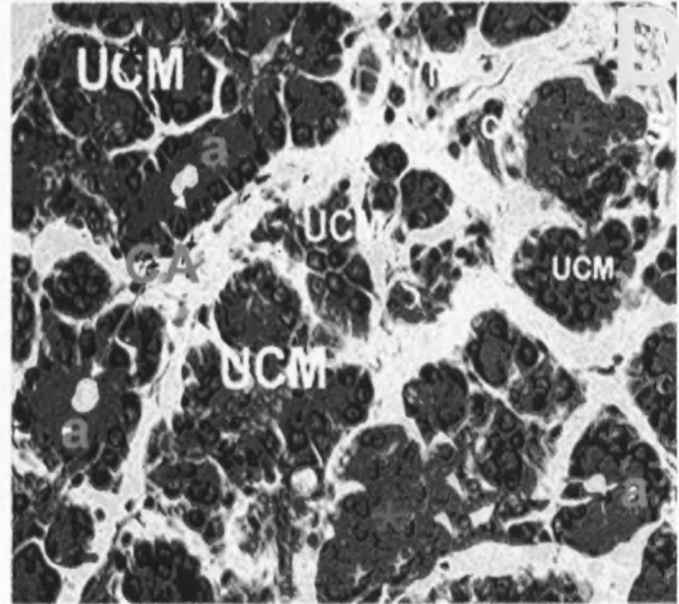
Control (head and neck regions)



Alcohol treated group (head and neck region)



control (body and tail region)



Alcohol treated group (Body and tail regions)

3.2.1.1 Effect of alcohol on acini cytoplasmic granules and staining

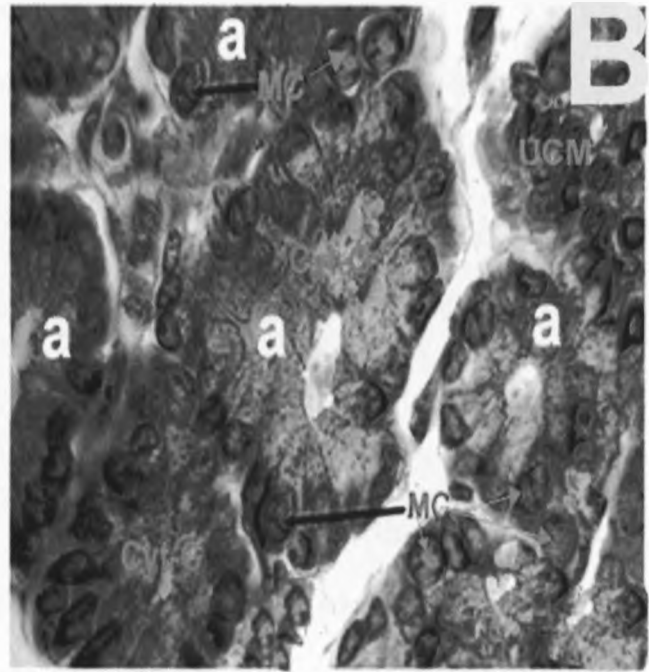
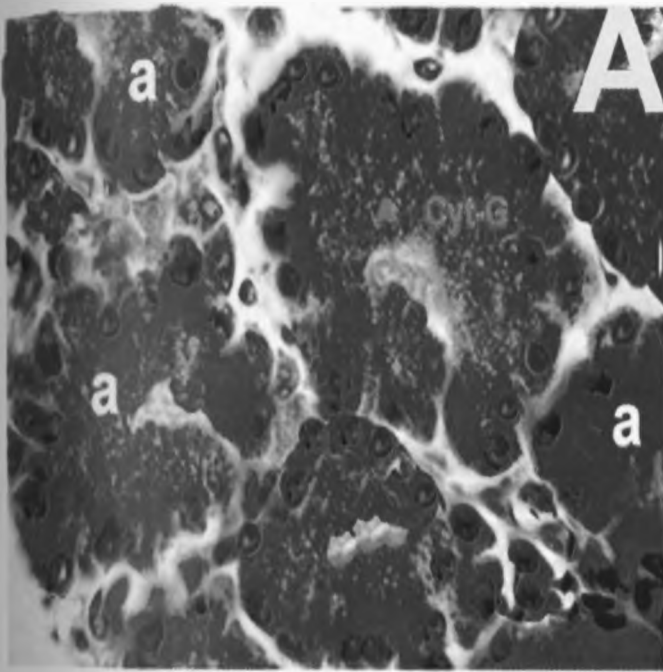
Alcohol was observed to interfere with the uptake of acidophilic stains making the cytoplasm of the alcohol treated groups appear basophilic as compared with the control cytoplasm that appeared acidophilic (*Figure 8a& b*).

Figure 8A & B: Sections of a-20-day old fetal acini illustrating acidophilic cytoplasm in the control compared with basophilic cytoplasmic staining in the alcohol treated group

8A: A section of control fetal acinar cells showing acidophilic cytoplasmic granulations (Cyt-G) in the acini (a). Note the well organized acini cells. *H&E Magx1000*

8B: A section of alcohol treated fetal pancreas showing basophilic cytoplasmic granulations (Cty-G) in the acini (a). Note the presence of cell showing mitotic activity (MC) with enlarged nucleus. Note the presence of undifferentiated cell mass (UCM). *H&E Mag x1000*

Figure 8A & B:



3.2.1.2 **The ultrastructural effects of alcohol on the morphology of fetal acini cells**

Ultrastructurally, alcohol was observed to interfere with the secretory granules and the morphological organization of the organelles in the acini cells (*Figure 9B, D & F*). It was seen to cause structural disintegration of the membrane binding the organelles responsible for the metabolic functions and sequestering of secreted granules including the rough endoplasmic reticulum (RER), and the golgi complex apparatus (*Figure 9D*). This disintegration of the cytoplasmic organelles was marked by the presence on focal points of cytoplasmic degenerations that contained myloid structures or homogenous dense bodies (*Figure 9D*).

Alcohol also caused lysosomal fragility and disorganization of the cell membrane as well as increase in lipid like vacuolations (*Figure 9F*). Alcohol also caused mitochondrial membrane fragmentation as well as interfering with inner folding of the mitochondrail membrane in the formation of cristae (*Figure 12a-b*).

The impaired secretory functions of the cell were also displayed by the reduction of cytoplasmic secretory granules (zymogen granules-ZG) that were poorly developed and depicted irregular binding membrane the "immature form" as compared with the "mature" form that were well developed and membrane bound in the control groups (*Figure 10a-d*). The numbers of "immature" granules

were also seen to increase linearly with increasing alcohol doses (*Figure 10a-d*). Alcohol also caused an increase in cytoplasmic lipid vacuolations (*Figure 10a-d*). In the ductular cells, it was observed to cause both cytoplasmic and nucleoplasmic vacuolations and disintegration of cell organelles (*Figure 9f, and Figure 11b,c&d*).

Figure 9A-F: The fetal acini cells illustrating alterations to the sub-cellular organelles in the alcohol treated groups compared with the controls

- 9A:** Pancreatic acinar cell from a control fetal pancreas showing the densely packed cytoplasmic membrane bound zymogenic granules (ZG) with closely parked stacks of granular rough endoplasmic reticulum (RER). Note the well developed cell nucleus (C-N). *Mag X13,800*
- 9B:** Pancreatic acinar cell of an alcohol treated fetal pancreas illustrating the presence of sparsely distributed immature zymogenic granules (ZG). Note the disintegrated rough endoplasmic reticulum (RER) near the cell nucleus (C-N). *Mag X13,800*
- 9C:** Pancreatic acinar cell from a control fetus. Note a well arranged parallel lamellae of the granular endoplasmic reticulum (RER) that occupy the basal region of the cell and conspicuous mitochondria (M). Also note the cell nucleus (C-N). *Mag x13,800*
- 9D:** Pancreatic acinar cells of an alcohol treated group showing the focal points of cytoplasmic degeneration (FP-D), note the disintegrated rough endoplasmic reticulum (RER) with free ribosomes (*indicated by the red arrows*) and lysosomal fragility (LYS) alongside sparsely parked and inconspicuous Golgi apparatus (GA). *Mag 13,800*
- 9E:** Pancreatic acinar cell from a control group. Note the numerous and well developed zymogenic granules (ZG) Mitochondria (M), Golgi apparatus (GA), cell nucleus (N). *Mag x13, 800*
- 9F:** Pancreatic acinar cell from an alcohol treated group. Note the high presence of lipid vacuolations (LV) and cell membrane degeneration (*shown by the red arrows*). Note the presence of lysosomal fragility (Lys-F) sparsely distributed Zymogen granules (ZG) and the irregularly bound membranous structures including Mitochondria. *Mag X13,800*

Figure 9 A-F:

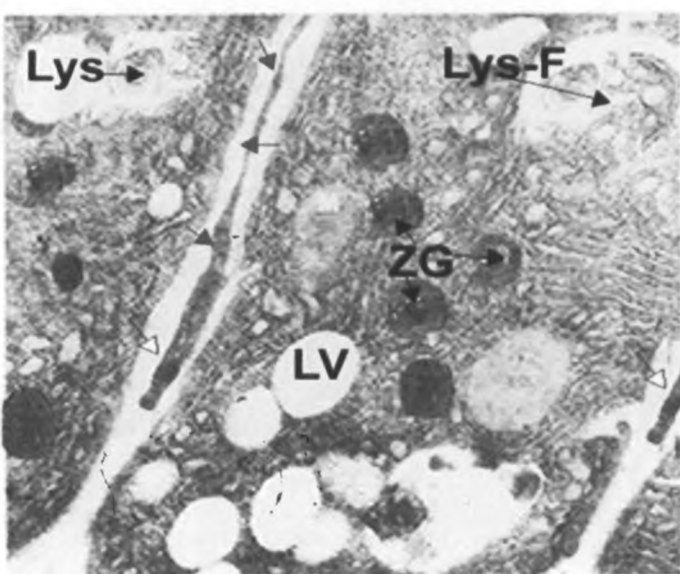
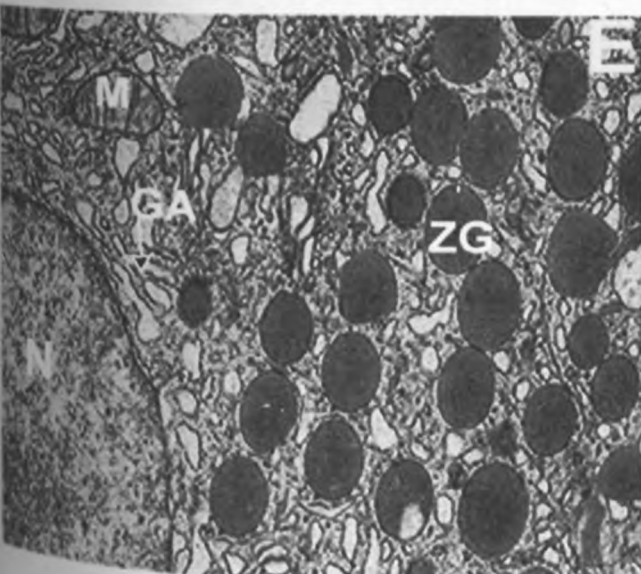
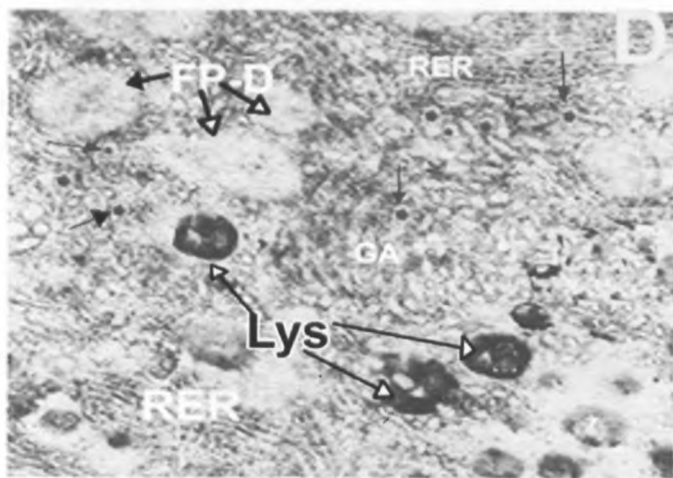
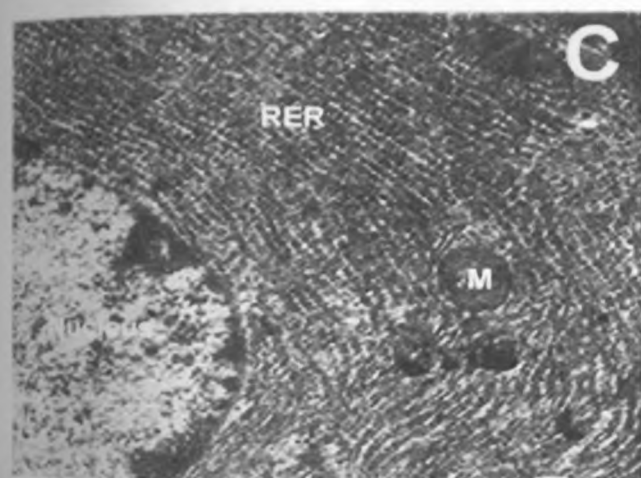
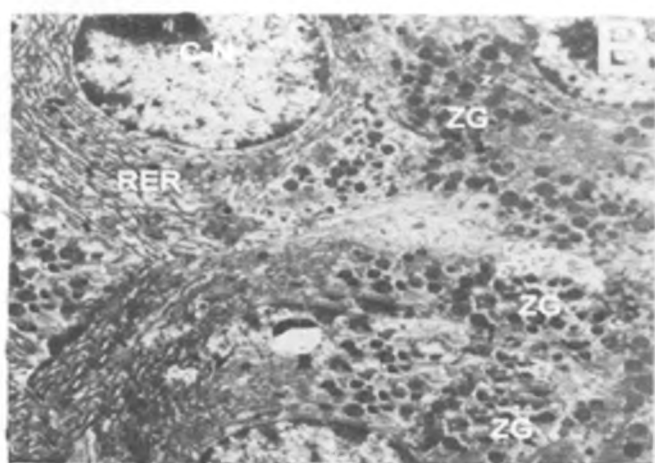
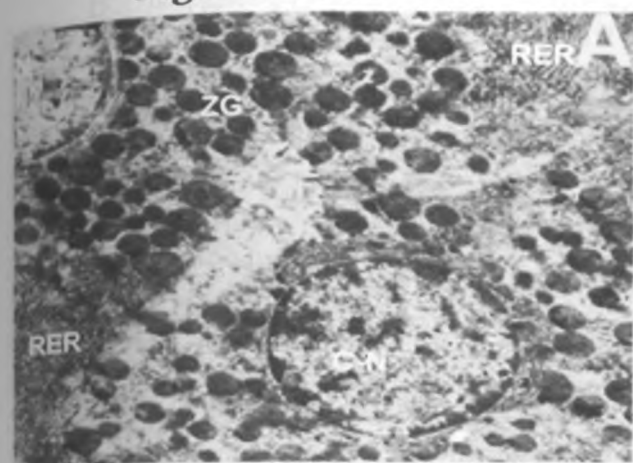
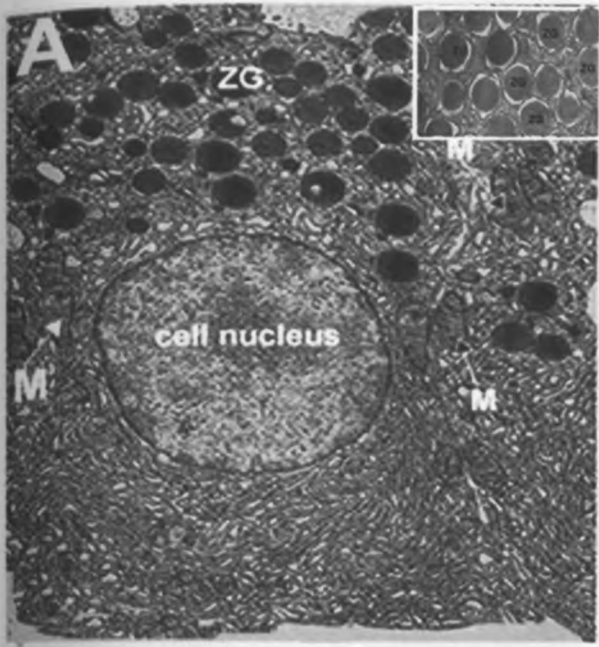


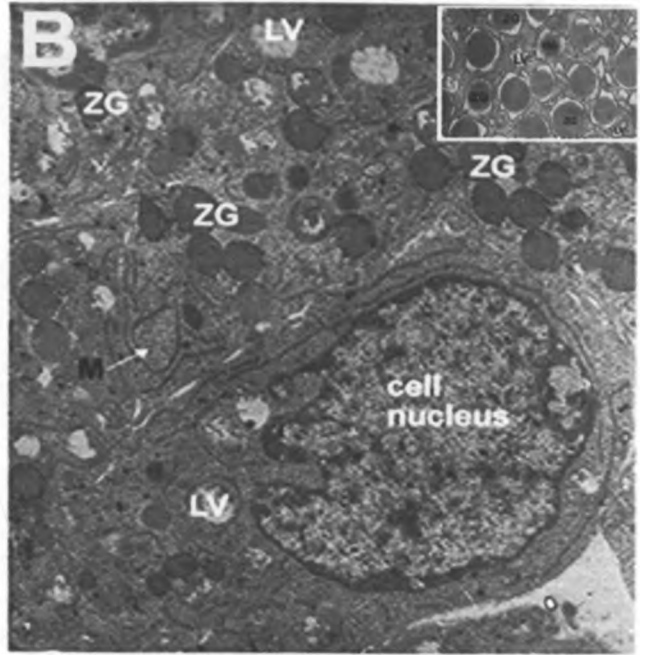
Figure 10 A-D: Fetal acini cells illustrating alterations to secretory granules in the alcohol treated groups compared with the control.

- 10A:** The ultrastructure of a control fetal acini cell. Note the numerous zymogenic granules (ZG) at the apical portion of the cell. Also note the presence of the mitochondria (M). Note the "mature" form of the secretory granules that are bound by a continuous membrane as shown in the insert. *Mag x 13,800*
- 10B:** An ultramicrograph of a fetal acini cell from low alcohol group (LAG). Note the presence of the lipid vacuolations in the cell cytoplasm (LV), the paucity and "immature" form of zymogenic granules that are bound by a discontinuous membrane as shown *in the insert*. *Mag x13,800*.
- 10C:** Ultramicrograph of a fetal acinar cell from medium alcohol group (MAG) showing high presence of lipid vacuolations (LV), many irregularly bound zymogenic granules (ZG)-(*in the insert*) and discontinuous basal plasma membrane(BaPM). Note the presence of chromatin granules in the nucleoplasm a sign of mitotic activity or arrested development. *Mag x13,800*.
- 10D:** A ultramicrograph of a fetal acinar cell from high alcohol group (HAG). Note the high presence of lipid vacuolations (LV), highly reduced zymogenic granules (ZG) *as shown in the insert*. The cell organells are inconspicuous a sign of interfered development. *Mag x 13,800*.

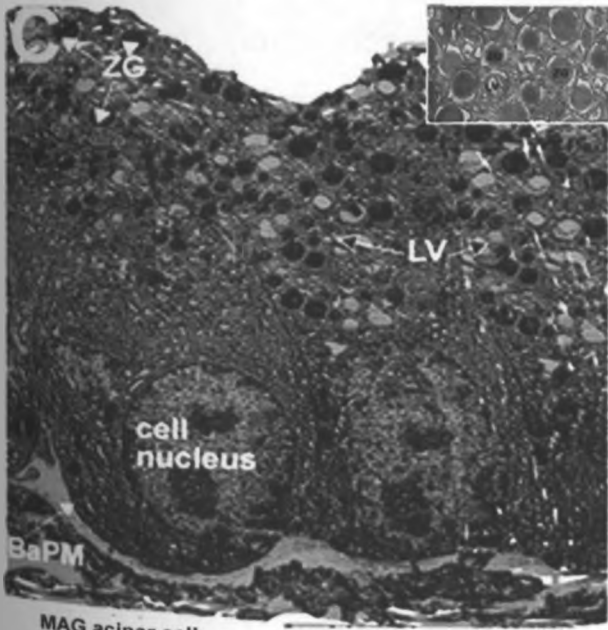
Figure 10 A-D:



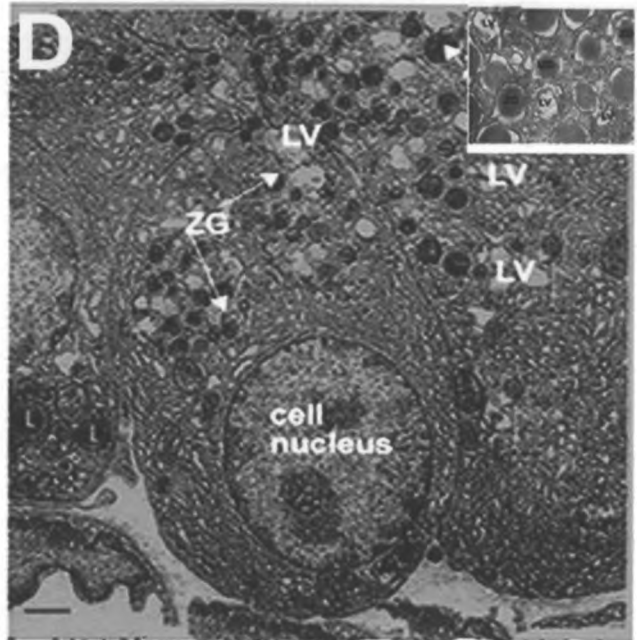
control fetal acinar cell



LAG group acinar cell



MAG acinar cell



HAG acinar cell

Figure 11A &B: Sections of fetal pancreas showing effects of alcohol on fetal ductular cells.

11A: Electron micrograph of an alcohol treated fetal ductular cell showing nucleoplasmic and cytoplasmic lipid vacuolations (LV). Note the chromatin granules in the nucleus as sign of mitotic activity (ma). The mitochondria (M) and highly reduced in these ductular cells that are tightly bound by the lateral plasma membrane (LaPM). Note the Zymogen granules (ZG) in the apical portion of the acini cells. TEM-Mag $\times 13,800$

11B: Electron Micrograph of an alcohol treated group showing disintegration of intracellular organelles of a ductular cell. Note the disorganized golgi complex apparatus (GA), rough endoplasmic reticulum (RER), Mitochondria (M), lipid Vacuolations (LV), immature secretory granules (SG) and disintegrating Lysosomes (Lys) . TEM-Mag $\times 13,800$

11C: Ultramicrograph of a fetal pancreas from an alcohol treated group showing disorganization of cytoplasmic cell organelles with fragmentation of the cell membrane (*red arrows*), disintegrating of lysosomal fragility (Lys-F), and disorganization and fragmentation of the Golgi complex apparatus (GA-f). Note the extensive lipid vacuolization in the cytoplasm (LV). TEM-Mag $\times 13,800$

Figure 11A-D:

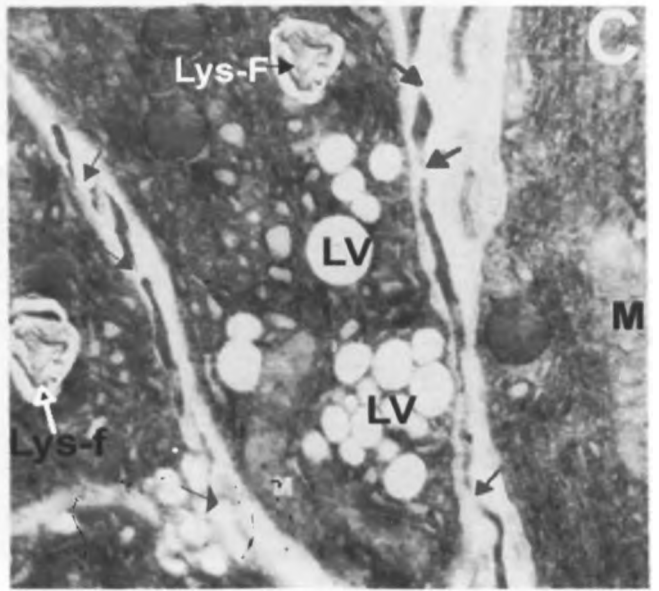
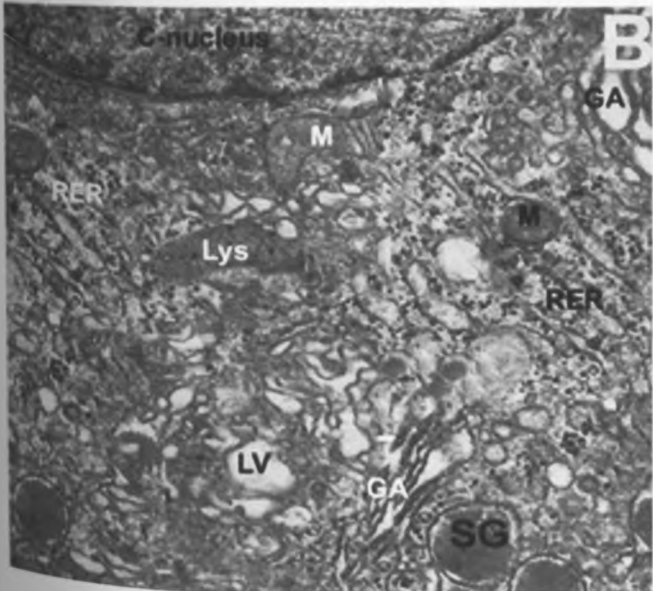
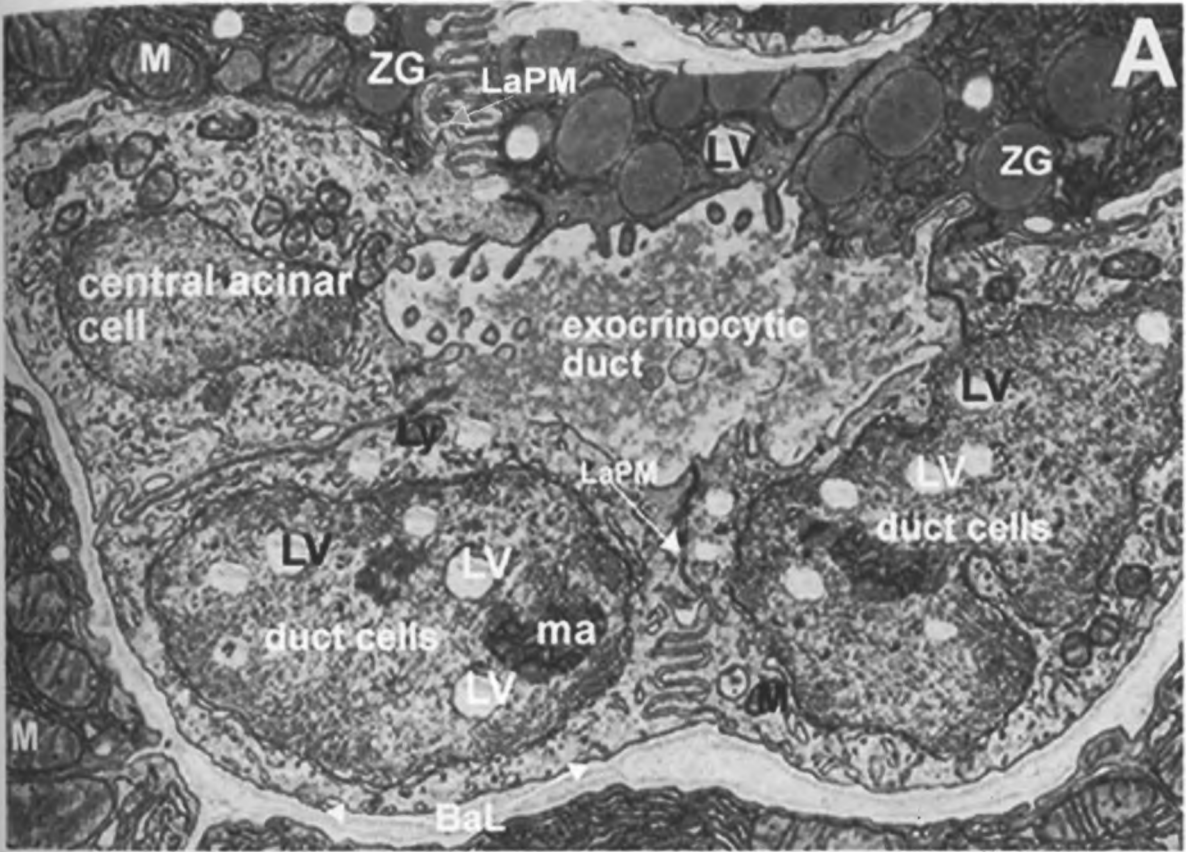


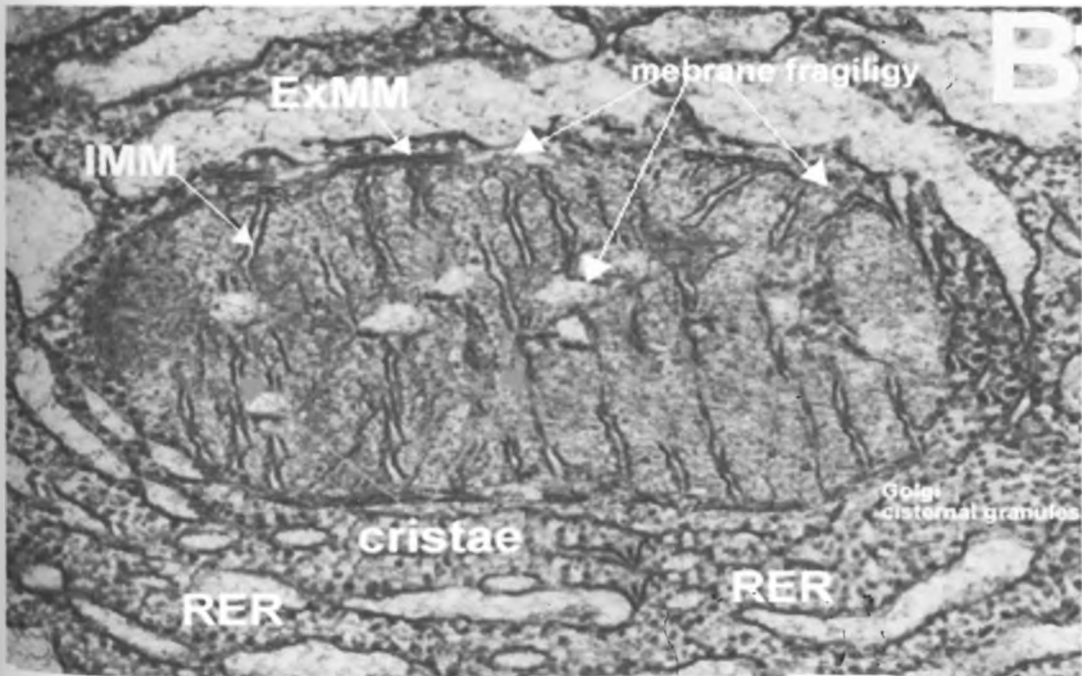
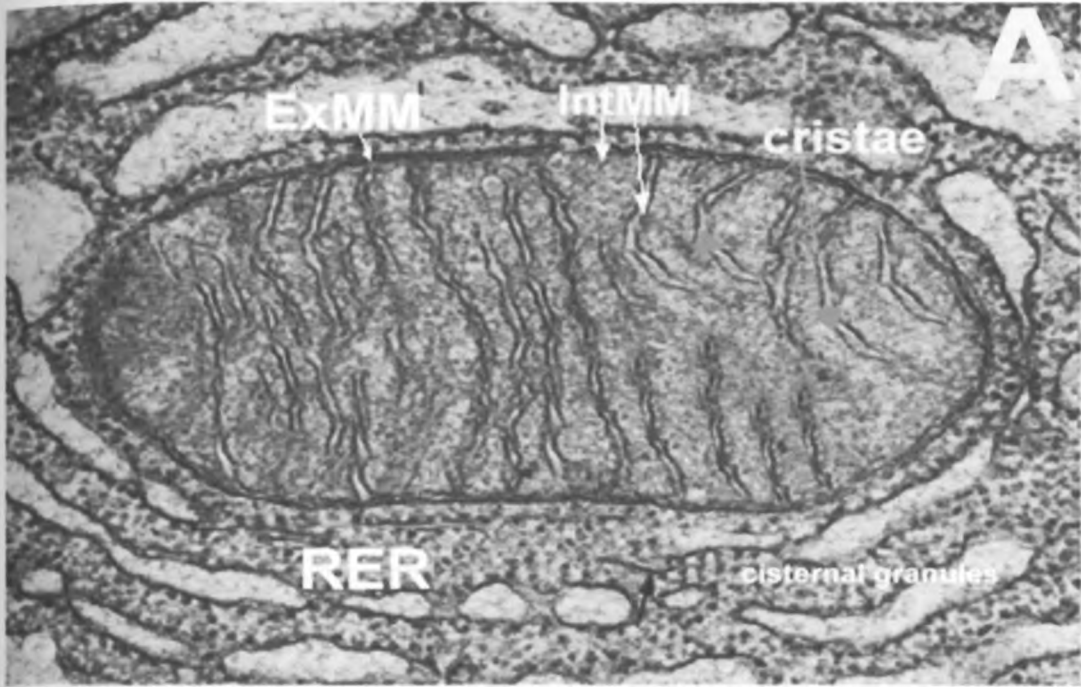
Figure 12A &B:

Mitochondria of the control (A) and that of an alcohol treated group (B). illustrating membrane fragility

12A: -A control fetal mitochondria showing a well developed internal and external mitochondrial membranes (*IntMM* and *ExtMM*), a well developed rough endoplasmic reticulum (RER) with conspicuous intra cisternal granules. *TEM-Mag x 52,200*

12B: The alcohol treated fetal mitochondria. Note the membrane fragility in the cristae as well as to both the external and internal mitochondrial membranes (*IMM* and *ExMM*). Note the disorganized rough endoplasmic reticulum (RER) adjacent to the mitochondria. *TEM-Mag x52,200*

Figure 12 A&B:



3.2.2. Effects of alcohol on the fetal pancreas duct system

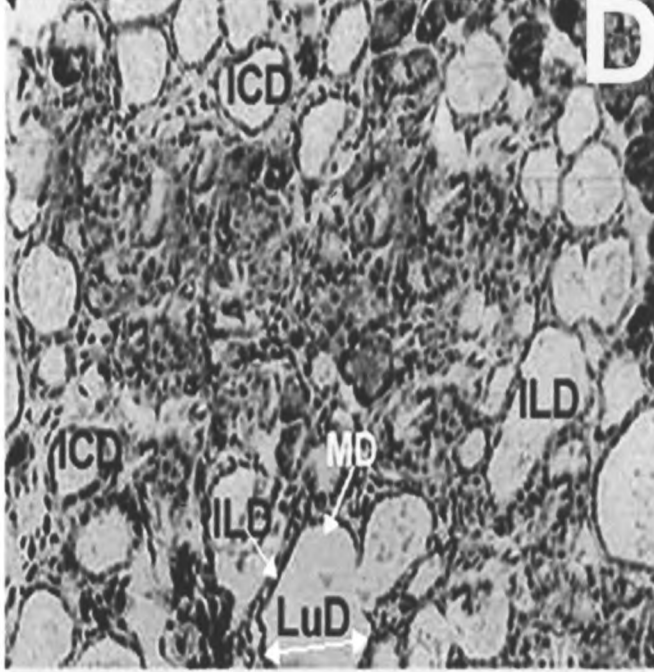
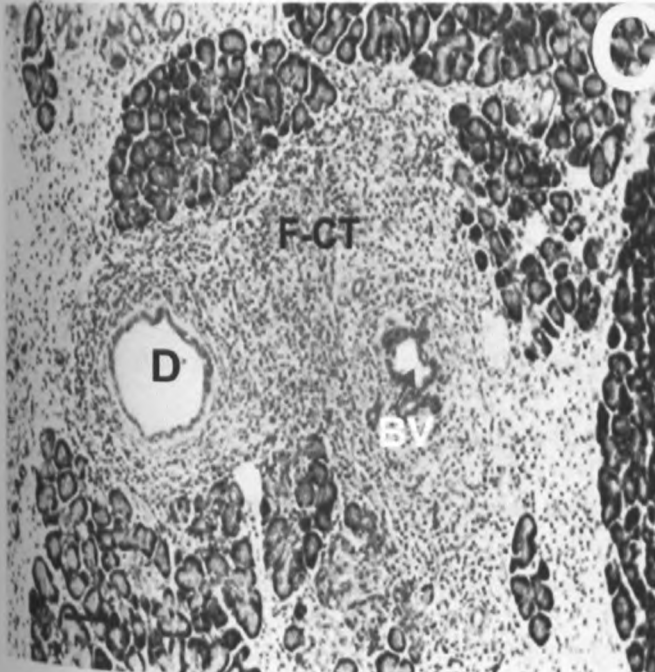
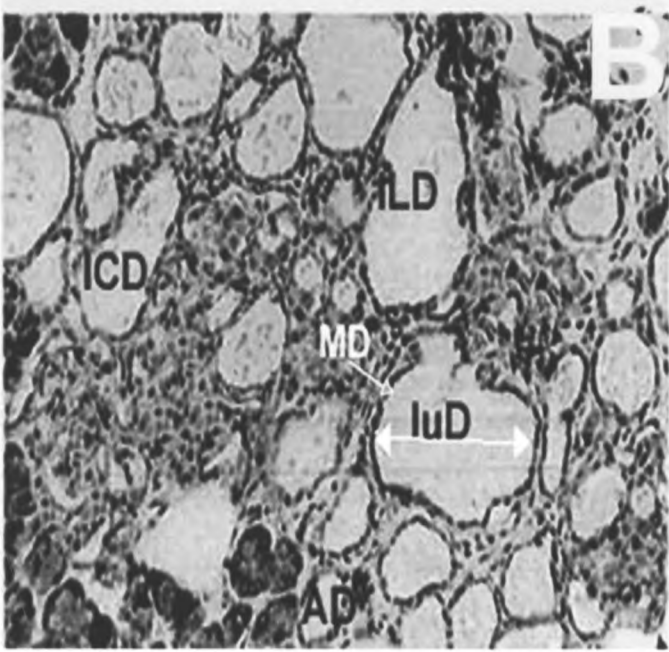
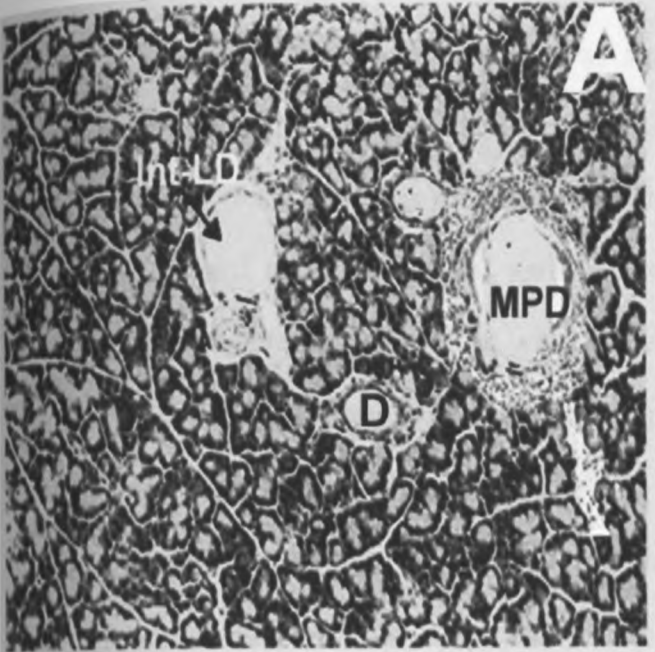
Prenatal alcohol exposure caused disorganization of the normal pattern of the duct system and reduced the luminal diameters of intralobular, interlobar and the main pancreatic ducts (*Figure 13A-D*). The luminal diameters of the intralobular ducts ranged between 1.34-1.87mm in the control and 1.01-1.44 mm in the alcohol treated groups while the luminal diameters of interlobular ducts ranged between 2.0-2.75mm in the control and 1.89-2.55 mm in the alcohol treated groups. The main pancreatic ducts recorded the widest diameters of between 4.80-5.01mm in the control and 4.61-4.93 mm in the alcohol treated groups. The intra and intergroup comparisons of the mean luminal diameters between the control and the alcohol treated groups were statistically significant ($P < 0.005$) by both ANOVA and the chi square test (*Table 8*).

Table 8: The mean luminal diameters of the ducts in the alcohol treated groups compared with the control.

Duct parameters	Main pancreatic ducts		Inter-lobular ducts		Intralobular ducts	
	control	Alcohol group	control	Alcohol group	control	Alcohol group
	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Luminal diameters (mm)	4.92±0.04	4.43±0.02	2.54±0.02	2.22±0.03	1.52±0.01	1.33±0.01

Strong positive linear correlations [$\rho(r)=0.517$; $\rho(r)=0.704$; $\rho(r)=0.712$] in the low, medium and high alcohol groups respectively was established between the luminal diameters of the duct and their volume densities i.e. the luminal diameters of the ducts reduced linearly with their reducing volume densities. On the other hand, a strong negative Pearson correlations [$\rho(r)=-0.712$; $\rho(r)=-0.619$ $\rho(r)=-0.679$ respectively] was established when luminal diameters and the volume densities were compared with the alcohol doses.

Figure 13A-D:



3.2.3 Effects of alcohol on fibrous connective tissue deposition

Prenatal exposure to alcohol was seen to influence increased deposition of fibrous connective tissues in the inter-acinar spaces and in the periductular areas of the fetal pancreas (*Figure 14C*). The deposition of the stromal tissue was observed to have regional variation, with the body and the tail suffering the greatest effects as compared with the head to the neck regions (*Figure 14D*).

At ultra-structural level, the fibrous stromal deposits in the inter-acinar spaces was observed in areas with “*activated*” stellate cells that appeared enlarged and triangular in shape occupying the entire inter-acinar space. Alcohol seemed to prime the stellate cells to change from their “*quiescent*” state to an “*activated*” state leading to secretion and deposition of fibrous connective tissues in the inter acinar space (*Figure 15*).

The volume densities of the connective tissue deposit increased significantly ($p < 0.05$) in the alcohol treated group when compared with the control recording a mean increase in the deposition of connective tissues of 17.09% (*Table 9*).

An inverse linear correlation ($\rho(r) = -0.689$; $p < 0.001$) between the volume densities of fibrous connective tissue deposit and the reduction in the volume density of the acini was found to exist in the alcohol treated groups. A Pearson correlation between the fibrous stromal deposits and the fetal pancreatic parenchymal parameters was seen to exist (*Table 10*).

Table 9: The mean comparative percentages of fibrous connective tissues deposits in the high alcohol treated groups compared with the control

Connective tissue (CT) parameters	Control	Alcohol group	P-values
	Mean+SE	Mean+SE	
Point counts per section	8.2+1.04	11.4+0.02	0.037
Volume densities of CT percentage (%)	22.90+0.01	35.67+0.09	0.042

NB> There was a 33.17% increase in fibrous connective tissue deposition in the high alcohol treated groups compared with the control.

Table 10: Pearson correlations between volume densities of the fibrous connective tissues and the pancreatic parenchyma in the alcohol treated groups

Time of Alcohol exposure N=24		Parenchymal Parameters			
		Pancreas weight	Total parenchymal volume[mm ³]	Volume density of acini	Volume densities of islets
Trimester 1 (TM1)	Pearson corr Sig(2 tailed)	-0.691** 0.0001	-0.739** 0.001	-0.712** 0.003	-0.624** 0.001
Trimester 2 (TM2)	Pearson corr Sig(2 tailed)	-0.619** 0.0003	0.773** 0.0012	-0.899** 0.0001	-0.681* 0.001
Trimester 3 (TM3)	Pearson corr Sig(2 tailed)	0.317 0.059	0.591* 0.0171	0.310 0.196	0.399 0.311

**correlation is significant at 0.01 level(2tailed)

*correlation is significant at the 0.05 level (2 tailed)

NB> Note the linear relationship between the time of exposure and changes in the pancreas morphometric parameters.

Figure 14A&B: Sections of a control and the alcohol treated fetal pancreas showing fibrous connective tissue deposits.

14A: A section of a normal fetal pancreas showing distribution of stromal and connective tissue deposits with well developed acini clusters. Note the evenly distributed connective tissues and ducts (D) in the section. *Weighat stain Mag x40*

14B: A longitudinal section of a control fetal pancreas showing the regional distribution of connective tissues including Blood vessels (BV) from the head (H), Neck (N), body (B), and the tail (T) regions. H &E. *Mag x10*

14C: A section of a fetal pancreas showing the extensive deposition of stromal tissues in the interacinar spaces and in the periductular area (F-CT) following alcohol treatment. Note the reduction in the number of the acini clusters (a) in the lobules and presence of stromal tissues in the interacinar spaces. *Weighat stain Mag X 40*

14D: A longitudinal section of an alcohol treated fetal pancreas. Note the increasing fibrous stromal (S) tissue deposition in the body and the tail region as compared with the head and the neck region. Note the presence of undifferentiated cell mass (UCM) and smaller blood vessels (BV) and ducts (D). Note also the corresponding paucity of the acini (a) in the body and tail regions. Note the proportional relationship between the stromal tissue deposit and the number of acini clusters in each region. *H&E Magx10*

Figure 14 A -D:

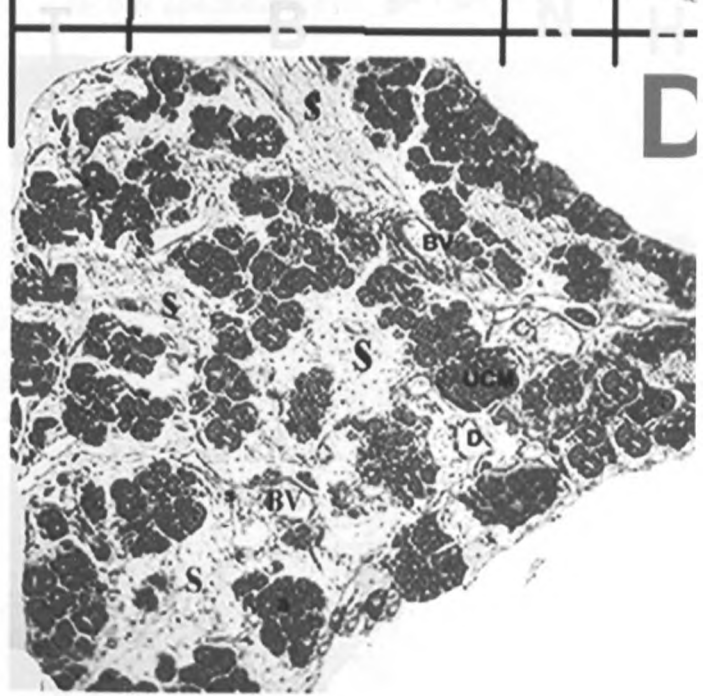
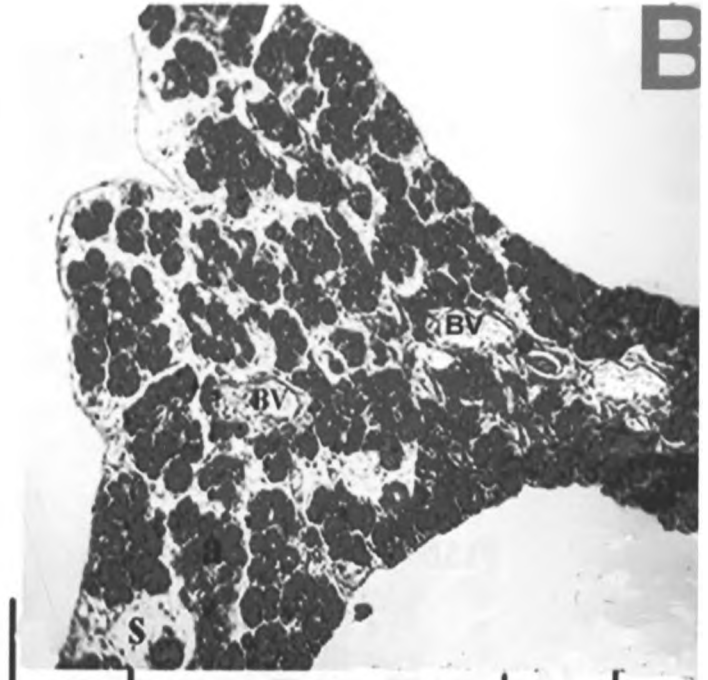
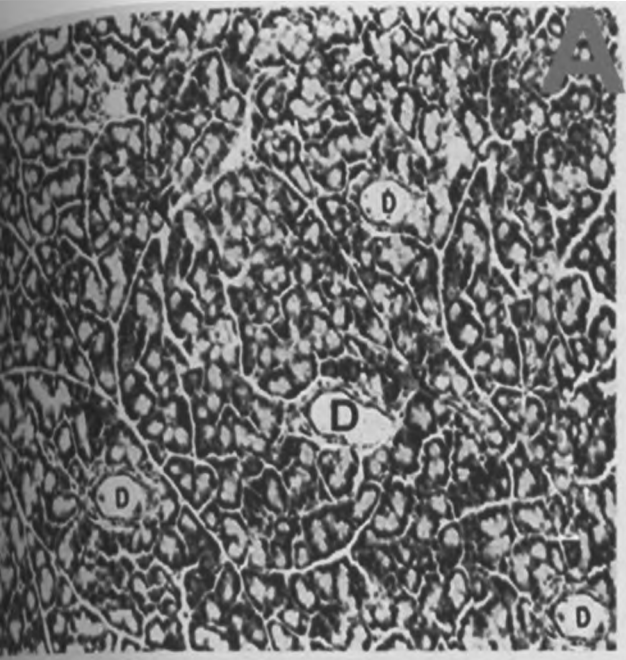
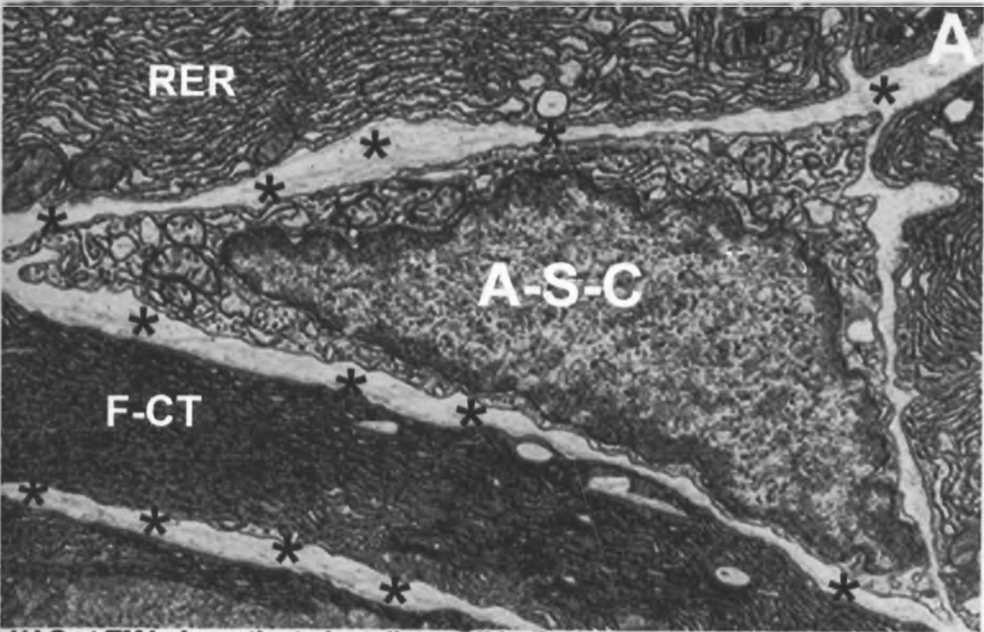


Figure 15 A&B: The “*activated*” or active stellate cells in the alcohol treated group compared with the inactive or “*Quiescent*” stellate cell in the control

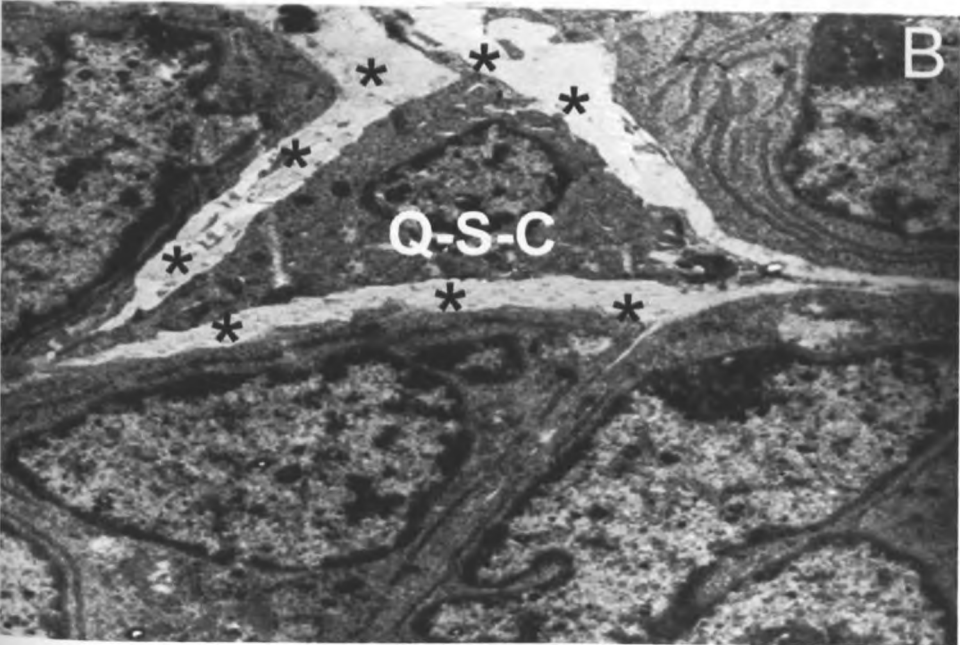
15A: A section of a fetal pancreas from an alcohol treated group showing an “*activated*” stellate cell (A-S-C) and deposits of the fibrous connective tissues (F-CT) in the interacinar spaces (*indicated by the stars*). Note the size of the stellate cell that fills the whole of the inter-acinar space: *TEM-Mag x8,200*

15B: A section of control fetal pancreas showing a “*quiescent*” stellate cell (Q-S-C). Note that there are no deposits of the fibrous connective tissues in the interacinar spaces (*indicated by the stars*): Note the small size of stellate cell as compared to (A) above. *TEM-Mag x8,200*

Figure 15A&B:



HAG at TM1: An activated stellate cell in the interacinar space. EMx54190



These findings were found to be statistically significant ($P < 0.005$) by both Chi-square test and with ANOVA when intra group and inter group comparisons between the controls and the alcohol groups were done.

The non parametric Mann Whitenney U-test done to compare the histomorphometric parameters between the control and the alcohol treated groups showed statistically significant U and U-Calculated values between the alcohol treated groups compared with the control. (Table 12)

Table 11: The comparative ranges and means of the beta cell counts per islet cluster for the alcohol treated groups compared with the control

Study group	Parameters	Regional distribution in the parts of the pancreas							
		Head		neck		body		tail	
		Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE
Controls group	No. of beta cell per islet cluster	55-75	69.2 \pm 0.5	82-97	88.7 \pm 0.8	80-120	96.2 \pm 0.7	50-80	59.7 \pm 0.8
Alcohol Treated groups	No. of beta cell per islet cluster	35-60	47.9 \pm 0.6	60-75	69.7 \pm 0.5	65-90	77.4 \pm 0.5	35-50	45.3 \pm 0.7
	P-values		0.036		0.029		0.033		0.017

Table 12: The non parametric Mann Whitennys U- test on the median values of the endocrine stereological parameters for the alcohol treated group compared with the control

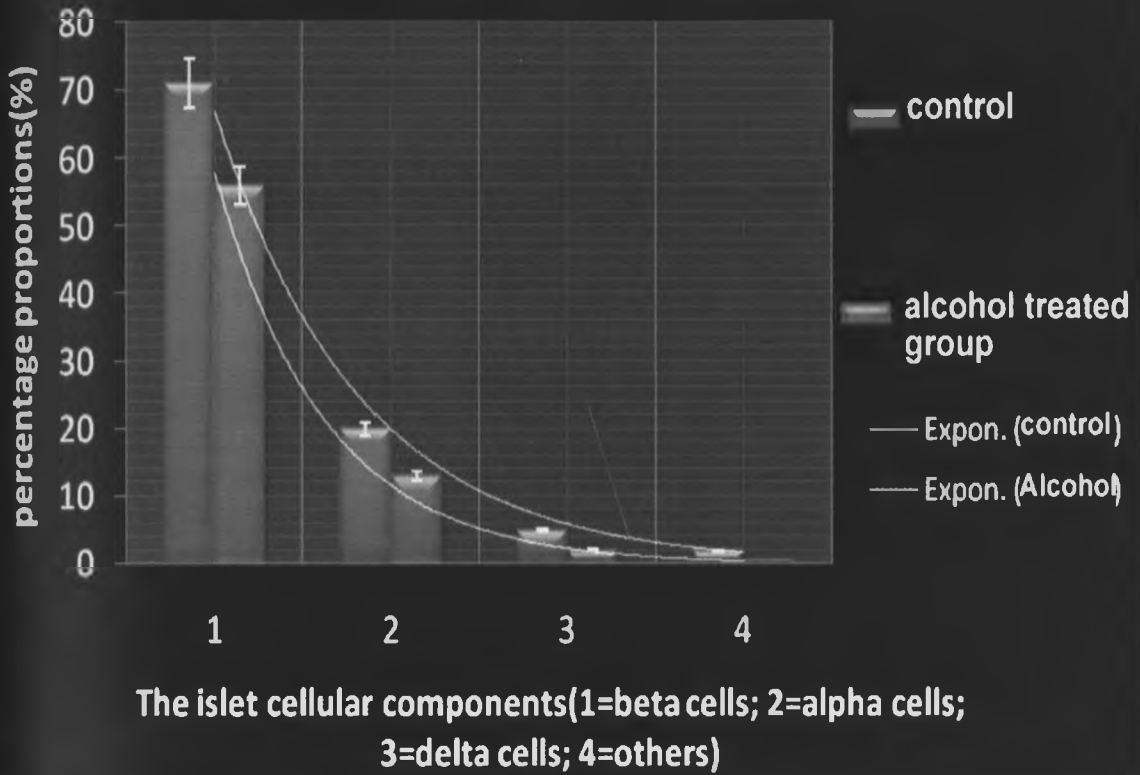
Parameter	Control	Alcohol group	Mann whitenny U-test [alcohol versus control groups]			
			Median values used		[U]calc	P[U]
			control	alcohol		
Islet mass (mgs)	2.2±0.003	1.83±0.012	2.22	1.80	1*	0.0032
Beta cell mass (mgs)	1.6±0.001	1.41±0.002	1.61	1.40	0*	0.004
Vol. density of Beta cell/islet (%)	73.4±1.5	67.2±1.1	73.1	67.01	2*	0.0016
Total B-cell Vol. (mm ³)	0.12±0.003	0.10±0.02	0.12	0.10	2*	0.0042
Volume density of Islet/pancreas (%)	2.87±0.08	2.49±0.24	2.88	2.50	6*	0.004
Total islet volume(mm ³)	0.93±0.002	0.90±0.005	0.923	0.901	0*	0.006
Volume weighted mean islet volume (mm ³)	0.95±0.006	0.89±0.002	0.947	0.891	9	0.321
Total islet numbers (10 ³)	2.67±0.012	2.41±0.041	2.670	2.40	0*	0.004

* values whose P- value was less than or equal to 0.05 at 95% confidence interval

U-calc= calculated Mann-whiteny U-value

P[U]= probability of occurrence of calculated U value

Graph 3: Percentage proportion of the islets cellular components for the alcohol treated groups compared with the control



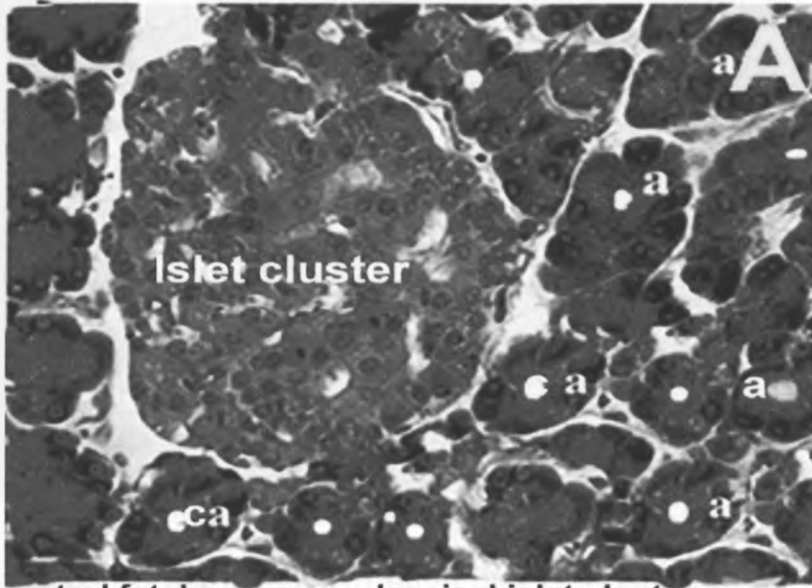
NB> Note the exponential change in the percentage proportions of the islet cell in the alcohol treated groups compared with the control as shown in the exponential lines in the graph.

Figure 16 A-B: Sections of fetal islets showing a classical islet cluster in the control (A) and disaggregated islets in the alcohol treated group (B).

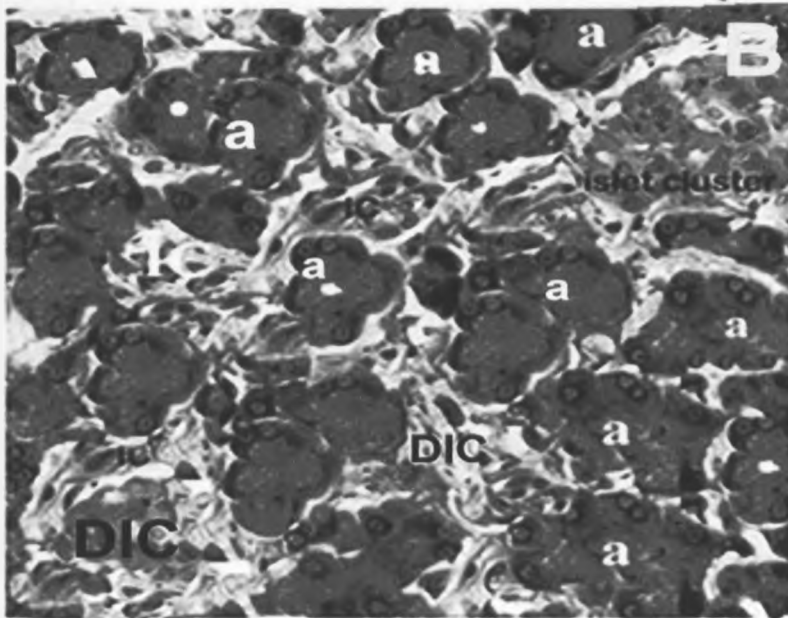
16A: The histological structure of a control fetal islet of langerhans. Note the features of a classical islet that is well developed and mature within the acini clusters. Also note the mature and canalized acini (Ca). *Haematoxyline phloxine stain, Mag x400*.

16B : Histological features of islets from an alcohol treated group. Note the extensive disintegration of the islet clusters and presence of disaggregated islet cells (DIC) in the inter-acinar spaces. observe the scattered and poorly developed acini (a). *Haematoxylin Phloxine stain Mag x400*.

Figure 16 A & B:



control fetal pancreas: classical islet cluster



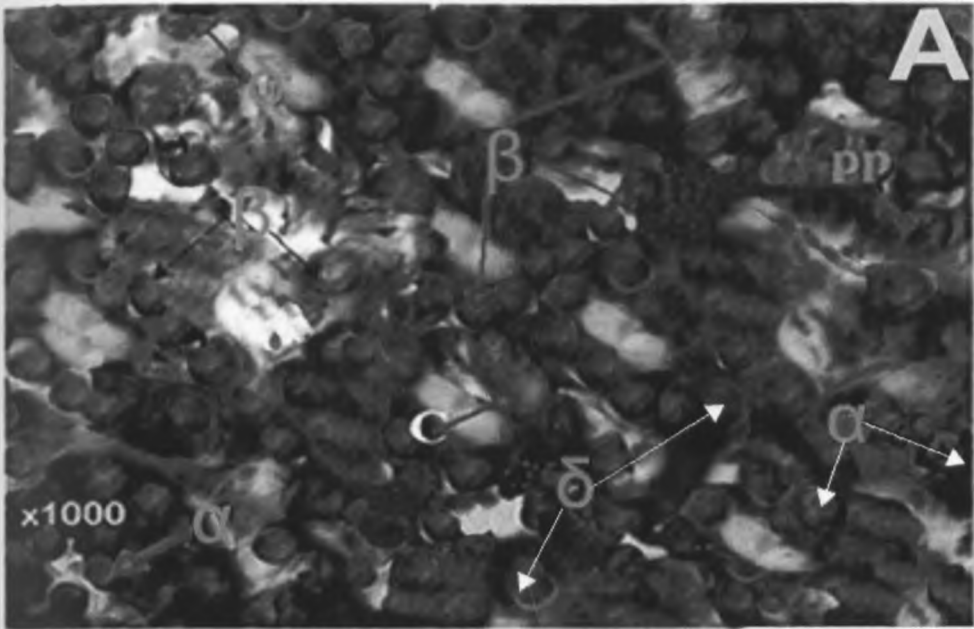
NB> note the classical islet cluster in the control and the disaggregated islets in the alcohol treated group. See the disaggregated cell in the inter acinar spaces (DIC)

Figure 17 A & B: Sections of 20-day old fetal islets showing distribution of cellular components in the alcohol treated groups (B) compared with the control (A)

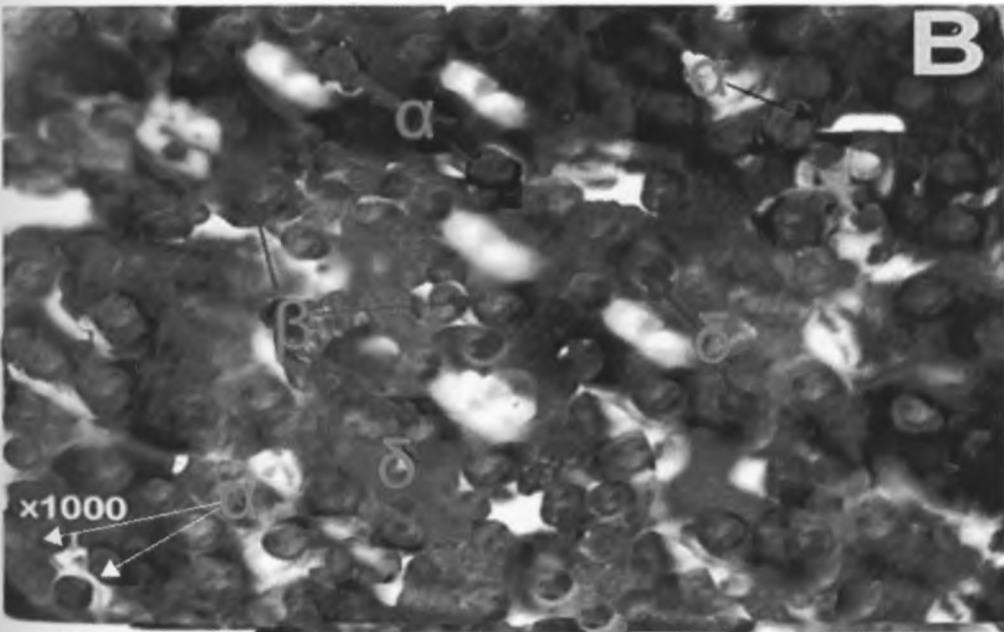
17 A: Section of a fetal pancreas illustrating the distribution of the various cellular components in an islet cluster; Note the high proportion of Beta (β) cells in relation to other cell types. Observe that they are arranged in cord like arrays of 8-16 cells lying side by side. Observe that the alpha (α) cells and delta (δ) cells distributed throughout the islet unlike for the PP and C-cells that are peripherally located. *Mordified aldehyde Fucshin stain: Mag x1000*

17 B: Section of a fetal pancreas from an alcohol treated group illustrating the distribution of the various cellular components in an islet cluster. Note paucity of all cells including the beta (β), alpha (α) and delta(δ) cells. Note the the PP and C-cells can rarely be seen. *Mordified Aldehyde Fucshin stain: Mag x1000*

Figure 17 A&B:



Control fetal islet showing the cell compositions



islets of an alcohol treated fetus showing the cell composition

NB> Note the paucity of the islet cells including β , α , δ and the disappearance of the pp and C-cells in the alcohol treated group (Fig 15B)

3.2.4.1 Effects of alcohol on the ultra-structure of the fetal islet cells

a) The Beta (β)-cells:

β -cells of control fetuses were polyhedral in shape, with membrane bound secretory granules "the mature granules" that had electron dense core of crystalline material. The secretory granules had a loosely fitting membrane with a spacious empty hollow between the membrane and the electron dense material forming the core. These cells had a high presence of rough endoplasmic reticulum (RER) that were scattered within the numerous secretory granules (*Figure 18A*). The secretory granules were also observed to be accumulated in a polar fashion away from the nucleus and to the surface facing the capillaries.

In the alcohol treated groups, alcohol was seen to alter or delay the secretory functions of the beta cells. At gestational day 21, some B-cell were still agranulated (*Figure 19A&B*). These granulated cells appeared columnar in shape with sparsely distributed secretory granules, the "immature form" that had little, or just a slightly electron dense content (*Figure 20B*). They also shown disorganization of the rough endoplasmic reticulum (RER), less Golgi apparatus and few polysomes as compared with the control groups (*Figure 20B*).

B) Alpha(α)-cells

The A-cells in the control appeared more columnar and smaller than the B-cells with many secretory granules in the cytoplasm that are electron dense with narrow hollow of less dense material and tightly fitting granule-limiting membrane (*Figure 18C*).

The alpha cells of the alcohol treated groups did not show great morphological differences when compared with the control in terms of cell shapes and the shapes of the secretory granules, however the secretory granules in the alcohol treated groups were fewer than in the controls (*Figure 20b*).

C) Delta(δ)-cells

In the control, the D-cells were observed to be smaller than any of the either of the previous types (B and A-cells) and were seen to be dendritic and well granulated. The granules contained a homogeneous high electron dense material that filled the granule limiting membrane. The RER were also numerous and prominent in these cells (*Figure 18B*).

It was observed that the number and electron density of the granules were quite variable between the control and the alcohol groups. The granules of the control groups had a high electron density while the ones from the alcohol treated groups were less dense and fewer in numbers (*Figure 21A*).

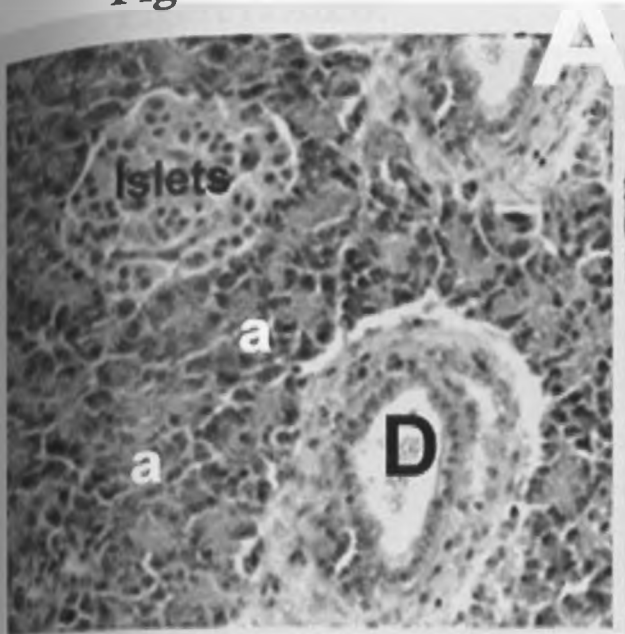
d) PP-cells

The PP-cells were observed to be variable in sizes and numbers in both the control and alcohol treated fetal pancreas. They were observed to be triangular and ovoid in shape and the secretory granules were less numerous in the alcohol treated groups compared with the controls (*Figure 21C-D*).

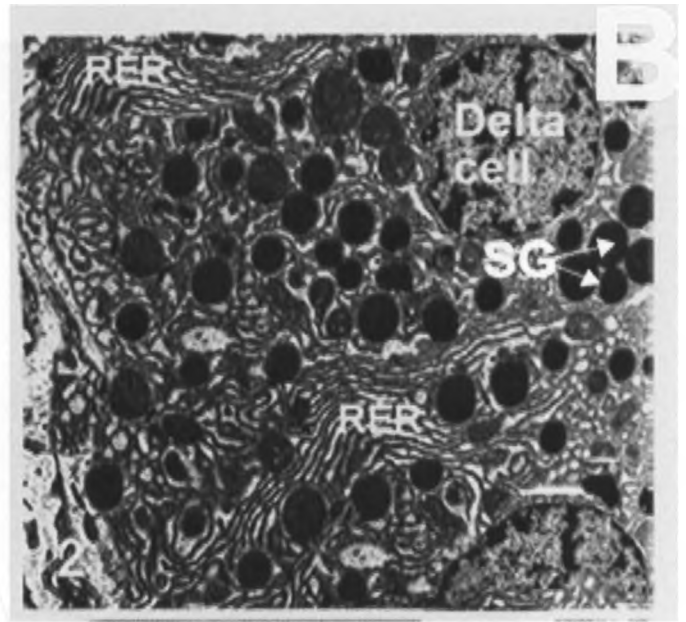
Figure 18A-D: A-20-day old fetal pancreatic islet cells showing the normal organization of the cells and the sub-cellular structures

- 18A:** Semi thin-micrograph of a control fetal pancreas illustrating a classical islet of langerhans in between the acini clusters (a). note the conspicuous ducts (D). *Mag X40*.
- 18 B:** A control fetal delta-cell. Note he conspicuous secretory granules that contained a high electron dense material that fills the secretory granules (SG) bound by a limiting membrane. Note the prominent rough endoplasmic reticulum (RER) that are highly prominent in these cells. *TEM-Mag x18,200*.
- 18C:** Ultra micrograph of a control fetal alpha cell. Note the cell nucleus with conspicuous chromatin granules. Note the numerous secretory granules in the cytoplasm that are electron dense with narrow hollow of low electron dense material that is tightly fitting within the secretory granule (SG)-limiting membrane. *TEM-Mag x18,200*.
- 18D:** Ultra micrograph of a control Beta cell: Note well granulated secretory "the mature granules " that had electron dense core of crystalline nature, with a loosely fitting secretory granule (SG) limiting membrane and had spacious empty hollow. Note the rough endoplasmic reticulum (RER) scattered among numerous secretory granules (SG). *TEM-Mag x18,200*.

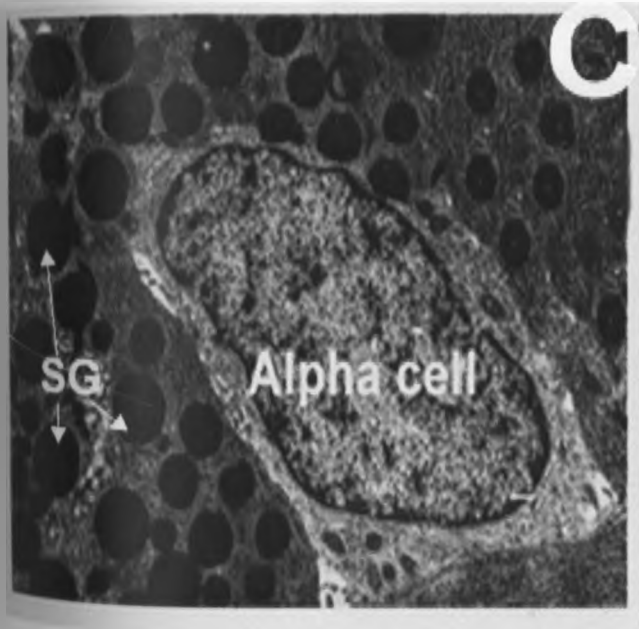
Figure 18 A-D:



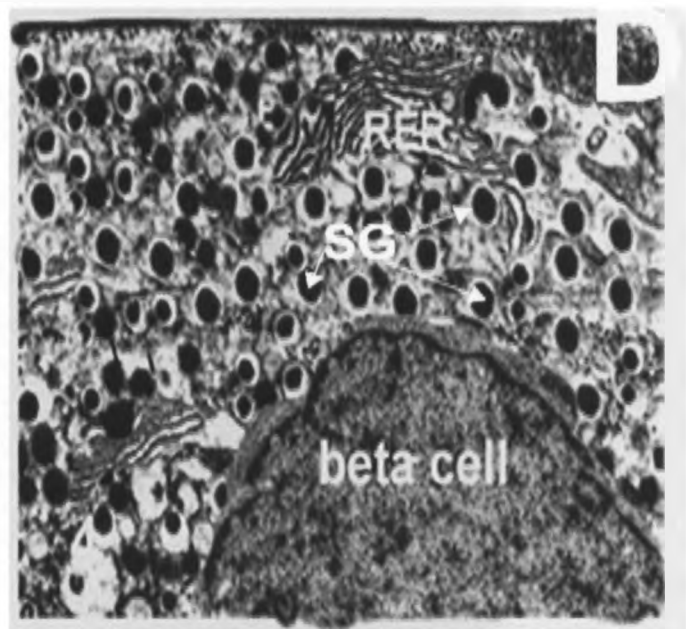
control :Islets, acini and ducts



Control: Delta cell



Control: Alpha cell



Control: Beta cell

NB> all these cells are from the control fetal islets and they depict characteristics of a protein synthesizing cells

Figure 19 A&B: A-20-day old fetal pancreas showing delayed granulation of the beta cells in the alcohol treated groups compared with the control

19A: A section of a-20-day old control fetal islet cell showing granulation of beta cells (G-B-C). Note the numerous and granulated secretory granules (SG) in the cytoplasm that they depict an electron dense core materials and an empty hollow between this dense electron core material and the limiting membrane. Note the close proximity to the blood capillary (B-c). Also note the cell nucleus (CN) and centerized nucleoli (N).
Mag x 8,200

19B: An alcohol treated fetal beta cell illustrating non granulated beta cells (NG-B-C). Note the absence of the cytoplasmic secretory granules in most of these cells. Note the presence of the cytoplasmic lipid vacuolations (CL-v), and mal-developed mitochondria (M) that are inconspicuous. *Mag x 8,200*

Figure 19A&B:

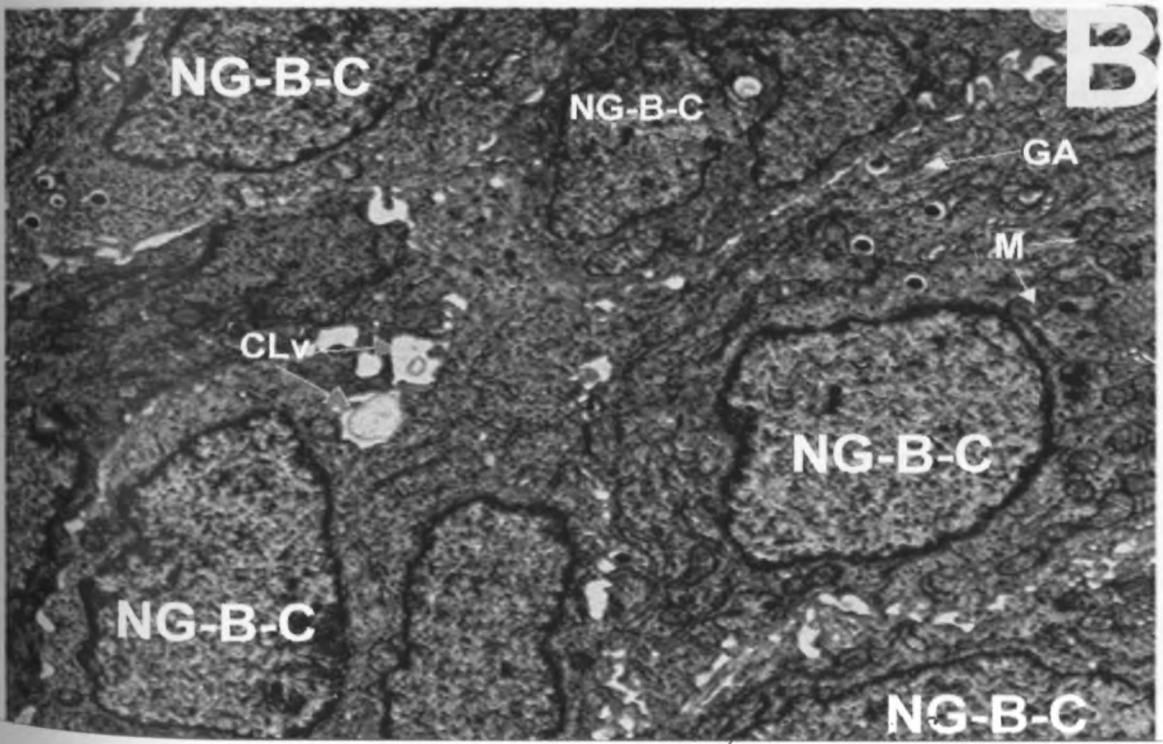
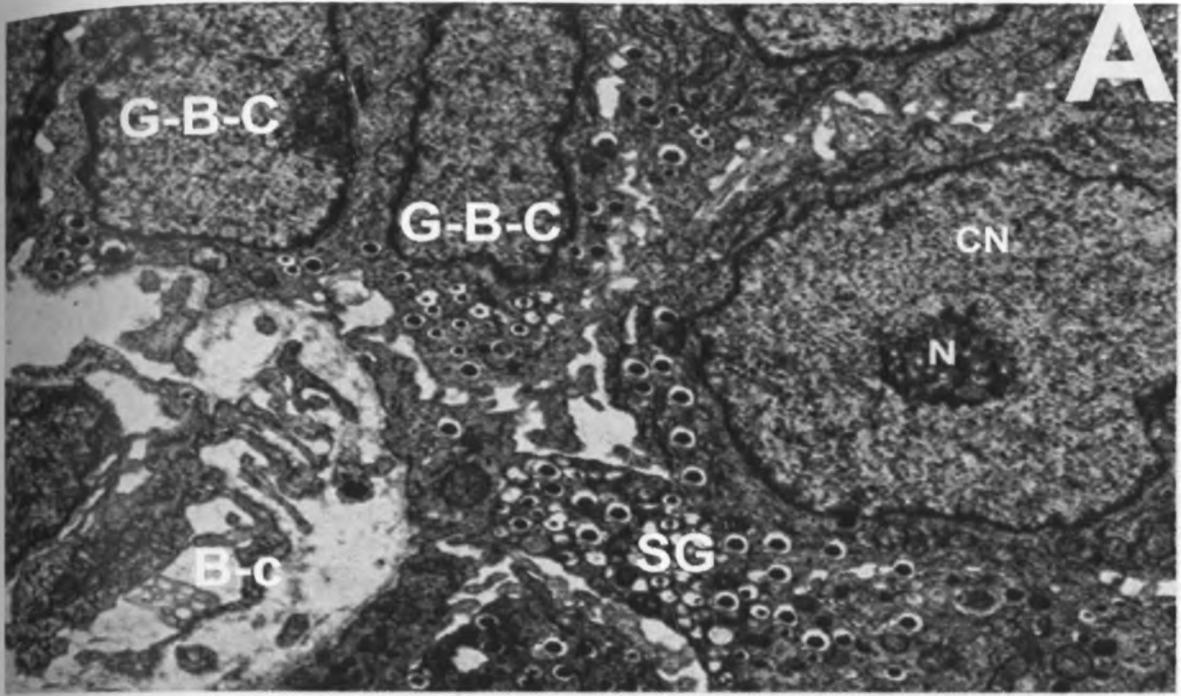
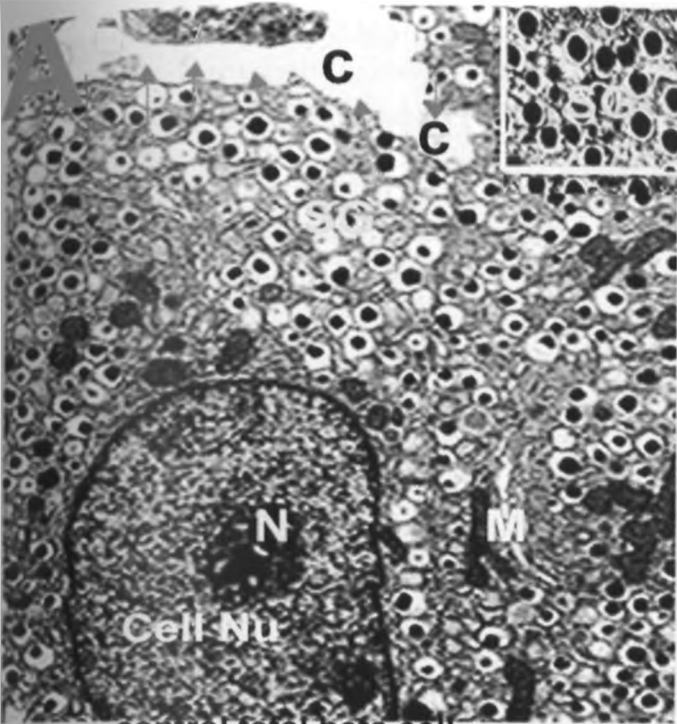


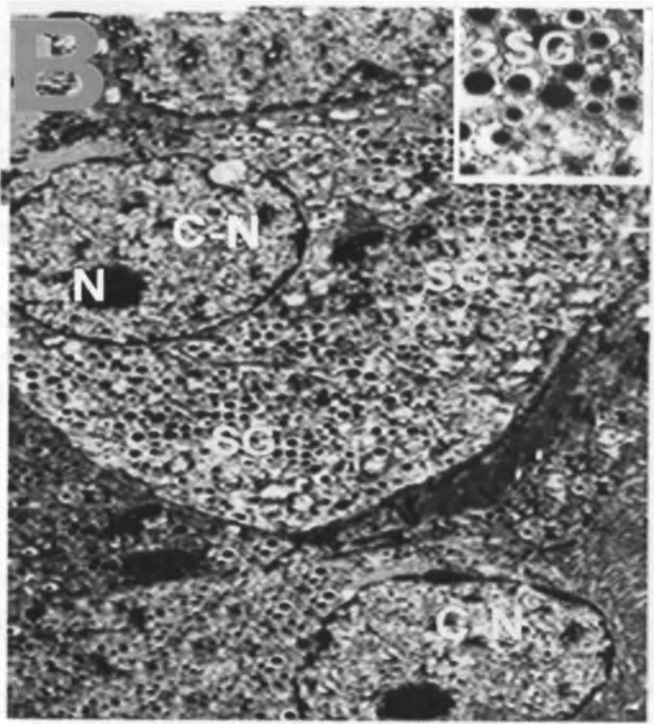
Figure 20A-D: Fetal islet cells showing the morphology and secretory alterations of β and α cells in the alcohol treated groups compared with the control

- 20A:** A control fetal beta cell: Note the numerous secretory granules (SG) that are densely distributed in the cell cytoplasm (*well illustrated in the insert*). Observe that the electron dense core granules have a space between the binding membranes. Observe a well developed nucleus (Nu), nucleolus (N) and conspicuous mitochondria (M). Note the apical part opens to a blood capillary(C) *indicated with green arrows. TEM-Mag x18,200*
- 20B:** An alcohol treated fetal beta cell. Observe the sparsely distributed secretory granules (SG) that appear immature as *shown in the insert*. Note the cell nucleus (C-N) and the nucleolus (N) that is densely stained. *TEM-Mag x18,200*
- 20C:** A control fetal alpha cell. Note the numerous secretory granules (SG) that are evenly distributed in the cell cytoplasm. Observe the electron dense granules that are membrane bound (*as shown in the insert*). Note the apical part of the cell opens to a blood vessel –capillary(c) *as indicated by the green arrows. TEM-Mag x18,200*
- 20D:** An alcohol treated alpha cell. Note the presence of many immature secretory granules (SG) that are sparsely distributed in the cell cytoplasm. Note the Cell nucleus (C-N) and inconspicuous secretory granules (SG) *in the insert. TEM-Mag x 18,200*

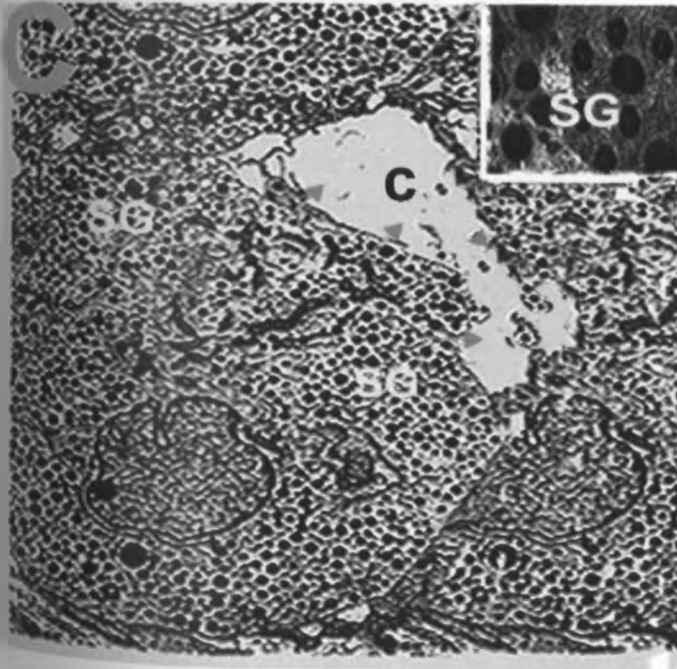
Figure 20A-D:



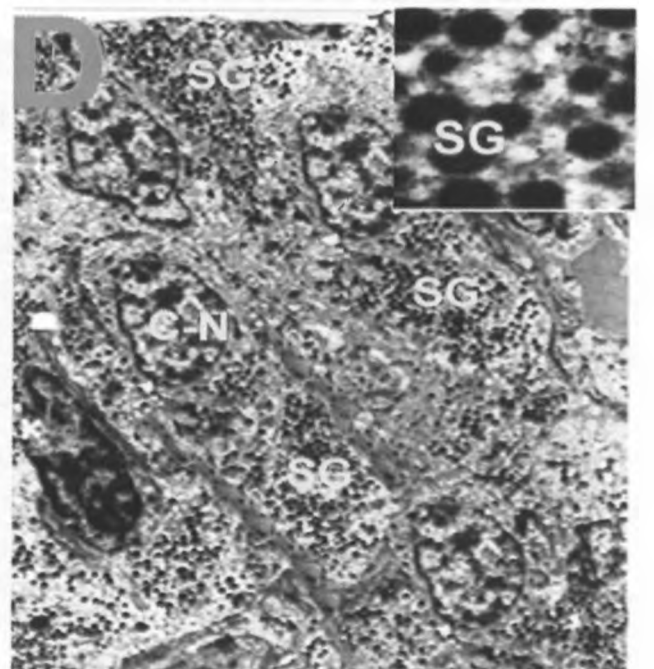
control fetal beta cell



Alcohol treated Beta cell



Control Alpha cell



Alcohol treated Alpha cell

Figure 21 A-D: Electron micrographs of a-20-day old fetal islet cells showing the morphology and secretory alterations of delta (δ) and PP-cell of the alcohol treated groups compared with the control

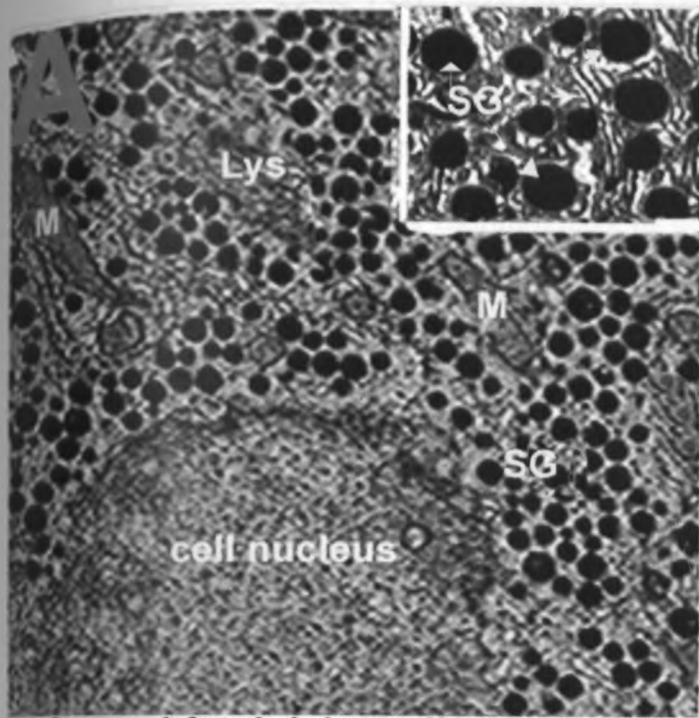
21a: A control fetal delta cell: Note the numerous secretory granules (SG), that are evenly distributed in the cell cytoplasm. They have an electron dense core granules and a space between the membrane and the electron dense core (*as shown in the insert*). Note the apical part that opens to a blood capillary. Note the Mitochondria (M) and the lysosomes (Lys). *TEM-Mag x18,200*

21B: An alcohol treated fetal delta cell. Note the presence of many immature secretory granules (SG) that are sparsely distributed in the cell cytoplasm that is faintly staining (*as shown in the insert*). *TEM-Mag x18,200*

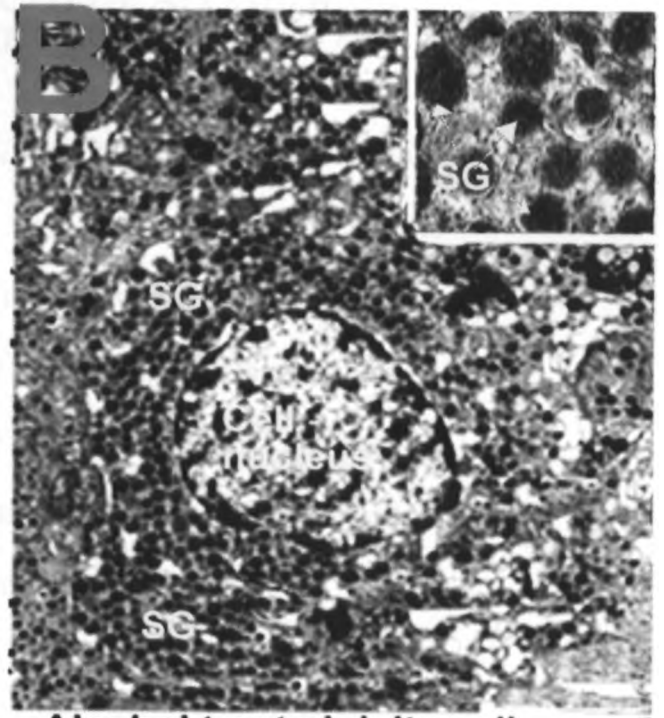
21C: A control fetal PP- cell: Note the numerous secretory granules (SG) that are evenly distributed in the cell cytoplasm. Note the electron dense granules that are membrane bound and filling the entire space (*as shown by the insert*). Note various sizes and shapes of the cell nucleus. *TEM-Mag x18,200*

21D: An alcohol treated PP- cell. Note the presence of many immature secretory granules (SG) that are sparsely distributed in the cell cytoplasm (*as shown in the insert*). Note cell nucleus C-N that is depicting mitotic activity with very conspicuous chromatin granules in the nucleoplasm: *TEM-Mag x 18,200*

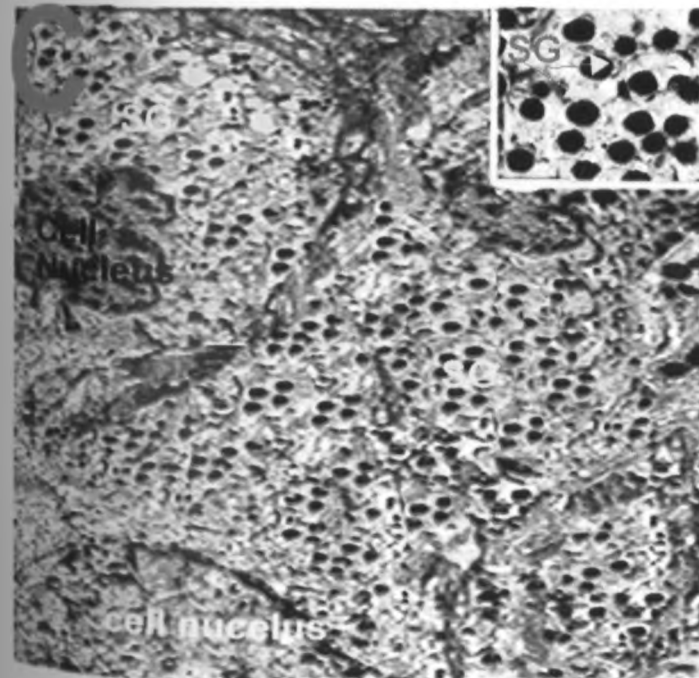
Figure 21 A-D:



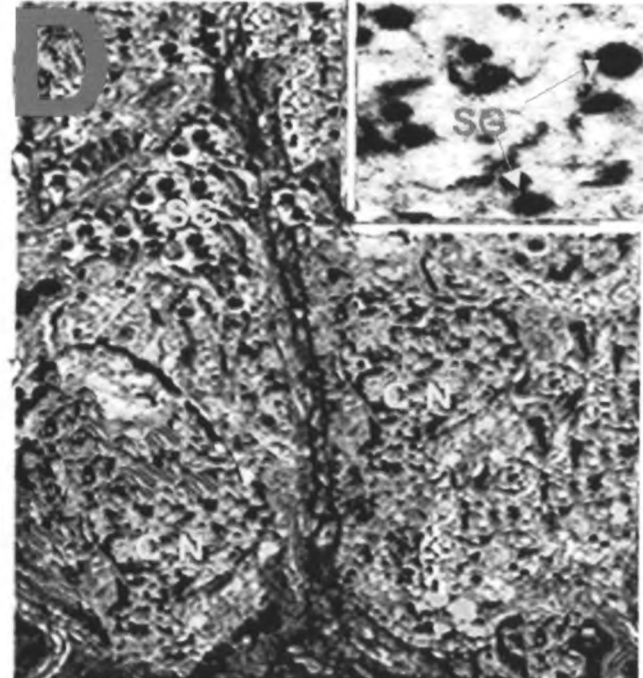
Control fetal delta cell



Alcohol treated delta cell



control PP -cell



Alcohol treated fetal PP-cell

3.2.5 THE VULNERABLE PERIODS OF ALCOHOL TERATOGENESIS TO THE FETAL PANCREAS

The alcohol exposure in the first trimester (TM₁) had the worst deleterious effect on the morphology and morphometry of the fetal pancreas. Exposure in the second trimester (TM₂) had an almost similar morphological alteration to both the endocrine and exocrine pancreas while the morphological effects following exposure in the 3rd trimester depended more on the dose than the time of exposure. The first trimester was then established to be the critical period of alcohol teratogenesis to the fetal pancreas histomorphogenesis.

3.2.5.1 The Vulnerable periods of the fetal exocrine pancreas

The characteristic alterations in exocrine pancreas that shown a time dependant relationship included, decrease in number and sizes of acini clusters (*Figure 22 a-d*), the acini cellular counts and the reduction in the number and sizes of secretory granules, increased lipid vacuolations (*Figure 10a-d*), disorganization of the intracellular organelles (*Figure 9b,d,f*) delayed cellular differentiation, acini canalization and maturation of the acini cells (*Figure 22 a-d*).

Alcohol was observed to cause reduction of cellular count per acini in a time dependant maner (*Tables 13&14*). At term, the fetal exocrine pancreases exposed to alcohol in the first and second trimester depicted fewer number of acini clusters with a handful being canalized. There was also a notable increase in the

undifferentiated cell masses as a sign of delayed differentiation of acini cells (Figure 22 a-d).

Quantitatively, there was a notable reduction in the mean numerical counts of the acini per field that was also statistically significant ($p < 0.005$) when they were compared with the controls and across different alcohol groups by both χ^2 and ANOVA (Table 13). A strong negative Pearson correlation ranging between [$\rho(r) = -0.980$ to $\rho(r) = -0.03$, (two tailed)] was also found to exist between mean numerical counts of acini per field and the time of alcohol administration.

The comparative analysis of the volume densities of the fetal exocrine parenchyma and connective tissues shown that alcohol exposure in the first trimester (TM₁) had the worst outcomes, while exposure in the third trimester resulted to very mild changes or no effects at all based on the alcohol doses administered (Graph 4).

3.2.5.2 The Vulnerable periods of the fetal endocrine pancreas

In the endocrine pancreas, alcohol was observed to cause disaggregation of the histo-cyto-architecture of the islet clusters. The structural alterations in the islet clusters were found to have a strong association with the time of exposure. It was seen that, when alcohol was administered in the first trimester, the islet clusters were greatly reduced in size with most of the disaggregated islet cells

appearing as single or groups of cells in the inter-acini spaces (*Figure 22a-d*). It was further observed that alcohol causes reduction of the beta cell counts per islet cluster in a time dependant manner (*Table 15*). Alcohol suppressed the development of the PP and C-cell particularly when the exposure was in the first and the second trimesters (*Figure 23 A & B; Figure 24 A-D*). A time dependant alcohol teratogenesis on the islet cells was observed to have an inverse relationship in the number of islet aggregations and beta cell counts at early exposures compared with those exposed in the third trimester and with the control (*Table 15*).

A time dependant effect in the reduction of the volume densities of islets, the beta cell mass, the islet volumes and total beta cell numbers in the alcohol treated groups exposed at different periods was observed (*Table 15*). It was noted that the proportionate reduction in the beta cell mass was highest in the high alcohol group exposed in the first trimester and least in the low alcohol group when exposure in the third trimester [*Graph 4 & 5*]. The fetal exocrine and endocrine parameters were seen to have strong negative Pearson correlations when compared with time of alcohol exposure (*Tables 16&17*).

Table 13: The mean numerical counts of acini per field correlated with time of alcohol exposure and the regional distribution in the parts of the fetal pancreas.

The mean numerical counts of acini per field \pm SE					
Animal group	Time of alcohol treatment	Head	Neck	Body	tail
Control	22.2+0.72	21.8+0.13	23.16+0.26	18.71+0.19
LAG	TM ₁	18.6+0.13*	17.9+0.01*	19.1+0.23*	19.1+0.02*
	TM ₂	19.1+0.10*	18.0+0.27*	19.7+0.15*	17.2+0.61*
	TM ₃	20.1+0.15	19.4+0.21	21.3+0.18	19.4+0.41
MAG	TM ₁	13.1+0.11*p	16.0+0.14*p	14.7+0.91*p	13.2+0.63*p
	TM ₂	12.7+0.13	15.7+0.18*p	12.0+0.97*	12.5+0.81*p
	TM ₃	17.4+0.17*	18.2+0.21*	18.6+0.82*	15.9+0.45*
HAG	TM ₁	9.1+0.12*p	10.4+0.13*p	13.7+0.34*p	12.3+0.13*p
	TM ₂	11.1+0.22*p	13.1+0.11*p	12.1+0.11*p	11.4+0.17*p
	TM ₃	15.1+0.10*p	14.3+0.21*	13.9+0.81*p	14.9+0.13
*shows the figures that have a statistically significant difference with the control(p<0.005) at 95% confidence interval					
*p-shows the figures that have a statistically significance difference compared with the control and other alcohol groups by both χ^2 test and by ANOVA					

Table14: The mean numerical counts of the canalized acini correlated with the time of alcohol exposure and the regional distribution.

Animal group	The mean numerical counts of canalized acini per field				
	Time of alcohol treatment	Head	Neck	Body	tail
Control	6.5±0.02	4.1±0.03	11.06±0.26	8.01±0.09
LAG	TM ₁	4.8±0.01*	3.9±0.01	9.1±0.03*	7.1±0.09
	TM ₂	4.9±0.05*	3.6±0.07*	9.7±0.01*	7.2±0.06*
	TM ₃	6.3±0.07	4.0±0.02	10.3±0.08	7.9±0.01
MAG	TM ₁	3.7±0.01*p	2.0±0.01*p	7.7±0.09*p	6.2±0.06*p
	TM ₂	4.7±0.03	3.7±0.02*p	9.1.0±0.97*	7.5±0.01*p
	TM ₃	5.7±0.01*	3.2±0.02*	8.6±0.02*	8.0±0.01*
HAG	TM ₁	3.1±0.01*p	2.9±0.01*p	6.7±0.04*p	3.5±0.03*p
	TM ₂	3.1±0.02*p	3.1±0.01*p	8.1±0.01*p	11.4±0.17*p
	TM ₃	5.1±0.10*p	3.3±0.02*	9.9±0.01*p	7.9±0.03

*shows the figures that have a statistically significant difference with the control (p<0.005) at 95% confidence interval

*p-shows the figures that have a statistically significance difference compared with the control and other alcohol groups by both χ^2 test and by ANOVA

Table15: The mean numerical counts of B-cells per islets correlated with time of alcohol exposure in the alcohol treated groups compared with the control.

Animal group	period of alcohol administration	Mean numerical counts of B-cells per islet \pm SE	P values
control	105.67 \pm 1.2	0.093
	TM1	93.41 \pm 0.93	0.041*
Low alcohol group(LAG)	TM2	101.51 \pm 0.8	0.037*
	TM3	103.09 \pm 0.7	0.068
Medium Alcohol group(MAG)	TM1	84.93 \pm 0.63	0.031**
	TM2	78.69 \pm 0.61	0.019*
High Alcohol group (HAG)	TM3	99.03 \pm 0.64	0.057
	TM1	71.27 \pm 1.09	0.012**
High Alcohol group (HAG)	TM2	73.19 \pm 0.82	0.039**
	TM3	88.97 \pm 0.19	0.046*

** shows p-values with statistical significance difference by both Chi- square test and ANOVA

* shows the P-values that were only significant with Chi-square test

Table 16: Pearson correlations between the time of alcohol exposure and fetal exocrine stereological parameters

Alcohol Period N=24		Parameters				
		Pancreas weight	Number of acini per field	Canalized acini per field	Numer of cells per acini	Volume densities of acini
Trimester 1 (TM1)	Pearson corr Sig(2 tailed)	-0.709** 0.000	-0.727** 0.001	-0.719* 0.001	-0.899* 0.001	-0.813** 0.001
Trimester 2 (TM2)	Pearson corr Sig(2 tailed)	-0.611** 0.000	0.564* 0.007	-0.537* 0.0012	0.513 0.008	-0.679 0.001
Trimester 3 (TM3)	Pearson corr Sig(2 tailed)	0.310 0.008	0.494 0.017	0.317 0.191	0.312 0.197	0.293 0.244

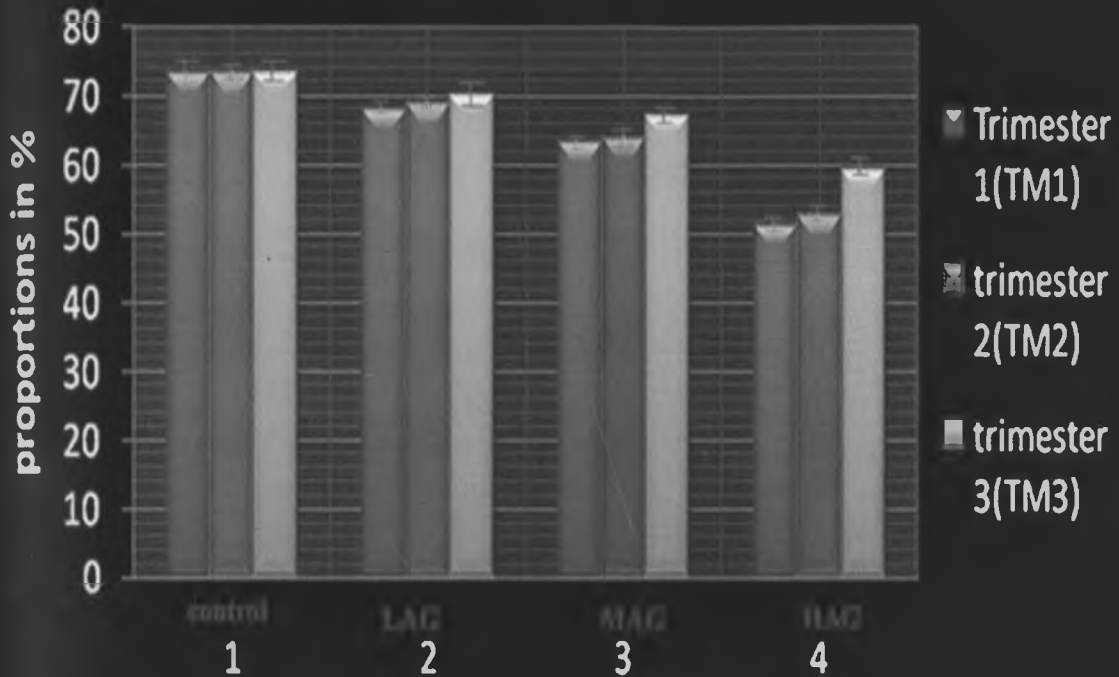
**correlation is significant at 0.01 level(2tailed)
*correlation is significant at the 0.05 level (2 tailed)

Table 17: Pearson correlations between the time of alcohol exposure and mean fetal endocrine stereological parameters

Alcohol Period N=24		Parameters			
		Pancreas weight	Number of islet clusters in a cross-section	Number of beta cell per cluster	Volume densities of islets
Trimester 1 (TM1)	Pearson corr Sig(2 tailed)	-0.778** 0.000	-0.739** 0.001	-0.812* 0.003	-0.824** 0.001
Trimester 2 (TM2)	Pearson corr Sig(2 tailed)	-0.615** 0.0001	0.666* 0.003	-0.591* 0.0012	-0.617* 0.001
Trimester 3 (TM3)	Pearson corr Sig(2 tailed)	0.314 0.009	0.494* 0.017	0.318 0.192	0.391 0.214

**correlation is significant at 0.01 level(2tailed)
*correlation is significant at the 0.05 level (2 tailed)

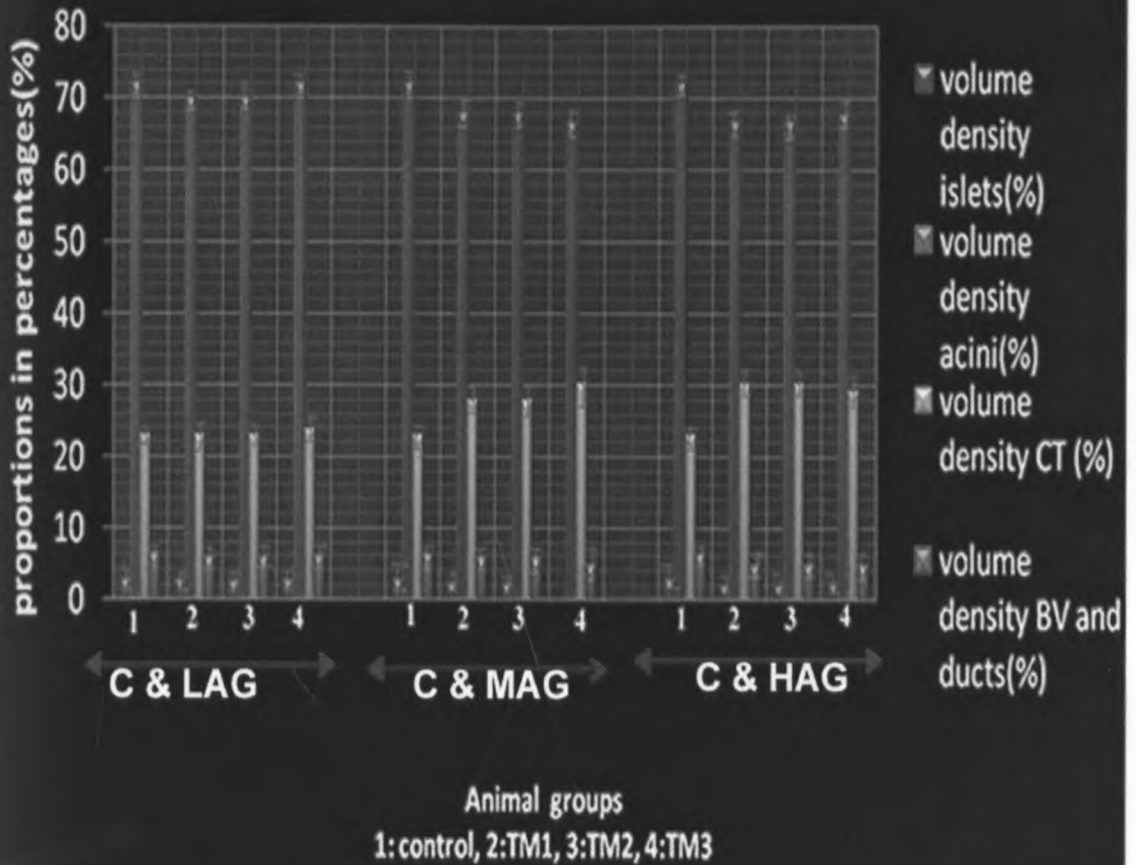
Graph 4: Shows the percentage proportions of the beta cell mass in the Alcohol treated groups against the control at TM1, TM2 and TM3



Animal groups: 1: control; 2: LAG; 3: MAG; 4: HAG

NB> Note the percentage proportion of the beta cell mass in all the alcohol treated groups reduced significantly in trimester one and two (TM₁ & TM₂) but was less affected when exposed in the third trimester (TM₃). Note that the beta cell mass in the control remained constant.

Graph 5: Comparative percentages of the volume densities of islets, acini, CT, Blood vessel and ducts in the alcohol treated groups compared with the controls at TM1, TM2, TM3



NB> Note that the volume density of the acini, islets, blood vessels and ducts in all the alcohol treated groups (LAG, MAG & HAG) reduced significantly when the alcohol exposure was in the first and the second trimester (TM₁ & TM₂). To the contrary, Note the increase in volume density of fibrous connective tissues within the same period. Note the inverse relationship between the time of alcohol exposure and reduction in volume densities of the acini, islets, blood vessels and ducts while there exists a linear correlation between the time of alcohol exposure and the fibrous connective tissue deposit.

Figure 22A-D: Sections of a-20-day old fetal pancreas showing delayed maturation and the reduced acini in the exocrine pancreas treated at TM1, TM2 and TM3.

22A: A section of a control fetal pancreas showing the distribution of acini (a). Note the numerous and evenly distributed canalized acini (a) in the field. *H&E Mag x40*

22B: A section of a fetal pancreas of an alcohol treated group at trimester one (TM1). Note the few numbers of canalized acini clusters that are also sparsely distributed in a field. Note the disaggregated islet (*marked by a star*) while acini clusters (a) appear immature with majority uncanalized. *H&E Mag 40*

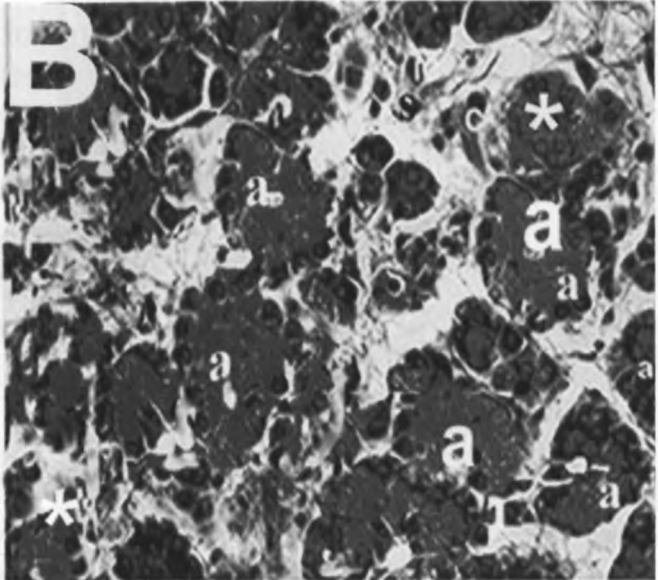
22C. A section of an alcohol treated fetal pancreas with alcohol treatment instituted in the second trimester (TM₂). Note the reduced number of canalized acini clusters (a) and disaggregated islet (*marked by a star*) similar to *Fig 21B*. *H&E Mag x40*

22D: A section of an alcohol treated fetal pancreas treated at trimester (TM₃). Note the relative increase in the number of canalized acini (a) and lesser disaggregation of islets (*marked by a star*) with features almost resembling those of the control (*Fig 20A above*). *H&E Mag x40*

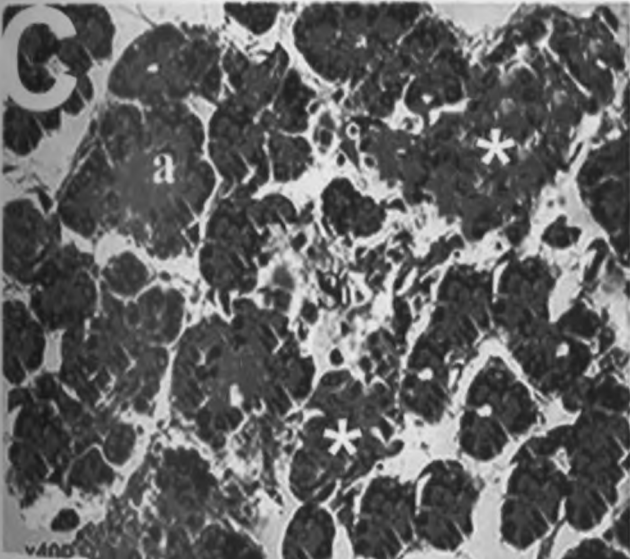
Figure 22A-D:



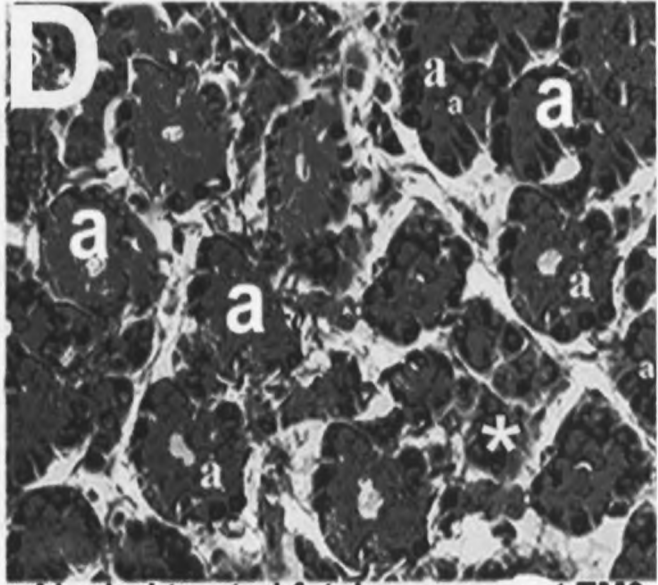
Control fetal pancreas (x400 H&E)



Alcohol treated fetal pancreas at TM1



Alcohol treated fetal pancreas at Tm2



Alcohol treated fetal pancreas at TM3

Figure 23 A-D: Sections of fetal pancreas showing disaggregation of the islets in relation to time of alcohol exposure compared with the controls

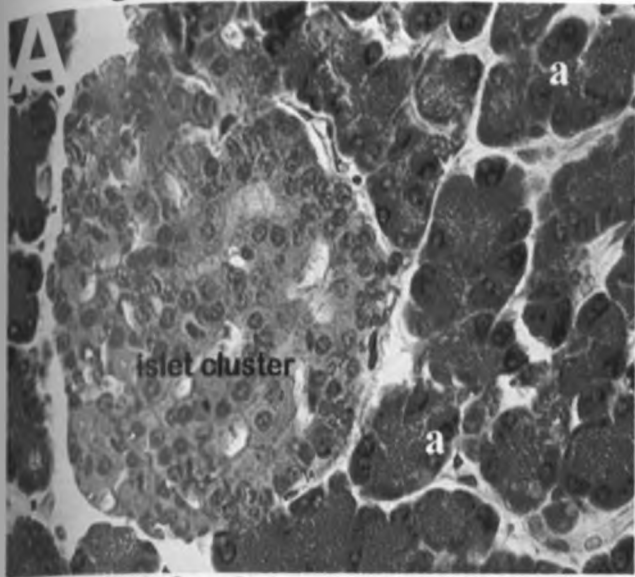
23A: A section of a control fetal pancreas showing the classical features of a spheroidal islet of langerhans in relation to acini clusters (a). *Haematoxyline phloxine stain Mag x40*

23B: A section of an alcohol treated fetal pancreas at trimester one (TM₁). Note the highly disaggregated islet clusters appearing irregular in shapes and small in size. Note the disaggregated islet cells (IC) appearing as single units or groups of cells in the inter-acinar spaces. *Haematoxyline phloxine stain, Mag x40*

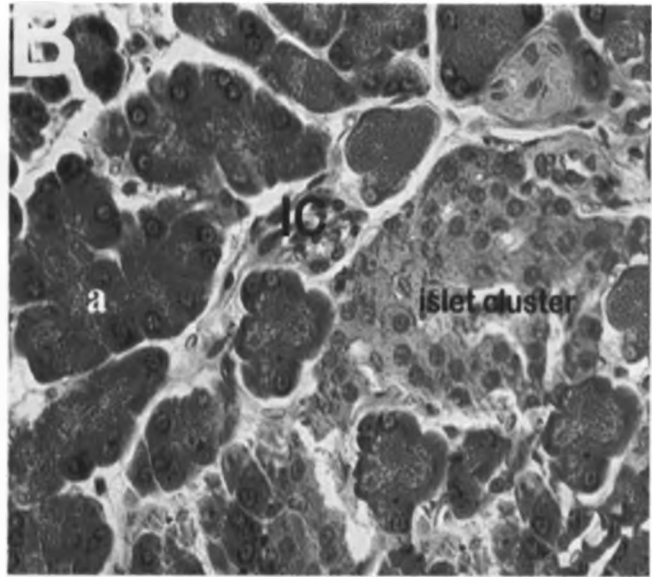
23C: A section of an alcohol treated fetal pancreas at trimester two (TM₂). Note the irregular shapes of highly disaggregated islet clusters with many islet cells (IC) resembling those in (22B) and appearing as single units or groups of cells in the inter acinar spaces. *Haematoxyline phloxine stain, Mag x40.*

23D: A section of an alcohol treated fetal pancreas at trimester three (TM₃). Note a relatively disaggregated islet cells (IC) appearing as small groups of islet clusters within the icini (a). *Haematoxyline phloxine stain, Mag x40.*

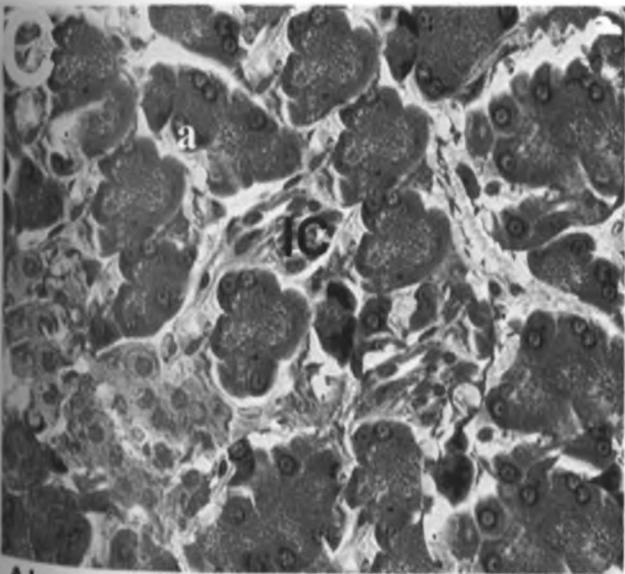
Figure 23 A-D:



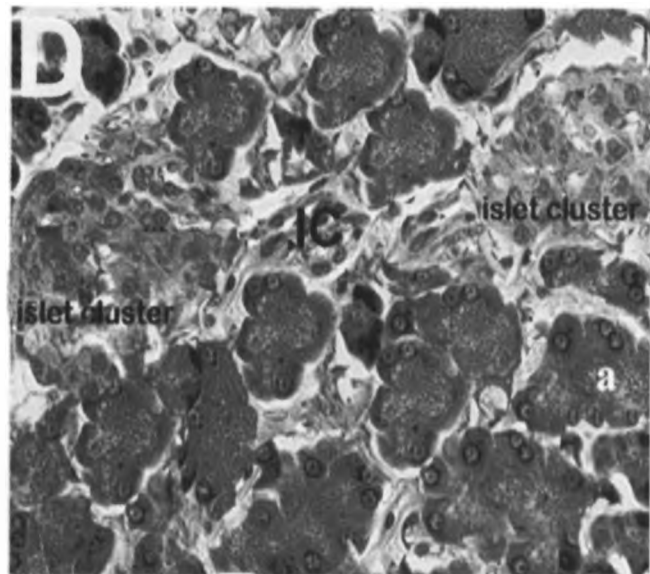
control fetal pancreas



Alcohol treated Islet at TM2



Alcohol treated fetal islets at TM 1

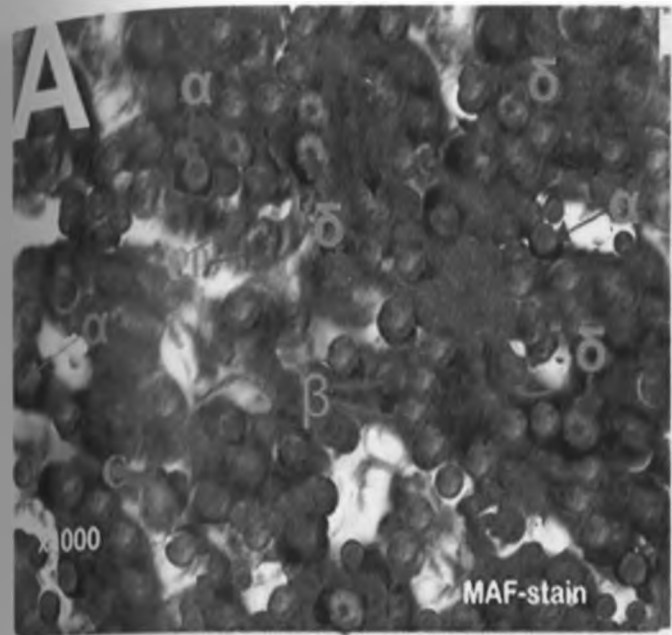


Alcohol treated fetal islet at TM3

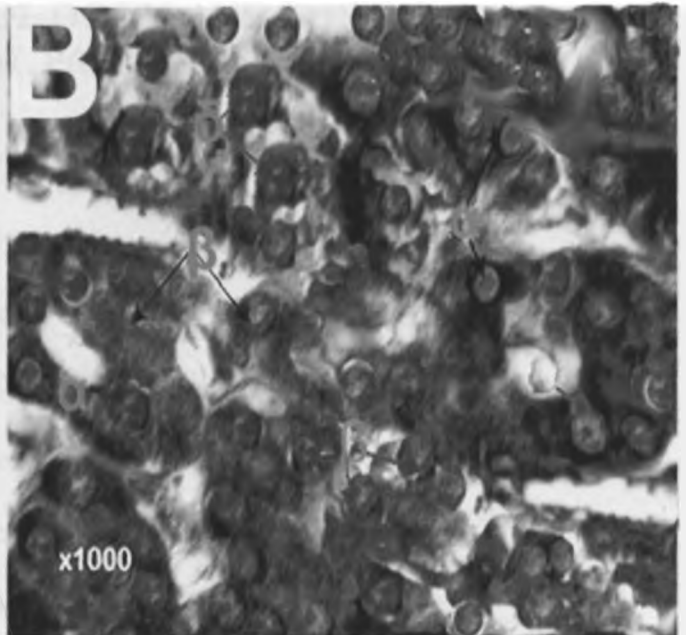
Figure 24 A-D: Sections of fetal pancreas showing the effects of alcohol on the composition of the islets cells in relation to time of exposure

- 24A:** A section a control fetal islet cells showing the composition of α , δ , β , PP and the c-cells in an islet of langerhans. *Modified aldehyde fuchsin stain (Mag x1000)*
- 24B:** A section an alcohol treated fetal islet cells exposed at trimester one (TM₁). Note the reduction in numbers of specific cells types including the α , δ , β cells and complete disappearance of the PP and the C-cells. *Modified aldehyde fuchsin stain (Mag x1000)*
- 24C:** A section of an alcohol treated fetal islet cells exposed at trimester two (TM₂). Note the reduction in numbers of specific cells including the α , δ , β and PP cells and complete disappearance of the C-cells. *Modified aldehyde fuchsin stain (Mag x1000)*
- 24D:** A section of an alcohol treated fetal islet cells exposed at trimester three (TM₃). Note some relative reduction in numbers of specific cells including the α , δ , β cells, PP and the C-cells there was no disappearance of any specific cell types. *Modified aldehyde fuchsin stain (MAFs x1000)*

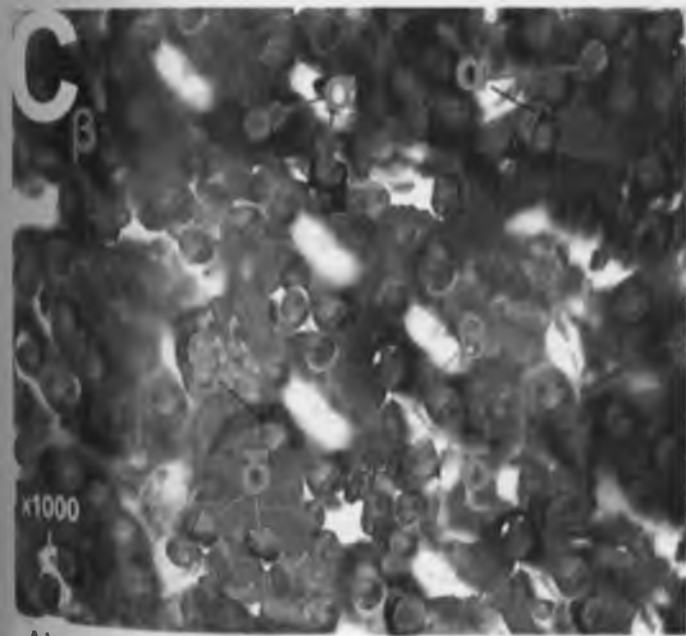
Figure 24 A-D:



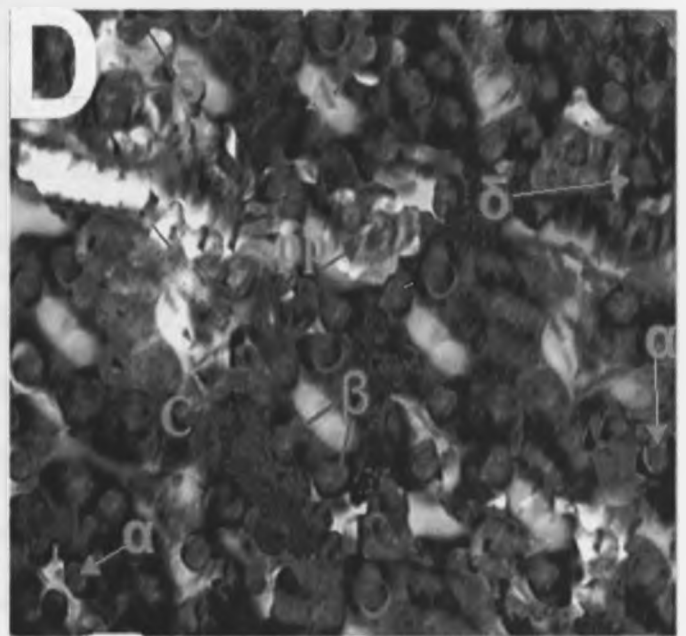
control fetal pancreatic Islet cells



Alcohol treated fetal islets at TM1



Alcohol treated fetal islets at TM2



Alcohol treated fetal islets at TM3

3.2.6 THE VARIOUS ALCOHOL DOSES AND THE EFFECTS ON THE FETAL PANCREAS

The prenatal alcohol dose of up to 6.5mgs/Kg/Bw was tolerated well by the pregnant rats and was established to be the critical dose with the greatest threshold of teratogenic effects to the fetal pancreas morphogenesis. The doses above this caused death to more than half of the mothers. It was however observed that there is no safe dose of alcohol exposure during pregnancy because even at low doses of 2.5gms/kg/Bw there were marked irreversible alteration in the morphology and morphometry of both the endocrine and exocrine pancreas as was observed in both the fetal and the in the pups pancreas.

In the exocrine pancreas, the concentrations of the alcohol inversely influenced the rates of maturation and canalization of the acini, as well as the cellular numerical counts of acinar cells per acini cluster (*Table 18*). High (6.5gms/kg/Bw) and moderate doses (5.0gms/Kg/Bw) were seen to cause shrinkage of acini clusters within volume densities (*Graph 6*). This reduction in acini was seen to be reciprocated with consequent replacement of the shrunk lobules with deposits of fibrous connective tissues (*Figure 14C&D*). The quantitative effects on the exocrine and connective tissues histomorphometric parameters that included the volume densities of acini, blood vessels and ducts reduced regressively with increasing alcohol concentrations while the fibrous

connective tissue deposits linearly increased with increasing alcohol doses (*Table 19*).

In the endocrine pancreas, the qualitative and quantitative effects depicted a dose dependant relationship. The endocrine cellular counts, sizes of islet clusters, and intracellular morphology of the islet cells showed an in an inverse relationship with increasing alcohol doses while the parenchymal parameters including, volume densities, the islet clusters per section, islet mass, islet volume, the Beta cell counts per islet, the beta cell mass and the beta cell volume reduced exponentially with increasing doses of alcohol (*Table 20; Graph 7*).

An inverse Pearson correlations between the total islet mass and the alcohol doses was observed to exist as follows {(LAG $\rho r=0.513$; $p=0.034$), (MAG $\rho r=-0.599$; $p=0.021$), (HAG $\rho r=-0.717$; $p=0.031$)}. Additionally, the volume density of the islets with the alcohol doses {(LAG $\rho r= -0.719$; $P=0.01$), (MAG $\rho r=-0.669$, $p=0.033$), (HAG $\rho r=-0.788$; $p=0.021$)} was seen to exist.

Table 18: Effects of various alcohol doses on canalization of acini, number of acinar cells per cluster and acini clusters per field in the alcohol treated groups compared with the controls.

Alcohol doses/ Study group	Number of acini per field	Canalized acini per field	No. of acinar cells per cluster
Control	39.10\pm1.12	11.23\pm0.16	27.23\pm0.92
2.5gms/Kg/Bw (LAG)	34.11\pm1.00	10.97\pm0.02	25.21\pm0.37
5.0gms/Kg/Bw(MAG)	31.01\pm1.01	7.10\pm0.09	22.11\pm0.41
6.5gm/Kg/Bw (HAG)	32.22\pm1.13	5.23\pm0.07	17.29\pm0.56

Values are Mean \pm SE. CoE for point counting in each measurement are less than 0.05

**P< 0.01 when compared with the control, low alcohol group and medium alcohol groups

*P<0.01 when compared with the control

^cP<0.01 when compared with the control and the low alcohol group

Table 19: Shows effects of various alcohol doses on the exocrine and connective tissues histomorphometric parameters.

Study group	Pancreas weight (mgs)	Vol. density of acini (%)	Volume density: CT (%)	Volume density: BV and Ducts (%)	Pancreas volume (mm ³)
control	11.14±0.012	67.35±1.97	27.90±0.92	5.49±0.053	12.02±0.03
2.5gms/kg/Bw	10.62±0.01*	65.31±0.81*	29.34±0.78*	5.19±0.003*	11.02±0.02
5.0gms/Kg/bw	10.34±0.003*	62.9±0.61**	32.67±0.19**	4.23±0.009*	11.91±0.016*
6.5gms/Kg/Bw	9.06±0.002* ^c	58.39±0.43 ^c	36.31±0.71* ^c	4.08±0.007* ^c	8.94±0.013**

Values are Mean±SE. CoE for point counting in each measurement are less than 0.05

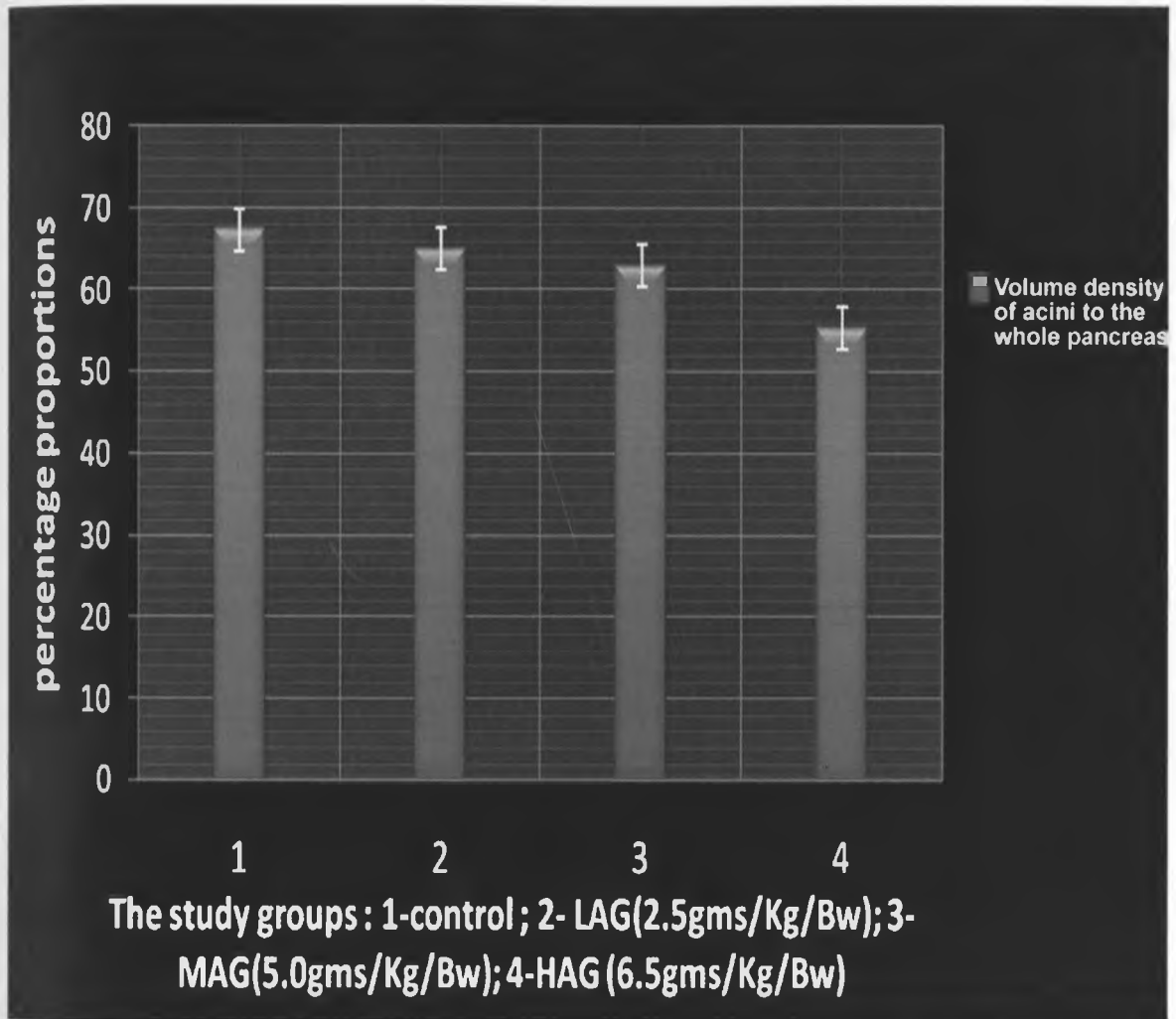
*P< 0.01 when compared with the control, low alcohol group and medium alcohol groups

**P<0.01 when compared with the control

^cP<0.01 when compared with the control and the low alcohol group

NB> Note that the reduction in fetal exocrine and connective tissues that is inversely proportional to the increasing alcohol doses.

Graph 6: A bar graph showing the percentage volume densities of the acini between the various experimental groups compared with the controls



NB> *Note the reducing proportion in volume density of the acini with increasing alcohol doses*

Table 20: The effects of various alcohol doses on the fetal endocrine pancreas quantitative parameters in the alcohol treated groups compared with the controls

Doses of alcohol/Study group	Islet mass	Beta cell mass	Volume density: Beta cell/islets (%)	Volume density: Islets/pancreas	Total islet volume
control	2.2±0.07	1.60±0.014	73.4±1.12	2.87±0.006	0.93±0.001
2.5gms/kg/Bw	1.9±0.01*	1.51±0.089 *	69.81±1.16*	2.69±0.0013*	0.88±0.0012
5.0gms/Kg/bw	1.61±0.018 *	1.33±0.023 **	64.23±1.11**	2.22±0.001*	0.71±0.0033*
6.5gms/Kg/Bw	1.37±0.009 * ^c	1.28±0.008 * ^c	52.67±1.34* ^c	2.09±0.001* ^c	0.71±0.0010**

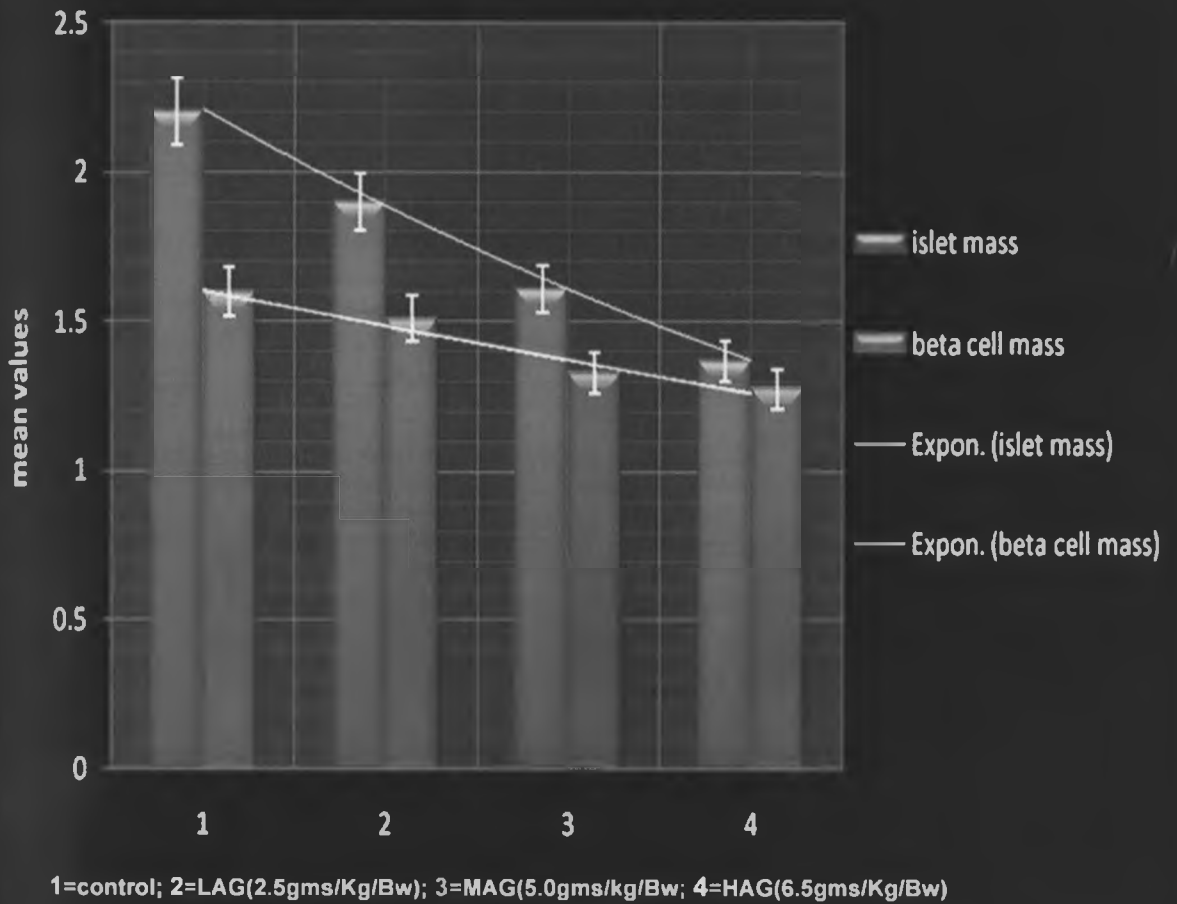
Values are Mean ±SE. CoE for point counting in each measurement are less than 0.05

*P<0.01 when compared with the control, low alcohol group and medium alcohol groups

**P<0.01 when compared with the control

*^cP<0.01 when compared with the control and the low alcohol group

Graph 7: A bar-graph with exponential trendlines showing the change in islet mass and the beta cell mass for the alcohol treated groups compared with the control



NB> Note the exponential reduction in both the islet mass and the beta cell mass in the alcohol treated groups compared with the control

3.3.0 THE POSTNATAL EFFECTS OF PRENATAL ALCOHOL EXPOSURE ON THE PUPS PANCREAS

3.3.1. General observations on the pups pancreas

The weight of a four weeks old pup ranges between 85.5 to 96.2gms while its pancreas weight ranges between 56.5 and 64.0mgs with a mean weight of 51.14 ± 0.82 mgs. The pancreas volume ranges between 52.00 - 53.09 mm³ with a mean volume of 52.02 ± 0.001 mm³. The exocrine parenchyma constitutes 67.5% of the total pancreas while the endocrine parenchyma and the connective tissues stroma constitutes 2.9% and 29.5% respectively (*Table 21*). The pup pancreas depicts similar morphological appearance to the fetal pancreas by having an expanded head, a narrow neck, broad body and a tapering branched tail (*Figure 25A*). It is also contained in a thin fibrous capsule that sends in septa dividing it into lobules (*Figure 25b*). Each lobule comprises of numerous evenly distributed acini that are clustered in the head, neck and the body, and relatively scattered in the tail region (*Figure 25a*). The acini clusters depicted regional variation with the head and neck recording 28-36 clusters per field, the body with 30-40, and the tail recording between 20-25 clusters per field (*Table 22*). The volume density of acini is 68.1%, followed by 28.9% of connective tissue stroma, then 5.67% being blood vessels and duct and lastly 3.1% islets (*Table 23 & 24*).

The beta cells contributes the highest proportion of the cellular constituents in an islet cluster of between 68 to 77% followed by alpha cells with between 20-25%,

and lastly the delta cells with 2-5% (*Table 25*). The neck and the body recorded the highest number of B-cell per cluster compared with the head and the tail (*Table 26*).

The parenchyma and stromal tissue comparisons between the fetal and pups pancreas did not show a statistical significance ($p=0.0691$).

These structural alterations including changes in size, shape, cellular composition and the patterning of parenchyma to both the exocrine and endocrine cells in the fetal pancreas that was caused by prenatal exposure to alcohol was also observed to be sustained in the pups pancreas. These structural and morphometric alterations in the pups' pancreas were also seen to have a bearing to the time of alcohol exposure and with the alcohol doses administered to the mother during pregnancy.

Table 21: The percentage proportions of the parenchyma and the stromal tissues in relation to the pancreas and body weight of a normal pup.

Study parameter	The percentage range between Min and max	Mean \pm SE
Body weight (gms)	85.5 - 96.2	91.63 \pm 1.13
Pancreas weight (mgs)	56.5 - 64.0	57.14 \pm 0.82
Percentage endocrine tissue(%)	63.0 - 71.1	67.5 \pm 0.15
Percentage exocrine tissue(%)	2.1 - 3.3	2.91 \pm 0.03
Percentage stroma(%)	27.2 - 31.3	29.51 \pm 0.51

Table 22: The ranges and the mean numerical counts of the acini clusters per field with their regional distribution in pups pancreas.

Acini distribution according to Parts of the pups pancreas								
Parameters	Head		neck		body		tail	
	Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE
No. of acini per field	28-37	33.9 \pm 0.6	30-40	31.1 \pm 0.2	20-31	25.6 \pm 0.7	20-25	21.3 \pm 0.3

NB> the mean values on the regional distribution of acini clusters per field were statistically significant ($P < 0.05$) across the regions by Chi-square test at (95%) confidence intervals when compared across the parts of the pancreas.

Table 23: The normal ranges and means of the exocrine and connective tissues morphometric parameters in relation to pups pancreas and body weights.

parameters	The normal ranges of the Fetal stereological parameters	
	Ranges	Means \pm SE
Fetal Body weight (gms)	85.5 - 96.20	93.57 \pm 0.11
Pancreas wet weight (mgs)	56.5 - 64.00	61.14 \pm 0.03
Pancreatic volume(mm ³)	52.00 - 67.05	62.02 \pm 0.01
Volume density acini (%)	65.00 - 75.0	71.31 \pm 0.96
Volume density Connective tissues(%)	25.00 - 29.0	25.90 \pm 0.07
Volume density Blood vessels and ducts (%)	4.00 - 7.00	5.47 \pm 0.001

Table24: The range and the means of the normal pups endocrine pancreatic stereological parameters

parameters	The ranges and the means of the Endocrine stereological parameters	
	Ranges	Means \pm SE
Islet mass (mg)	5.6 - 6.75	6.22 \pm 0.07
Beta cells mass(mg)	2.01 - 3.00	2.6 \pm 0.01
Volume density: Beta cell /islet (%)	66.0 - 79.00	75.4 \pm 1.06
Total B-cell volume(mm ³)	1.88 - 1.95	1.92 \pm 0.09
Volume density: Islet/pancreas (%)	3.80 - 4.01	4.87 \pm 0.03
Total islet volume (mm ³)	1.50 - 2.0	1.93 \pm 0.01
Volume -weighted mean islet volume(mm ³)	2.30 - 2.50	2.35 \pm 0.02
Total islet number (x10 ³)	2.30 - 3.15	2.67 \pm 0.012

NB> In this study positive correlations were observed as follows:- ($r=0.69$, $p<0.013$) between the islet mass and the beta cell mass, ($r=0.719$, $p<0.05$) between volume density of the beta cell per islet and the volume density of islets to the total pancreas mass, and ($r=0.69$; $p<0.017$) between the total islet volume and the total islet numbers. It was however noted that, the volume-weighted mean islet volume did not show an intra-group or inter-group statistical significance difference ($P>0.05$). In addition, the pancreatic endocrine tissue per unit body weight and the unit body

volume also remained constant when comparisons were done within and across the study groups.

Table 25: The ranges, means and the percentage cell composition of islet cells in the control pups pancreas.

Cell type	The ranges of cell counts per islets	Mean numerical counts \pm SE	The ranges of Percentage (%) compositions
Beta cells(β)	64 - 135	107.11 \pm 2.09	68 -77%
Alpha cells (α)	15 - 39	22.47 \pm 1.29	20-25%
Delta cells (δ)	4 - 13	5.61 \pm 0.08	2 - 5%
Others (PP, Cells)	1 - 5	1.79 \pm 0.01	0.1- 0.9%

Table 26: The ranges and the mean cellular counts of β -cells per islet cluster with their regional distribution.

Parameters	Islet distribution according to the parts of the pups pancreas							
	Head		neck		body		tail	
	Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE
No. of beta cell per islet cluster	63-79	71.3 \pm 0.9	84-101	96.3 \pm 0.7	79-119	97.5 \pm 0.2	51-76	63.2 \pm 0.3

NB>

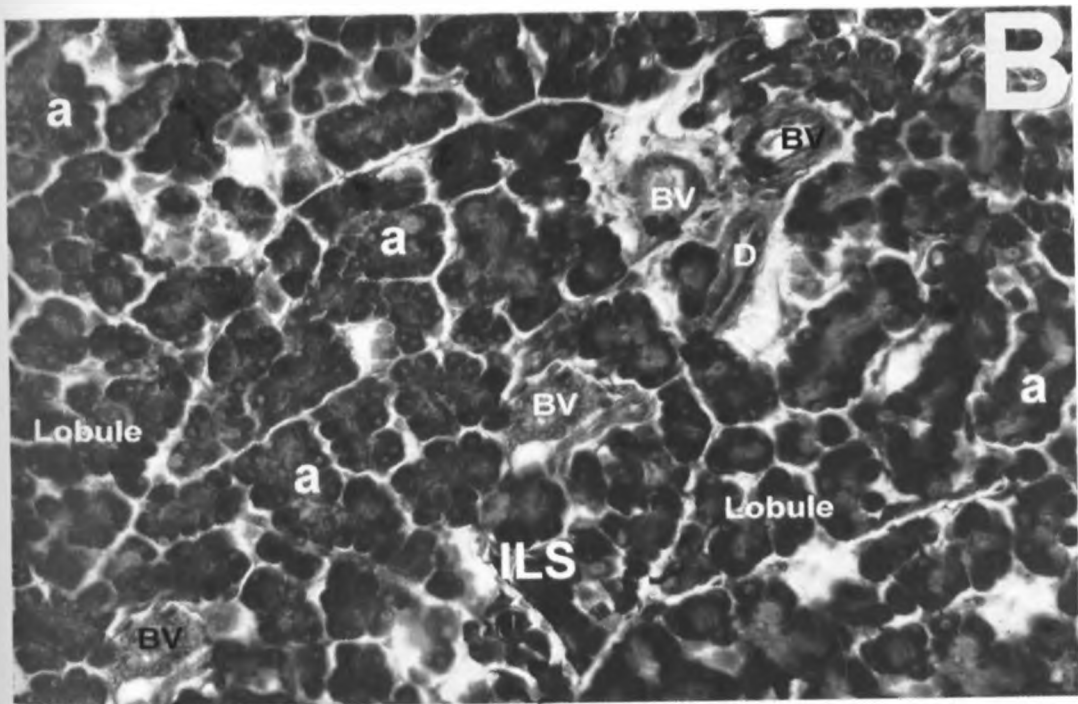
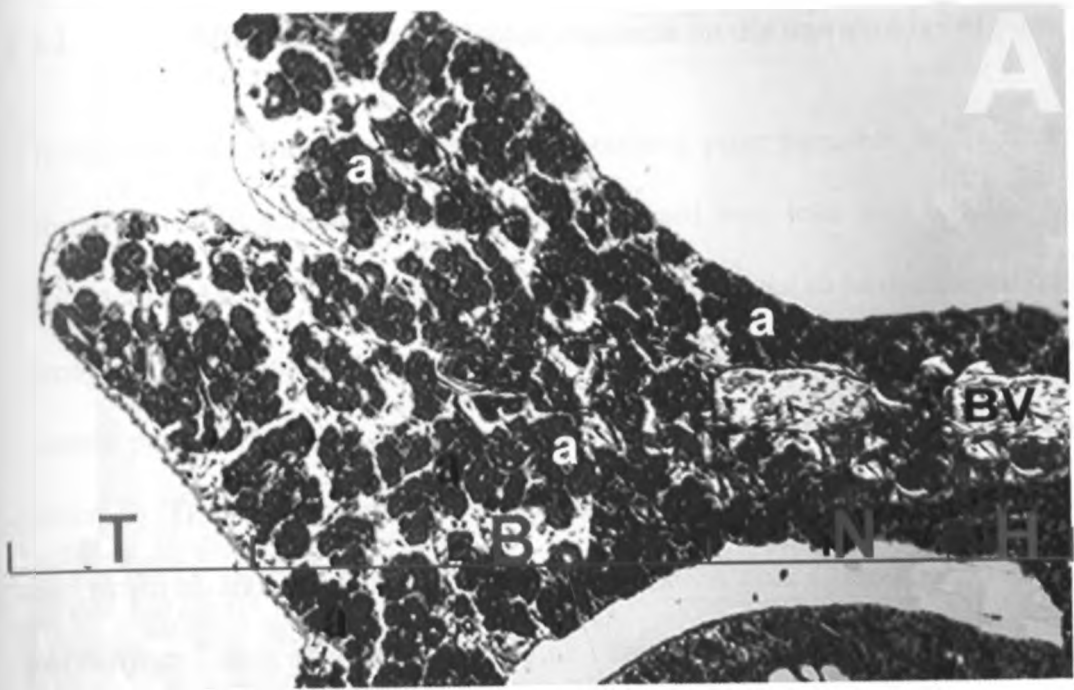
The mean values on the regional distribution of the numerical counts of beta cells per islet are statistically significant ($P < 0.05$) across the regions by Chi-square test at (95%) confidence intervals when compared across the parts

Figure 25 A&B: Sections of pup pancreas showing the parts and distribution of acini within the lobules.

25A: A transverse section through the middle of a pup pancreas showing the parts. Note the slightly expanded head (H) as it joins the second part of duodenum, the narrow neck (N) joining the head and body, and the broad body(B) and the branched tail (T). Note the regional distribution of acinar clusters (a) across the section. Note the high clustering of the acini in the head (H) and the neck (N) region with a the relative scattering of the acini clusters in the body (B) and the tail (T) regions.
H&E Mag x10

25B: A section of pup pancreas showing the organization of the acini clusters into lobules. Note the interlobular septa (ILS) that are an extension of the outer fibrous covering capsule. Note the organization of the acini (a) into lobules. *H&E Mag x 40*

Figure 25A&B:



3.3.2 Effects of prenatal alcohol exposure on the morphology of pups exocrine pancreas

Though the exocrine parenchyma in the control pups pancreas had attained substantive development by being well organized into lobe and lobules, the exocrine tissue in the alcohol exposed groups was observed to have attained only some relative maturation with some acini remaining uncanalized, fewer acini clusters per field as well as fewer acinar cells per cluster (*Figure 26A&B, and Tables 27*). The mesenchymal tissue was highly reduced in all the study groups (experimental and the control) due to proliferation and differentiation of the parenchyma (exocrine and endocrine tissues) though some traces of undifferentiated mesenchymal tissues were still found in the interlobular and inter-acinar spaces and seen as aggregations of undifferentiated cell masses with cells depicting delayed or arrested development (*Figure 26A&B*). The numerical counts of acinar cells per acini cluster in the alcohol treated pups were also fewer compared with the control as shown in *Table 28*. These effects on the acini were also seen to have regional variations with the tail and body suffering the highest reduction in the number of acini while the head and neck were not as greatly affected (*Figure 27 A-D*)

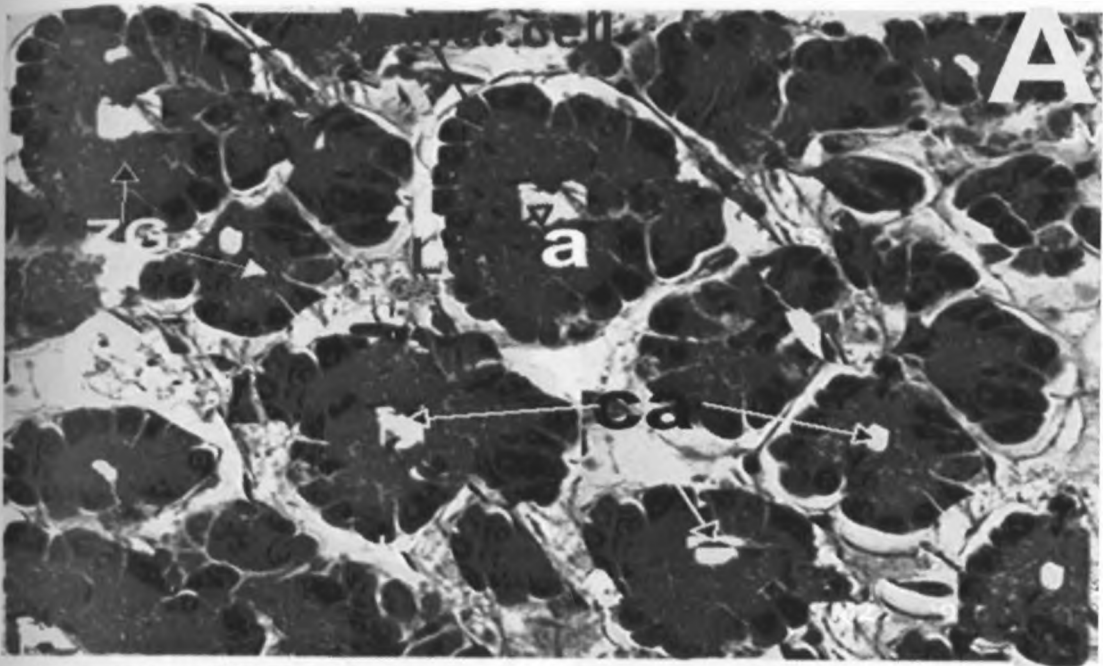
The volume densities of the acini, blood vessels and ducts regressed linearly with increasing alcohol doses. To the contrary, the fibrous connective tissue deposits increased linearly with increasing alcohol doses (*Table 29, 30 & Graph 8*).

Figure 26 A&B: Sections of pups pancreas showing reduced acini clusters in the alcohol treated groups (MAG) compared with the control.

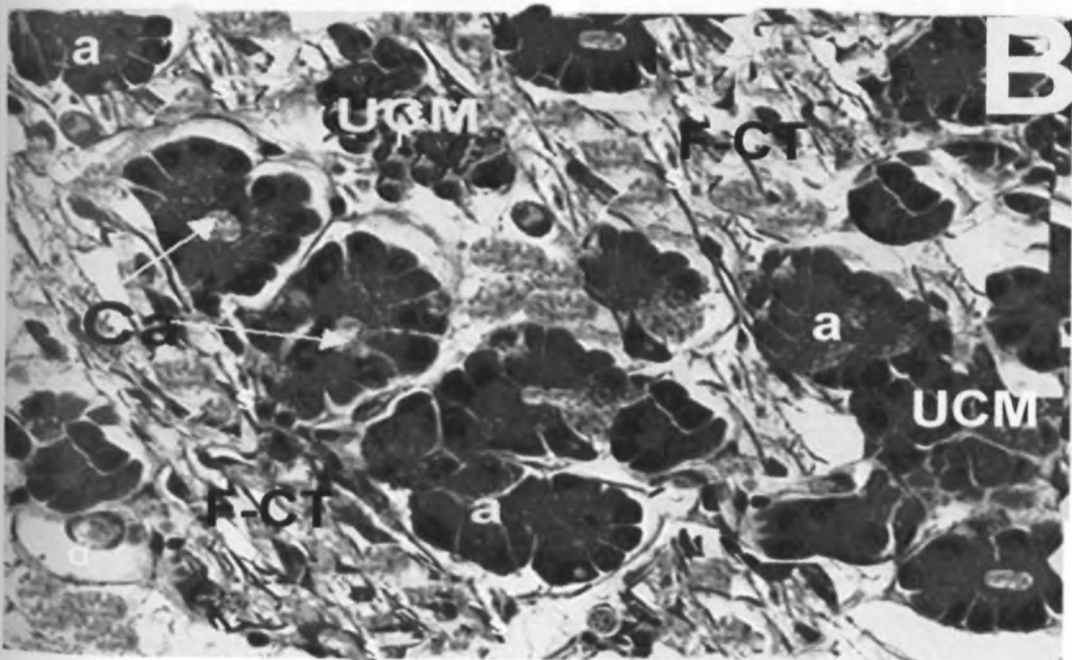
26A: A longitudinal section of a control pup pancreas showing the distribution of acini clusters (a) across the section. Note the even distribution and canalization of the acini (Ca). They depict conspicuous acidophilic zymogenic granules (ZG). The acinar cells are pyramidal in shape with basally located nuclei while the apical side that bears the cytoplasmic granules faces the lumen (Lu) of the acini cluster. *H&E Mag X40*

26B: A section of an alcohol treated pup pancreas showing the distribution of acini clusters (a) in a field. Note the relative reduction in the number of acini (a) that are sparsely distributed in the field with high deposition of fibrous connective tissue (F-CT). Note traces of cells with arrested or delayed cell differentiations that are seen as undifferentiated cell masses (UCM). Note the reduced number of canalized acini (Ca), and the small sizes of acini clusters with less number of acinar cells per cluster. The acinar cell are pyramidal in shape with basally located nuclei and the cytoplasm facing the apical side of the lumen (Lu) of the intra-acini duct. *H&E Magx40*

Figure 26 A&B:



Control pupa exocrine tissue



The alcohol treated exocrine tissue




Figure 27A-D: Sections of the pups pancreas showing the effects of alcohol exposure on the morphology of islets in the alcohol treated group compared with control

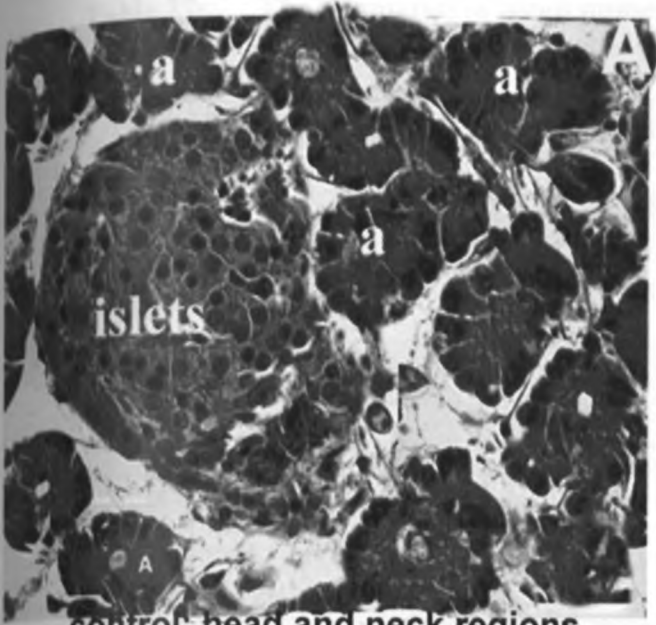
27A: A section of pup pancreas taken between the head and neck region showing the morphology of well developed islets. Note the high number of acini clusters(a) that are well developed. *H&E Mag x40*

27B: A section of an alcohol treated pup pancreas taken at the level of the head and neck region. Note the disaggregated islet clusters, increased stomal tissue deposits (S), and the reduction in the numbers of the acini clusters (a). *H&E Mag x40*

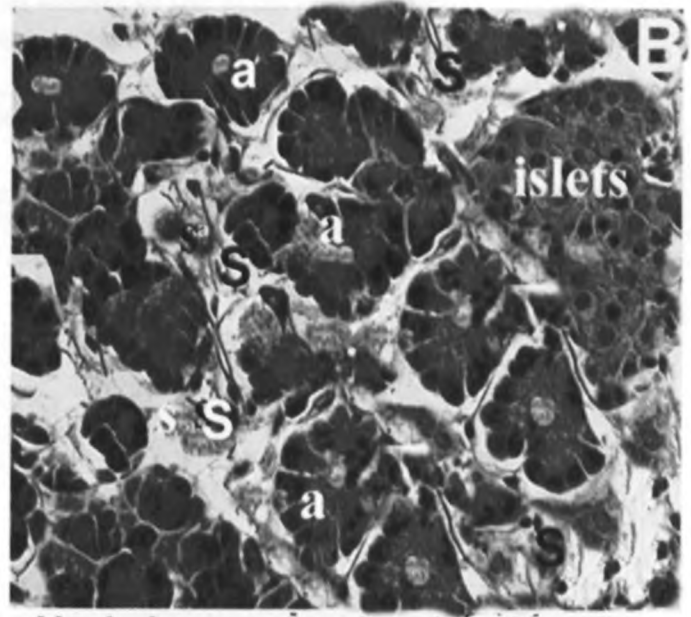
27C: A section of a control pup pancreas taken at the level of the body and the tail region showing a well developed islets in an even distributed acini (a). Note the large sizes of the acini clusters with numerous acinar cells per cluster. *H&E Mag x40*

27D: A section of an alcohol treated pups pancreas taken at the level of the body and the tail region. Note the relative reduction in the the size of the islet cluster as well a reduction in numbers of the acini clusters (a) and deposition of stroma (S) *H&E Mag x40.*

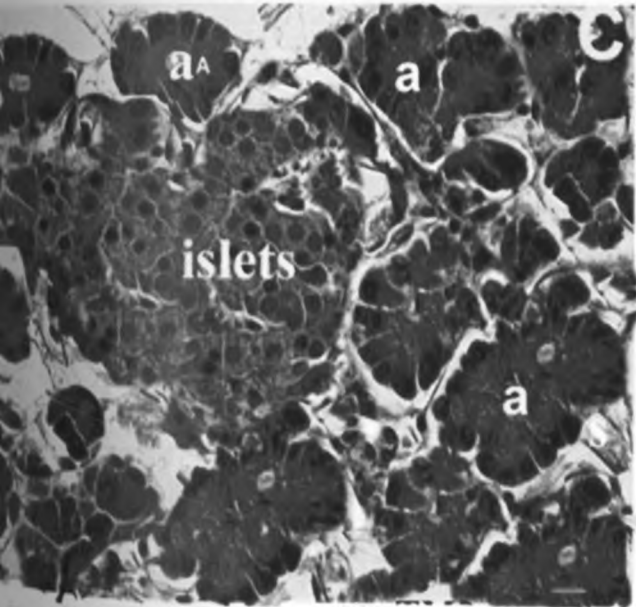
Figure 27 A-D:



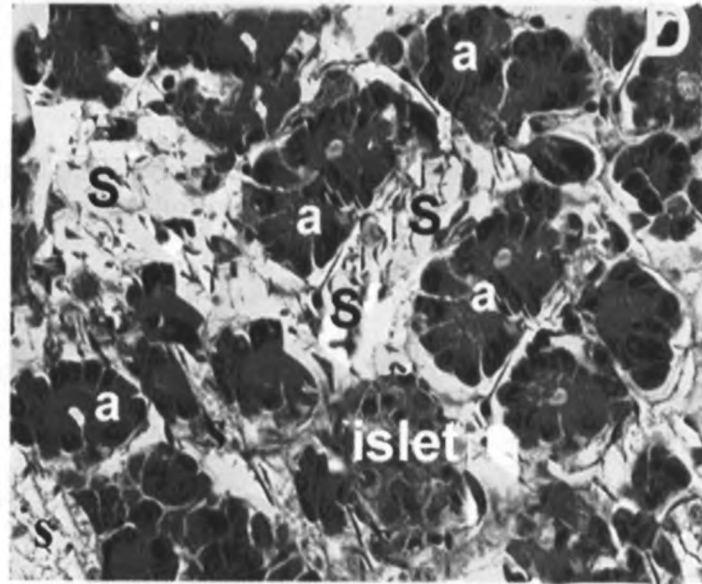
control: head and neck regions



Alcohol group: head and neck region



control: Body and tail region



Alcohol group: body and tail region

Table 27: The comparative mean numerical counts of the acini clusters per field for the alcohol treated pups compared with the control

Study group	Parameters	Acini per field with regional distribution							
		Head		neck		body		tail	
		Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Controls group	No. of acini per field	29-37	27.2±0.5	24-32	28.1±0.9	20-27	21.2±0.2	15-19	17.7±0.1
Alcohol Treated groups	No. of acini per field	19-28	26.92±0.3	17-27	26.3±0.3	19-27	21.1±0.4	11-16	13.3±0.9
	P-value		0.032		0.037		0.017		0.013

Table 28: The range and the mean canalized acini per field across the head, neck, body and the tail regions of the alcohol treated pups compared with the controls

Study group	Parameters	The ranges and means of canalized acini per field							
		Head		neck		body		tail	
		Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Control:	Canalized acini per field	5-10	7.6±0.3	5-12	7.1±0.4	10-16	12.6±0.2	8-15	11.8±0.9
Alcohol Treated groups	Canalized acini per field	4-7	5.2±0.5	4-9	6.7±0.8	8-14	9.6±0.7	2-6	5.7±0.8
	P-values		0.0477		0.016		0.021		0.039

Table 29: The comparative mean numerical counts of the pups acinar cells per acini cluster in the alcohol treated group compared with the controls

Study group	Parameters	Acini cells per cluster with regional distribution							
		Head		neck		body		tail	
		Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE	Range	Mean \pm SE
Controls group	No. of acini per field	27-39	30.1 \pm 0.3	25-31	28.3 \pm 0.9	22-35	25.9 \pm 0.1	19-24	18.6 \pm 0.4
Alcohol Treated groups	No. of acini per field	23-34	28.1 \pm 0.9	18-26	24.1 \pm 0.9	18-29	19.6 \pm 0.2	12-19	14.1 \pm 0.2
	P-value		0.032		0.048		0.032		0.049

Table 30: The non parametric Mann Whitennys U- test on the pups' acini, CT, the pancreas and body weights of the alcohol treated group compared with the control

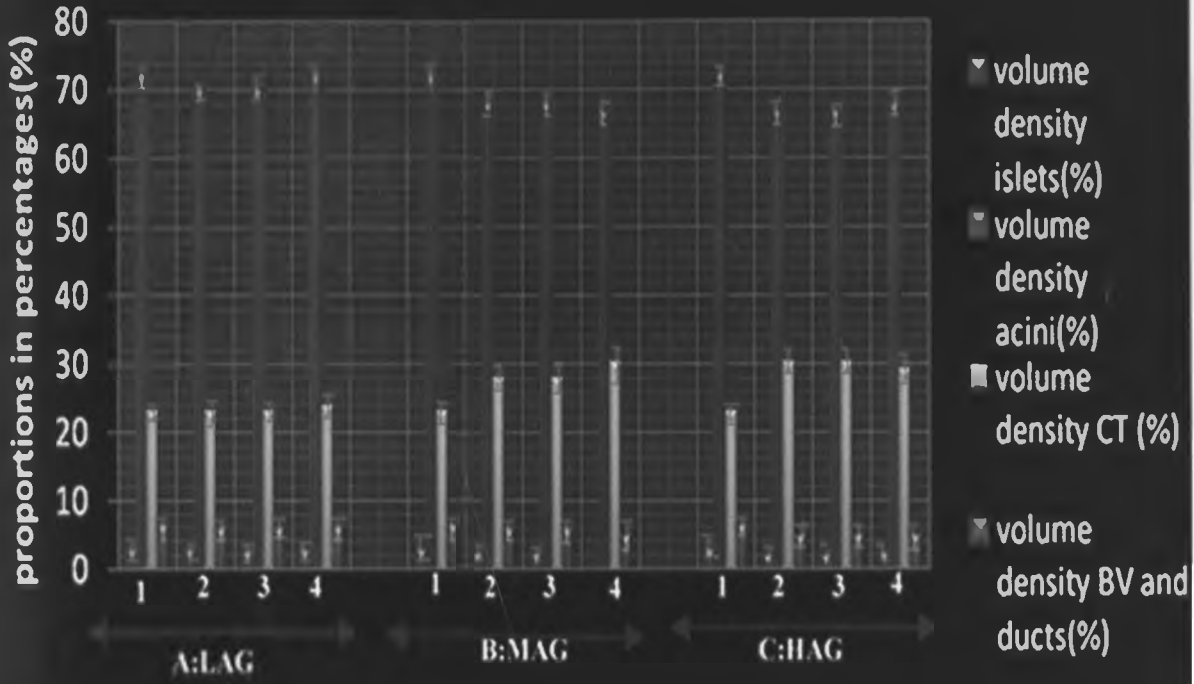
	Means \pm SE		Mann whitenny U values-Alcohol group versus the control group			
	control	Alcohol group	The median values Used		[U] Calc	P[U]
			control	Alcohol		
Volume density of acini (%)	71.31 \pm 0.96	62.91 \pm 0.63	67.12	63.4	0*	0.004
Volume density BV and ducts (%)	5.47 \pm 0.03	4.83 \pm 0.002	5.33	5.00	0*	0.004
Vol. density fibrous CT (%)	25.91 \pm 0.07	33.61 \pm 0.07	29.27	31.35	2*	0.038
Pancreas wet weight	61.14 \pm 0.03	59.29 \pm 0.04	11.01	10.53	2*	0.034
pups body weight (gms)	5.67 \pm 0.19	4.93 \pm 0.16	5.63	5.12	0*	0.004

* - values whose P- value was less than or equal to 0.05

U-calc = calculated Mann-whiteny U-value

P[U]= probability of occurrence of calculated U value

Graph 8: Comparative percentage proportions in the volume densities of islets, acini, CT, BV and ducts of the alcohol treated groups against the controls at TM1, TM2 and TM3



Animal groups
1: control, 2:TM1, 3:TM2, 4:TM3
A:LAG; B:MAG; C:HAG

3.3.3. The ultra- structural effects of alcohol on the morphology of pups acini

Ultrastructurally, alcohol interferes with subcellular morphological structures of the pups acini cells. It impaired the secretory process of the acini cells as was depicted by the reduced number and the sizes of the cytoplasmic zymogen granules and by the presence of lipid vacuolations in the cytoplasm

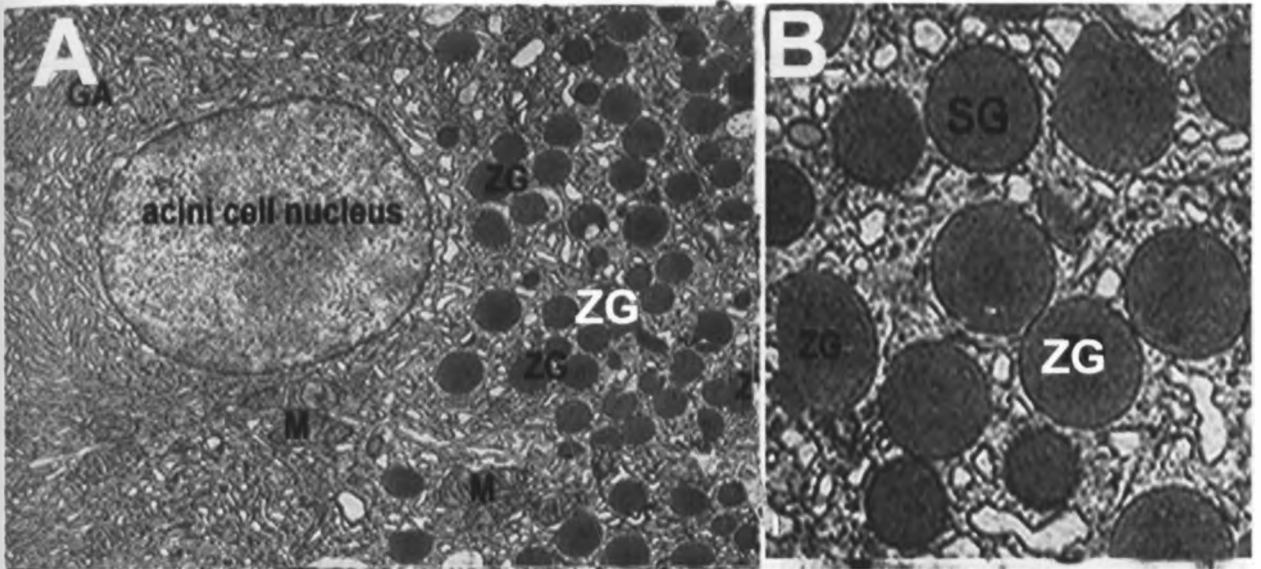
(Figure 28A-B).

The cytoplasmic vacuolations in the acini were observed to be filled with lipid droplets *(Figure 28b).*

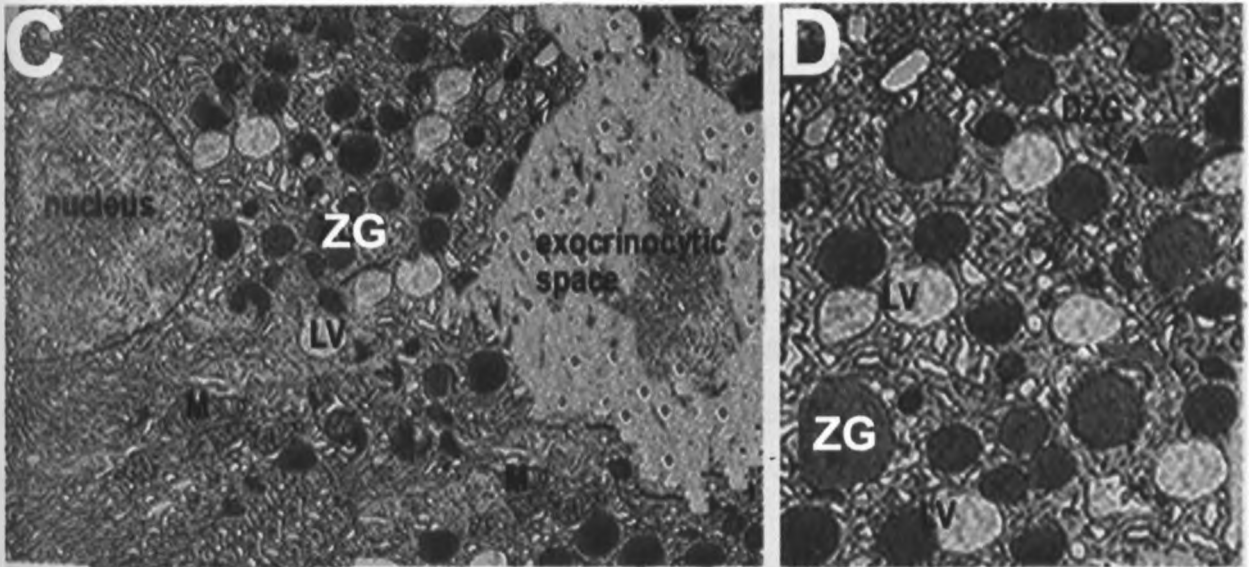
**Figure 28A -D: Electron micrographs of pups acinar cells showing
Altered secretory functions of the cells and
reduction in zymogen granules**

- 28A:** An electron micrograph of a control acinar cell depicting typical protein secreting cell with conspicuous zymogenic granules (ZG) that are electron dense and membrane bound. Note the presence of Mitochondria (M), Golgi apparatus (GA). *TEM-Mag x 18,200*
- 28B:** An electron micrograph showing mature and well developed zymogen granules (ZG) of the control fetal acini cells. Note that they are filled with an electron dense material. *TEM-Mag x 55,200*
- 28C:** An alcohol treated pups acinar cell showing cytoplasmic lipid vacuolations. Note the reduced number of zymogenic granules and the increasing numbers of lipid vacuolations (LV). Note the presence of Mitochondria (M) *TEM-Mag x 18, 200*
- 28D:** An electron micrograph showing immature and poorly developed zymogen granules (ZG) of an alcohol treated fetal acini cells. Note that they are small in size and sparsely distributed with some showing discontinuous binding membrane. Also note the presence of lipid vacuoles (Lv). *TEM-Mag x 55, 200*

Figure 28A& B:



control Pups acini cell and the Zymogenic granules



Alcohol treated pups acini cell and the zymogenic granules

3.3.4 **Effects of prenatal alcohol exposure on the morphology of the pups endocrine pancreas**

In the endocrine pancreas, the alcohol treated groups had fewer aggregations of islets clusters with poorly defined areola like capsule. They were also small in size and irregular in shape, with fewer numbers of β and α -cells and showing poor vascularization (*Figure 29A-D*). This was in variance with the control pups whose islets depicted a prototypical characteristics of well developed islets by being larger in size and encapsulated with the cells closely packed close to a rich capillary network. The β and α -cells being bigger in size numerous in numbers (*Figure 30a-d*). Further, the percentage of the total islet mass as well as the total beta cell mass in the alcohol treated groups was lower compared with the control (*Graph 7*).

The total islet mass and the beta cell mass were found to have a linear correlation ($p \leq 0.005$) with the pups pancreas weights as well as the pups body weights in both the alcohol treated groups and the controls. Such a similar linear correlation was also found to exist when the total islet mass was correlated with the volume weighted mean islet volume in the same group ($p \leq 0.05$) regardless of the study category in the experimental or the control group. However, a statistically significant ($p < 0.05$) linear regression in all endocrine histomorphometric parameters was noted when correlated with the alcohol concentrations and with the time of exposure. The ratios of the pancreatic endocrine tissue per unit body

weight and the volume weighted mean islet volume examined for both the alcohol groups and the control did not show statistical significance difference ($P > 0.05$). The percentage cellular constituents of the islets were however all reduced by the exposure to alcohol (Table 31).

Table 31: The range and the means of the islets cellular composition for the alcohol treated pups compared with the controls.

Cell type	controls		The alcohol groups		P- values
	Ranges of numerical β -cell counts per islets	Mean numerical β -cell counts \pm SE	Ranges of numerical cell counts per islets	Mean numerical counts \pm SE	
Beta cells (β)	81 - 158	112.06 \pm 2.91	70 - 129	103.09 \pm 2.67	0.011
Alpha cells (α)	24 - 56	32.49 \pm 1.33	16 - 37	27.26 \pm 1.33	0.034
Delta cells (δ)	12 - 22	13.20 \pm 0.19	7 - 16	9.20 \pm 0.10	0.039
Others (PP,C-cells)	7 - 13	5.01 \pm 0.07	2 - 6	3.00 \pm 0.01	0.0412

Figure 29A-D: Sections of the pups pancreas showing effects of alcohol on the disaggregation of the islet clusters

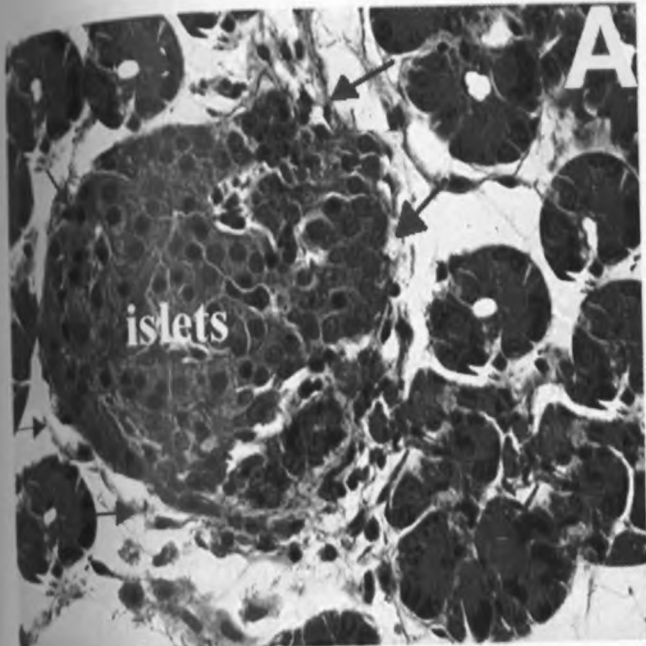
29A: A section of a control pups pancreas taken at the junction of the head and neck region showing a classical islet cluster that is contained in a capsular layer of areolar like capsule (*indicated by the blue arrows*). Note an islet cluster that is well developed between acini clusters. *H&E Mag x40*

29B: A section of an alcohol treated pups pancreas taken at the junction of the head and neck region at TM₁. Note the highly disaggregated islet clusters with reduced number of cells. The capsular is also disintegrated. *H&E Mag x40*

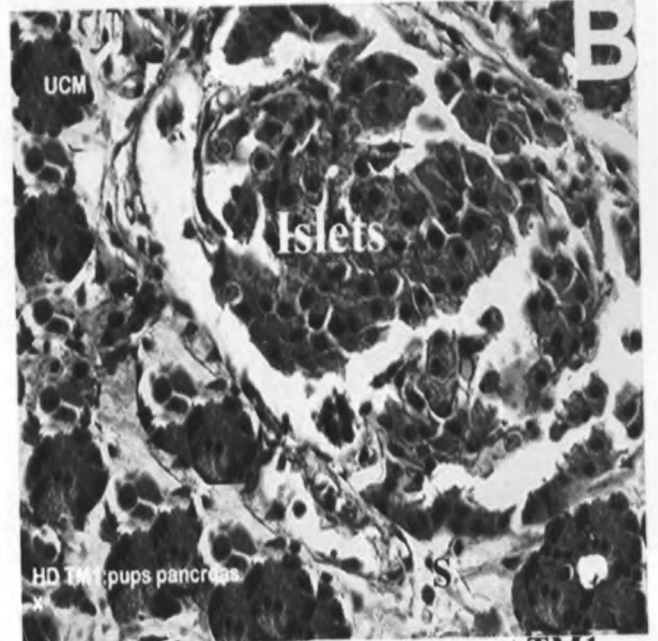
29C: A section of a control pups pancreas taken at the junction of the head and the neck region at TM₂. Note the disaggregated islet clusters that resembling those in *figure 29B above*. Note that the capsular covering is also poorly developed and unrecognizable. *H&E Mag x40*

29D: A section of an alcohol treated pups pancreas treated at TM₃. The section is taken at the junction of the head and neck. Note some slight disaggregation of the islet clusters but almost looking like the islets wqtyof the control. *H&E Mag x10*

Figure 29A-D:



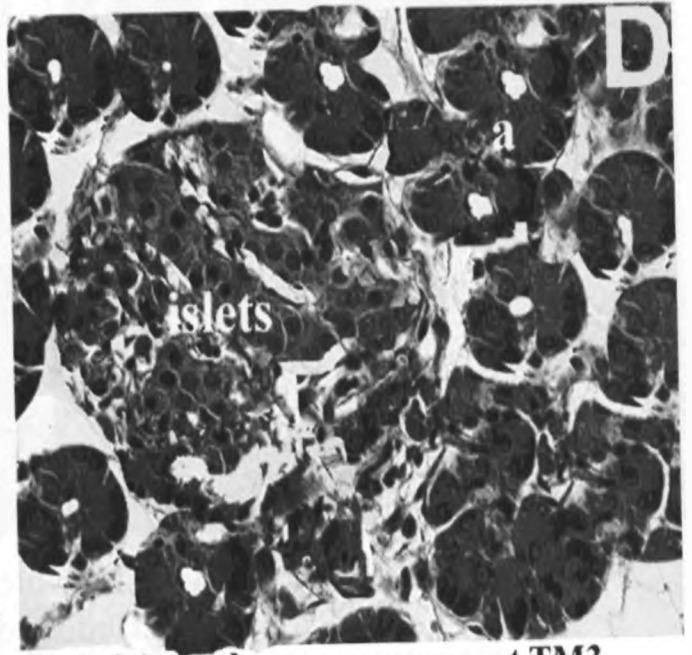
control Pups pancreas



Alcohol treated pups pancreas at TM1



Alcohol treated pups pancreas at TM2

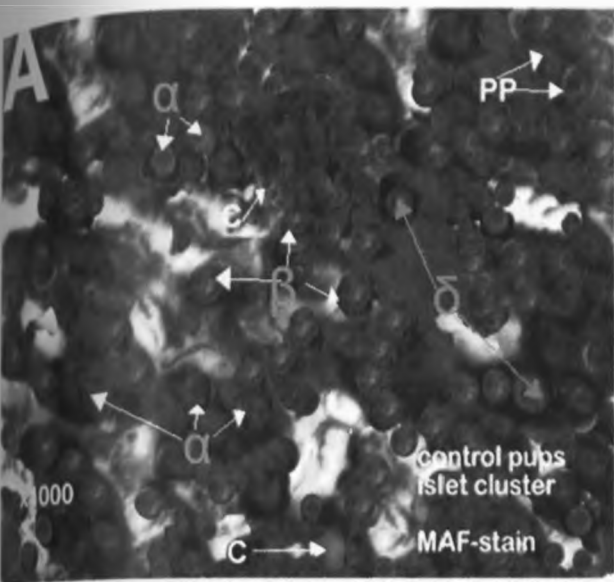


alcohol treated pups pancreas at TM3

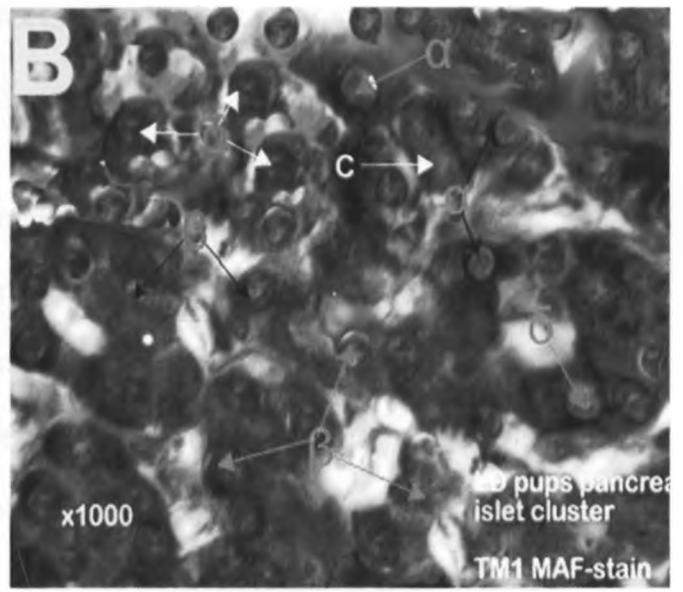
Figure 30 A-D: Sections of pup pancreas showing the effects of alcohol on the cellular composition of the islets.

- 30A:** A section of a control pup islet of langerhans showing the composition of the α , δ , β , PP and the c-cells in an islet of langerhans. Note the highly parked cells appering in cord like structures. (*MAFs x1000*)
- 30B:** A section of an alcohol treated pup islet of langerhans exposed to alcohol on trimester one (TM₁). Note the paucity in the specific cellular components including the α , δ , β cells and complete disappearance of the PP and the C-cells. Modified modified adehyde fucshin stain (*MAFs x1000*)
- 30C:** A section of an alcohol treated pups islet cells exposed at trimester two (TM₂). Note the paucity in the specific cellular components including the α , δ , β , PP and C-cells and complete disappearance of the C- cells. Modified modified adehyde fucshin stain (*MAFs x1000*)
- 30D:** A section of an alcohol treated pups islet cells exposed at trimester one (TM₁). Note some relative reduction in numbers of specific cells including α , δ , β cells, PP and the C-cells there was no disapparence of any of the cell types. (*MAFs x 1000*)

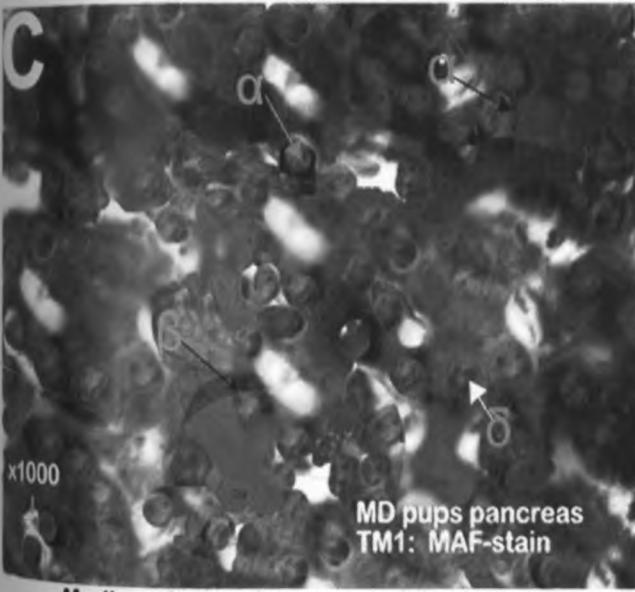
Fig 30 A-D:



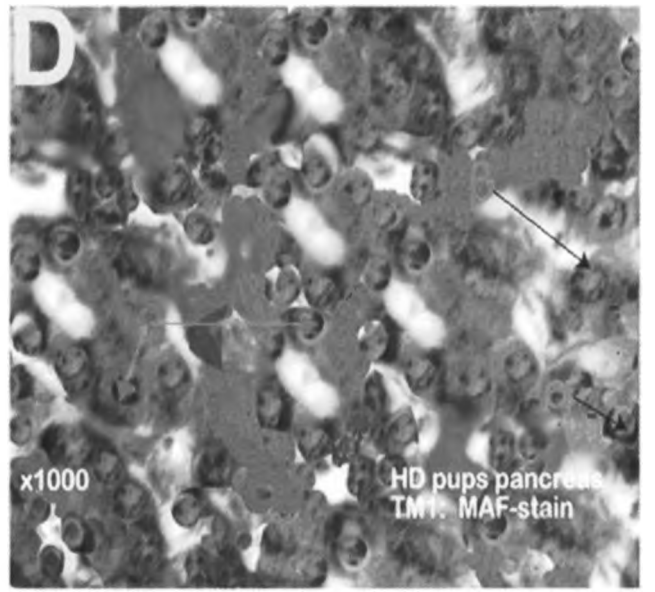
control pups pancreas



Low dose pups pancreas



Medium dose pups pancreas



high Dose pups pancreas

NB> *Note the paucity of the different cell types in the alcohol treated groups compared with the control.*

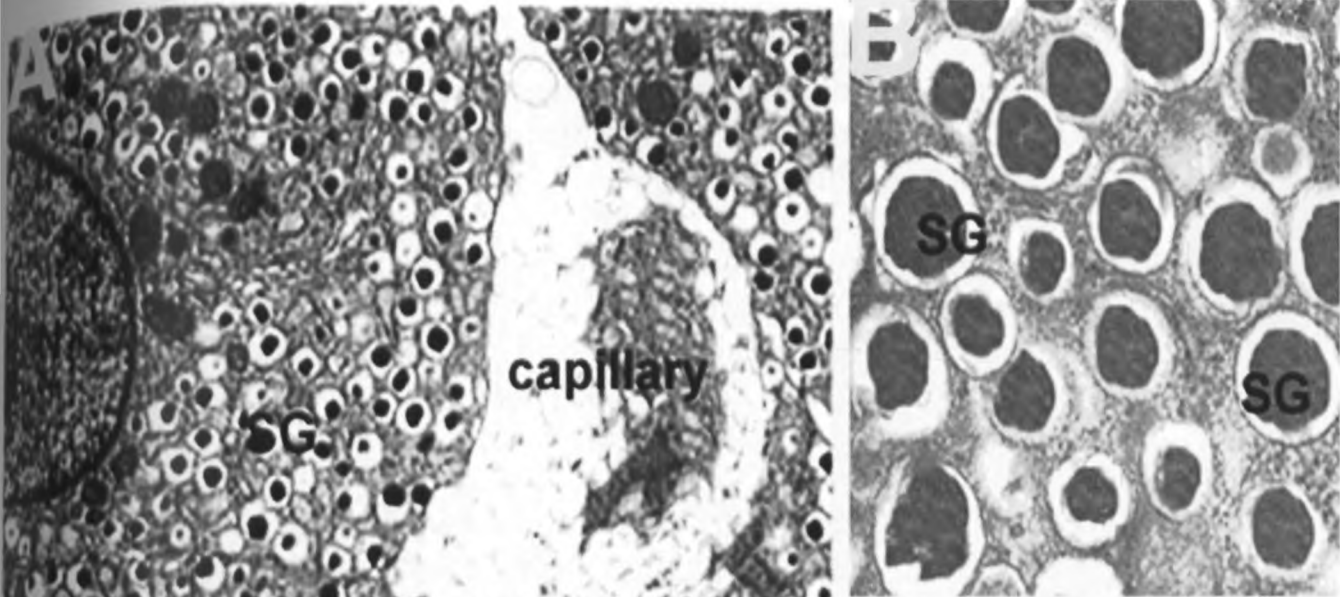
3.3.4.1 The ultrastructural effects of alcohol exposure on the pups endocrine cells

At electron microscopy level, the beta (β) and alpha (α) cells showed reduction in secretory granules. Some of the granules had their binding membrane distorted. These secretory granules were also fewer in number in the cytoplasm for the alcohol treated groups compared with the control (*Figure 31A&B, Figure 32A&B*). There was also disorganization of the rough endoplasmic reticulum (RER), Golgi apparatus and few polysomes in the alcohol treated groups as compared with the control groups. (*Figure 31A&B, Figure 32A&B*).

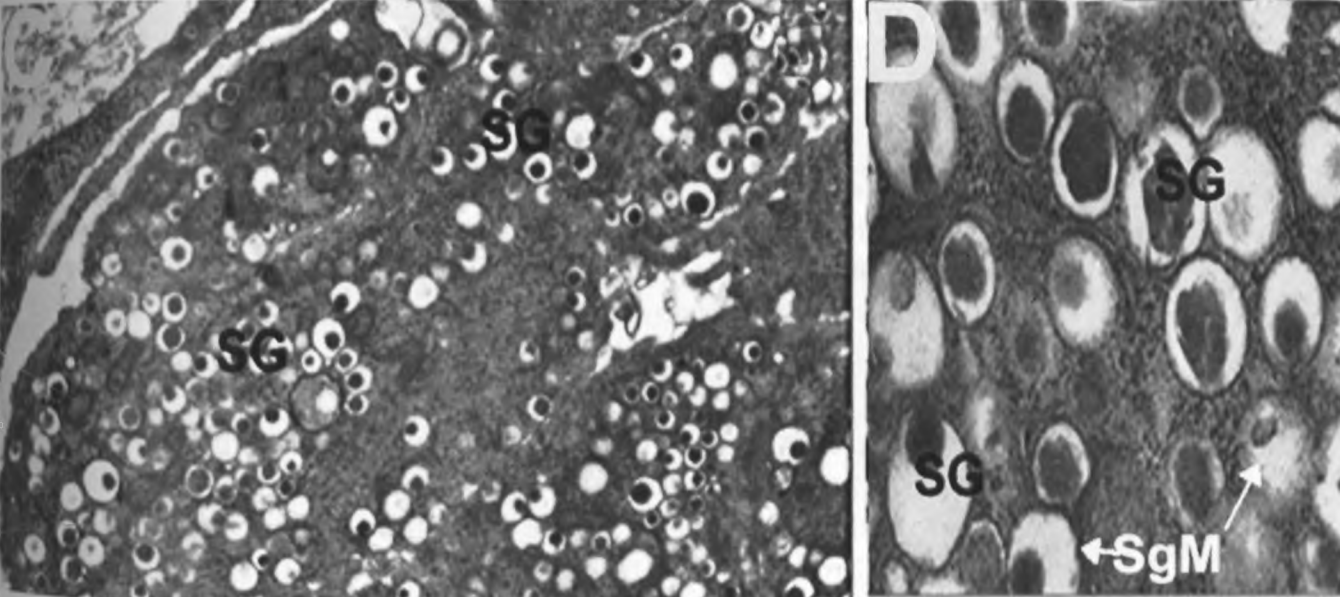
Figure 31A-B: Electron micrographs showing pupal β -cell morphology and secretory granules alteration in the alcohol treated groups compared with the control

- 31A:** An electron micrograph of a control pupal beta cell: Note the numerous secretory granules (SG) that are evenly distributed in the cell cytoplasm. Note the conspicuous electron dense core granules with a well defined space between the membrane and the electron dense material that form the core of the secretory granules. *TEM-Mag x18,200*
- 31B:** Electron micrograph of a control pupal cytoplasm showing the distribution and morphology of the secretory granules (SG) of a beta cell. Note the way they are densely packed with well developed binding membrane and a well defined space between the membrane and the electron dense material at the centre. *TEM-Mag x 55,200*
- 31C:** An alcohol treated pupal beta cell: Note the sparsely distributed secretory granules (SG) that are not evenly distributed in the cell cytoplasm. Note the reduced electron dense core material in the secretory granules with a widened space between the membrane and the electron core material in the granules. Note also the distorted shape of the membrane binding the secretory granules (SgM). *TEM-Mag x18,200*
- 31D:** Electron micrograph of an alcohol treated pupal cytoplasm showing the distribution and morphology of the secretory granules (SG) in a beta cell. Note the paucity of these granules that also have segmented binding membrane (SgM) and a highly reduced electron dense core material at the centre. *TEM-Mag x 55,200*

Figure 31A-B:



control pups beta cells and secretory granules



Alcohol treated pups beta cell and the secretory granules

Figure 32A-B: Electron micrograph of a pup alpha (α) cell morphology and alterations of secretory granules in the alcohol treated groups compared with the control

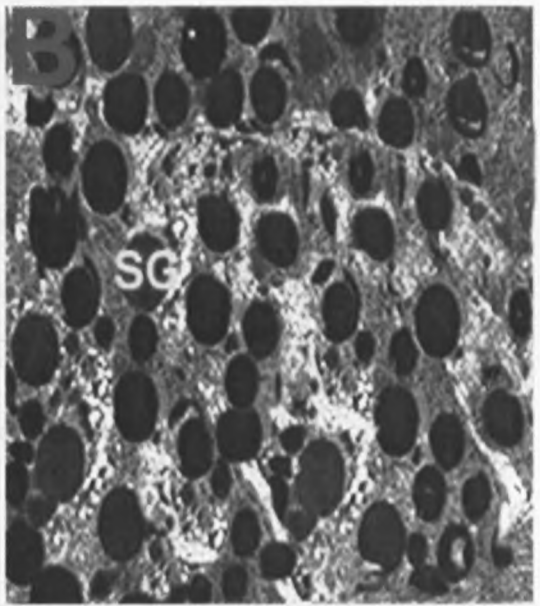
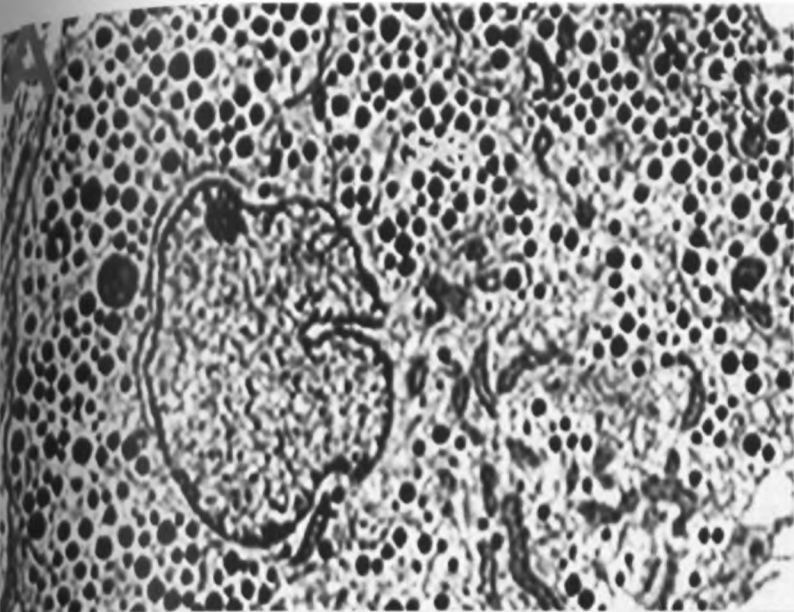
32A: A control pup alpha (α) cell: Note the numerous secretory granules (SG) that are evenly distributed in the cell cytoplasm. Note the conspicuous electron dense granules that are membrane bound. *TEM-Mag x18,200.*

32B: Electron micrograph of a control pup cytoplasm showing the distribution and morphology of the secretory granules (SG) of an alpha cell. Note the way they are densely packed with an electron dense material filling the central portion of the secretory granules. *TEM-Mag x 55,200.*

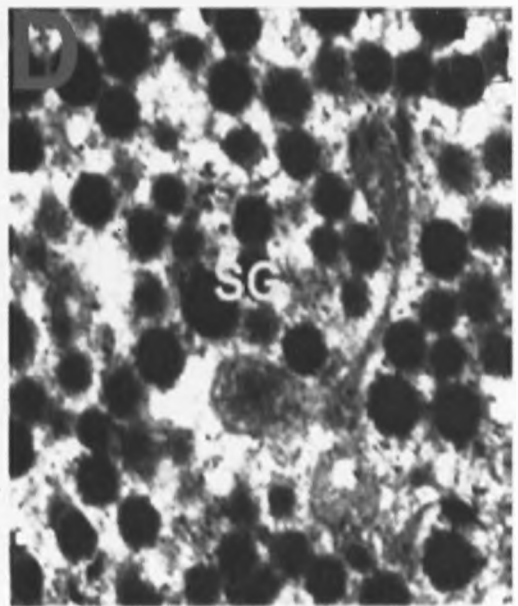
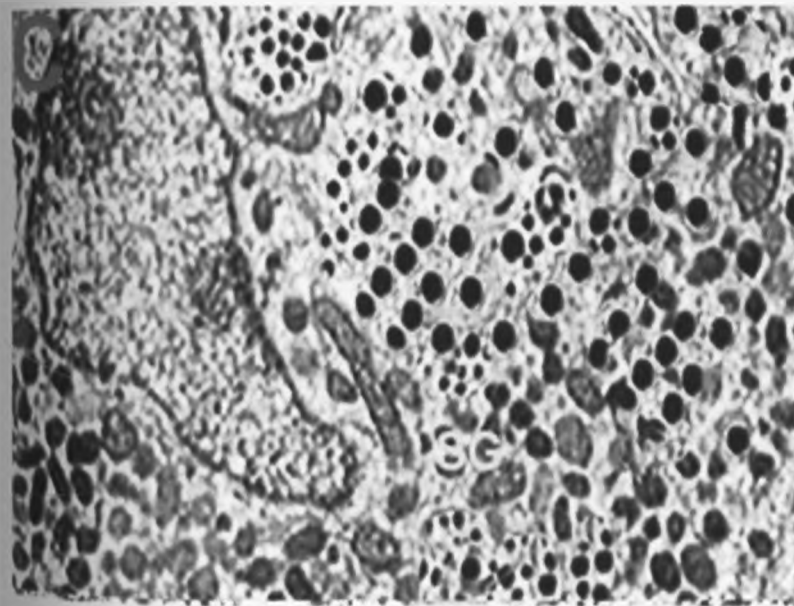
32C: An alcohol treated pup Alpha (α) cell: Note the sparsely distributed secretory granules (SG) that are not evenly distributed in the cell cytoplasm. Note the reduced electron dense core material in the secretory granules that is not as conspicuous like in the control. *TEM-Mag x18,200.*

32D: Electron micrograph of a control pup cytoplasm showing the distribution and morphology of the secretory granules (SG) of an alpha cell. Note the way they sparsely parked compared with the control with a poorly staining electron dense material. *TEM-Mag x 55,200.*

Figure 32 A-B:



control pups: alpha cells and granules



Alcohol treated group: pups Alpha cells and granules

3.3.5 Effects of prenatal alcohol exposure on the pups fibrous connective tissue deposition

Prenatal exposure to alcohol was seen to cause increased deposition of fibrous stromal tissues in the inter-acinar spaces of the pups pancreas. This deposition of the fibrous stromal tissues showed a regional variation with the body and the tail bearing the greatest effects as compared with the head to the neck regions (*Figure 33B&D*). This deposition of stroma was also observed to vary with the time of alcohol exposure, with those exposed in the first (TM₁) and second trimester (TM₂) depicting heavy deposits of stoma while those exposed in the third trimester showing only some slight changes (*Figure 33A-D*). The proportions of fibrous tissue deposits were also seen to have a strong Pearson correlation with the time of alcohol exposure and with doses of alcohol administered as shown in *Table 32* below.

Table 32: Pearson (r) correlations between the stromal tissue deposits, the various alcohol doses and time of exposure

exposure	Time of Alcohol	Parameters		
		Fibrous stromal deposits against the time of exposure	Dose of alcohol	Fibrous stromal deposits against the alcohol doses
Pearson corr Sig(2 tailed)	Trimester 1	-0.958** 0.000	LAG 2.5mgs/kg/Bw	0.699* 0.003
Pearson corr Sig(2 tailed)	Trimester 2	-0.915** 0.000	MAG 5mgs/Kg/Bw	0.737* 0.0012
Pearson corr Sig(2 tailed)	Trimester 3	0.314 0.090	HAG 6.5mgs/kg/Bw	0.815 0.001

**correlation is significant at 0.01 level(2tailed)
*correlation is significant at the 0.05 level (2 tailed)

Figure 33 A-D: Sections of pups pancreas showing deposition of stroma in the alcohol treated group compared with the control.

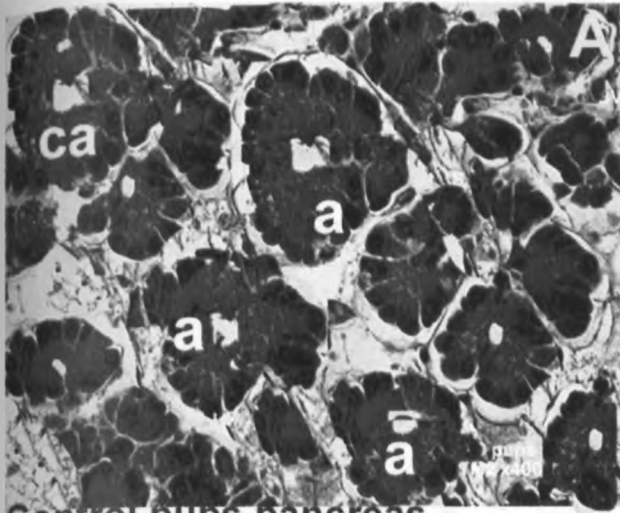
33A: A section of a four weeks old control pups pancreas showing the normal distribution of parenchymal acini (a) within the connective stromal tissues. *H&E Mag x10*

33B: A section of an alcohol treated pups pancreas showing high deposits of stromal (S) tissues when prenatally exposed to alcohol at trimester one (TM₁). Note the relative reduction in the numbers of the acini clusters (a). *H&E Mag x10*

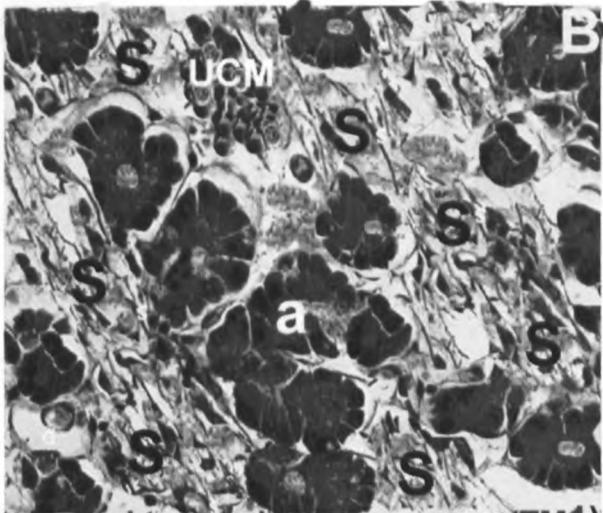
33C: Section of an alcohol treated pups pancreas showing stromal tissue deposits when prenatally exposed to alcohol in the second trimester (TM₂). Note the high stromal (S) tissue deposits with reduction of acini clusters (a) that is comparative to 33B above. *H&E Mag x10*

33D: A section of an alcohol treated pups pancreas showing stromal tissue deposits when prenatally exposed to alcohol at trimester three (TM₃). Note that the stromal tissue deposits (S) are relatively less compared to those exposed in the first and second trimester. Note that the number, the sizes and levels of maturity of acini (a) is comparable with the control. *H&E Mag x10*

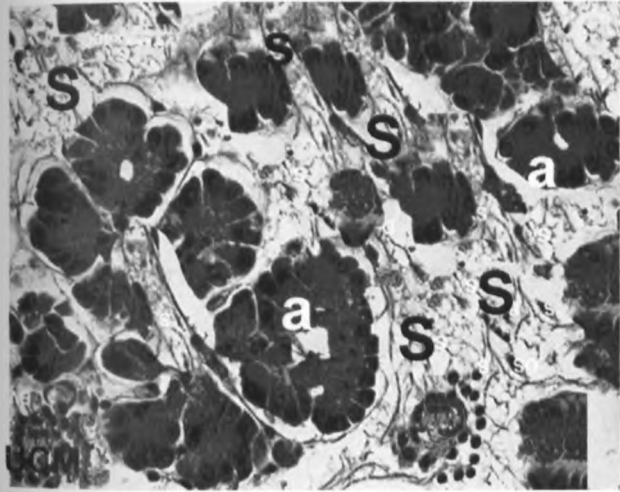
Figure 33 A-D:



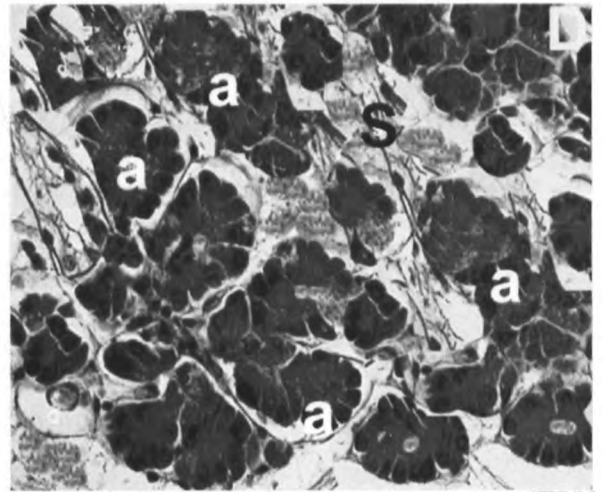
Control pups pancreas



Alcohol treated pups pancreas (TM1)



Alcohol treated pups pancreas (TM2)



Alcohol treated pups pancreas (TM3)

CHAPTER FOUR

DISCUSSION

4.1.0 General structure of the rat pancreas

The gross and microscopic appearance of the rat pancreas resembles that of humans and other animals like cats, horses, in that, it is a compound racemose gland that connects to the duodenum and has four distinct parts namely the head, the neck, the body and the tail (Dyson 1995; Mayhew 1999; Young and Moore and Persaud 2003; Patric 2005; Heath 2006). These four parts are also distinct in the human pancreas as well as in other animals like the guinea pig (Peterson *et al* 1966), the horse (Furuoka *et al* 1989), and the cat (Furuzawa *et al* 1992). However, the branching pattern in the tail region seen in the rat pancreas is in variance with the shape described in humans (Piper *et al* 2004, Beringue *et al* 2002), pigs (Bock *et al* 2003) in that the pancreases of these animals are prismatic or conical in shape with only one tapering tail.

Microscopically, the exocrine cyto-architecture in the rat pancreas is composed of acinar cells that are either prismatic or columnar in shape and contained in round or ovoid acini clusters of between 35 to 45 clusters per lobule. These cells depict a two zonal arrangement: an outer one, clear and finely striated next to the basement membrane, and an inner granular one next to the lumen. The outer

zone stained deeply with various dyes, whereas the inner zone stained slightly basophilic or depicting basophilic cytoplasmic granules. This morphological zonation of the rat exocrine tissue had also been observed in studies by Ekholm *et al* (1962), Weisblum *et al* (1962), and Haber *et al* (1998). This arrangement of acini cells is however divergent with what is reported in humans by Gupta *et al* (2002), where zonation of the acinar cells does not exist. This zonation in rats can be attributed to the continuity in the maturation process of the zymogen granules where the inner zone contains apically mature secretory granules whose content is similar to the content of the acinar lumen while the outer zone contained the immature granules in the process of maturation. This supports the hypothesis by Ekholm *et al* (1962) that zymogen granules are capable of opening at the cell surface and emptying their content into the acini lumen.

The clustering pattern of acini also showed regional variation in the rat pancreas. The acini clusters were highly concentrated in the head and the neck regions and relatively sparse in the body and the tail. This patterning is in contrast with what is reported in humans where the acini are highly clustered in the body and the tail and relatively sparse in the head and neck (Slack 1995, Biber *et al* 1998). Ultra-structurally, the sub-cellular organelles of the acinar cells depicts typical features of protein synthesizing cell with numerous golgi complex apparatus, numerous mitochondria, lysosomes and conspicuous electron dense granules in

the cytoplasm as described by Kaung (1994), Ahmed *et al* (1995), Young and Heath (2006).

The islets in the present study depicted a heterogeneous cellular composition where two types of islets were distinguishable, a PP-rich islet, seen near the head and neck region, and an alpha (α) rich islets predominant in the body and the tail region. This findings are in tandem with observations made by Orci *et al.* (1982); Abel and barbara, (1978); McEvoy (1981) and Orci *et al* (1981), and Ahmed and Abulbari, (2001). These two types of islets seems to relate to the developmental origin of the pancreas where it develops from a ventral and the dorsal bud. A significant differences in the numerical counts of α , β , δ and PP-cells per islet in the four pancreatic regions was also observed and could be attributed to the functional adaptations. The number of β and δ cells per islet showed a significant decrease in the body and tail regions as compared with the head and neck regions ($P < 0.05$) similar to observation made in naked mole rats by Kramer and Rochelle (2004). Moreover, there was a significant decrease in the number of α -cells per islet in the head and neck region compared with the body and the tail regions ($P < 0.05$). An explanation to these regional differences could relate to the embryological basis of the pancreas with a postulation of two different

arterial systems for irrigation of pancreatic islets from the ventral and dorsal buds (Orci *et al.* 1969; Syed-Ali, 1984). However, both hypotheses are insufficient to justify the differences between the regions of the dorsal pancreatic lobe, as observed in this study. Further investigations are required to elucidate the reasons for these differences in various regions of the dorsal lobe of the pancreas. These regional differences of the islet cells in the rat pancreas, observed in this study further emphasizes the need of applying stereological methods that rely on principals of validated mathematical and statistical principles in the pancreas morphometric and analytical studies.

The connective tissues including the fibrous stroma, nerves, blood vessels and ducts were embedded in loose areolar tissues. The blood vessels were highly concentrated in the head and the neck and relatively sparse in the body and the tail. Most of the arterial and nerve plexuses were also seen close to islet clusters along the midline while the duct depicted a branching tree with the main ducts concentrated in the head and neck regions. The fibrous stromal tissues constituting collagenous and reticulin fibres were seen forming the framework of the gland connected different components of the parenchymal tissues. This arrangement in the connective tissue stroma is in tandem with the prototypic arrangement described in humans as well as in other animals (Wessels and Cohen, 1967; Slack, 1995; Longnecker, 1996).

4.2.0 Effects of prenatal alcohol exposure on morphology of the fetal pancreas.

The results of this study showed that prenatal alcohol exposure causes significant structural and morphometric changes on both the exocrine and endocrine pancreas. These teratogenic effects that were both qualitative and quantitative ranged from gross reduction in fetal and pancreatic weights, disorganization of exocrine and endocrine parenchymal structures, that included alterations of the cellular shapes, sizes and numbers, reduction in cellular counts per islet and cellular compositions. It also caused disorganization of the duct system, plug formation in the ductal lumen and periductal fibrosis. At subcellular level it induced ultramicrostructural changes of pancreatic acinar and islet cells, including increased lipid droplets, myelinoid inclusion bodies, dilated and disorganized rough endoplasmic reticulums, mitochondrial degeneration, lysosomal fragility and diminished secretory granules. These general effects of alcohol on both the exocrine and the endocrine pancreas are in tandem to the findings by Pelletier (1977); Lickens and Nyomba (2003); McEvoy and Hegre, (1977) and Ahmed *et al*, (1995).

4.2.1 Effects of alcohol exposure on the fetal exocrine pancreas

The qualitative effects of prenatal alcohol exposure on the exocrine pancreas showed that alcohol is injurious to the development of the exocrine pancreas. In

this present study, alcohol was seen to delay the maturation and canalization of acini. This delayed maturation of acini was seen to be as a result of interference with the normal process of cyto-differentiation, development and maturation of the exocrine parenchyma (Pikkarainen and Riaha, 1967; Pictet *et al*, 1972; Slack *et al* 1995; LiZ *et al* 2004; Wu *et al*, 2008). This interference with the differentiation process in the exocrine pancreas was depicted by the heavy presence of undifferentiated cell masses between the acini clusters whose cells nuclei were enlarged showing mitotic figures.

The acini cell in the alcohol treated groups also depicted a mixed population of low-lying columnar “*immature form*” and the tall prismatic or columnar cells “*the mature form*”. The immature form constituted the greater proportion of cells that formed the acini clusters in the alcohol treated group with a proportion of between 65-75% as compared with 33-45% in the controls. The cell shapes in the alcohol treated groups also depicted a more or less cuboidal shapes that were different to the columnar shapes of acini cells in the control groups that depicted the prototypic columnar shapes of acini described in literature by (Mayhew, 1999; Moore and Persaud 2003; Young and Heath, 2006). This meant that the cells in the alcohol exposed groups were taking a longer-time to attain the normal shapes as a sign of arrested development.

The mean numerical counts of acinar cells per acini cluster as well as the numbers and the sizes of the acini clusters per field in the alcohol treated groups were significantly smaller ($p < 0.05$) compared with the control. Thus, the mean numerical counts of acini cell per cluster in the alcohol treated groups ranged between 25.3 ± 0.67 to 52 ± 0.34 compared with 49.1 ± 0.39 - 79.1 ± 0.93 in the control. Further, the mean number of acini cluster per field ranged between 13.8 ± 0.6 - 27.9 ± 0.7 in the alcohol treated group compared with 17.8 ± 0.9 and 28.7 ± 0.3 in the control. These mean numerical changes in the acini cells per cluster and the mean sizes of the acini were seen to be statistically significant ($p < 0.05$) by both ANOVA and the chi square test. A regional variation in the clustering patterns of the acini was also noted in accordance with the four parts of the fetal pancreas. The body and the tail were seen to be highly vulnerable to alcohol as compared with the head and neck. The number of acini clusters in the body and the tail in the alcohol treated groups ranged between 11-30 compared with 25-35 in the controls. This implies that the pancreases of the alcohol treated groups had significantly ($p < 0.005$) reduced exocrine tissue a phenomenon that is characteristic of chronic alcoholic pancreatitis reported by many author including Ammann, (2001); Frenzer *et al* (2002); Criddle *et al* 2006. This finding suggests that alcohol directly or indirectly suppress the cytogenesis of new pancreas acini cells by targeting some specific stages of the cell cycle responsible for growth and cell replacement in an acini cluster.

The observed delay in canalization of acini in the present study can be attributed to alcohol interfering with the normal process of cellular differentiation and patterning of acini. Pictet *et al* (1972) and Slack (1995) observed that early periods of organogenesis are critical in determination of the sizes, shapes, numbers and the functional attributes of the acini. Hence, perturbation from alcohol exposure in the period of organogenesis as in the case of the present study could be responsible of the delayed canalization and plug formation in the entire duct system in the alcohol treated groups. This preposition was supported by the observation made in this study where the higher the number of non canalized acini the higher was the level of plug formation in the duct system. Another explanation on the non patency of the acini and the duct system could be on the biochemical changes in the pancreatic acini cells leading to the accumulation of non oxidative ethanol metabolites that interfered with clearance of extra-cellular protein matrix responsible for opening of the duct system (Majumdar *et al*, 1986; Lugea *et al*, 2003). This suggestion by these authors on the biochemical changes occurring in acini cells following exposure to alcohol could be explained in this study following the observed alteration in the cytoplasmic uptake of stains where the alcohol treated groups depicted a basophilic cytoplasm compared with the control whose the cytoplasmic granules stained deeply acidophilic. It is therefore probable that, alcohol interferes with

the biochemical composition of the cytoplasmic inclusions and organelles responsible for uptake of stains and that are responsible in the clearance of the duct system.

4.2.1.1 The ultrastructural effects of alcohol on the morphology of fetal acini cells

At subcellular level, the acini cells depicted a wide range of ultra-structural changes including the disorganization of the organelles, increased lipid vacuolations. It also caused fragmentation of the membrane bound organelles like the rough and smooth endoplasmic reticulum, mitochondria and the lysosomes, decreased secretory granules, presence of abnormal intra-cisternal granulations, myeloid inclusion bodies with varied consistencies. The observed abnormal changes in the mitochondria, golgi complex , apparatus rough and smooth endoplasmic reticulum in alcohol treated rats was important from the standpoint of their role in protein and lipid bio-synthesis in the cell. The observations in this study that indicated degenerative changes in the endoplasmic reticulum and ribosomes may be an indication that alcohol perturbs the important site of protein and lipid synthesis in the pancreatic acinar cells. These current observations are in agreement with the reports of the studies by Percival and slack (1999) who showed that labeling of particular proteins was later found to be incorporated into proteins in the microsomes of the pancreas

and that the labeled proteins appeared in large amounts in the zymogen granules.

The appearance of the intra-cisternal granulations in the rat acini cells was considered abnormal in this study. Normally, granules of this type have been reported to occur exclusively in the guinea pig pancreas (Pettersson, 1966). Ekholm *et al* (1990) also demonstrated that granules of this type occurred in the pancreatic acini of the rat given ethionine. The appearance of these granules was considered by these authors to be disturbances in the synthesis of digestive enzymes. In this case therefore alcohol could be interfering with acinar components that directly or indirectly results into similar induced disturbances. The decrease in zymogen granules seen in the alcohol treated rats may therefore be attributed to defects occurring in the endoplasmic reticulum and its associated ribosomes.

Although the Golgi complex were not as greatly affected like the rough endoplasmic reticulum in the acinar cells of the alcohol treated rats, some structural aberrations were observed in which defective zymogen granules and focal cytoplasmic lesions were present. This observation is in agreement with the findings of Wilson and Apte (2003), Wu and Bhapale *et al* (2008), who found no discernible defects in the Golgi complex for a considerable period after alcohol treatment.

4.2.1.2 The morphometric effects of alcohol on the fetal exocrine pancreas

The exocrine pancreas of the alcohol treated groups had significantly reduced mean acini histo-morphometric parameters compared with the control. In the alcohol treated group, the mean volume density of acini (V_{va}), the mean acini mass and the mean acini volume reduced by 33.2%; 30.75% and 27.2% respectively when compared with the control. These findings are in line with previous studies by Apte *et al* (2000), Gukovskaya *et al* (2002), Araki *et al* (2003) who observed that the mean volume densities (V_{va}) of acini as well as the mean acini mass were also significantly reduced following a chronic exposure to alcohol and could be as a result of atrophy to the acini cellular components.

Strong positive Pearson correlations [$\rho(r)=0.719, P=0.037$; and $\rho(r)=0.563, P=0.048$] were seen to exist between the mean volume density and mean acinar cells per acini cluster as well as between the mean acini mass and the mean acini clusters per field. This correlation then implies that, the reduction in the mean volume density and mean acini volume can as well be as a result of the reduction in the mean numbers of acinar cells per acini cluster as well as the reduction in the numbers of acini clusters per field. This reduction in acini clusters and the acinar cells per cluster could probably have had the subsequent reduction in the mean cellular volume. These observations are in line with what

had previously been observed by Horb and Slack (2000), LiZ *et al* (2004), Perides *et al* (2005) following chronic alcohol exposure on pancreas.

Following the reduction in acini mass and acini volume, it is also possible that, an increase in total acini volume is caused by multiplication of cells from the existing acinar cells as the primary sources for the new cells hence, the effects of alcohol that caused delayed maturation of the acini cells also caused reduction in acini total volume. Such changes in pancreatic acini tissues was also previously reported (Nicholas *et al*, 1998; Araki *et al*, 2003; Lastra *et al*, 2006; Perides *et al*, 2005) following nutritional deprivations like in the case of proteins and in zinc deficiency. In view of this similarity with what was observed in these other studies it is also possible that perturbations on fetal pancreatic acini as observed in this present study could be related to perturbations of alcohol on the absorption of important nutrients necessary in the development of the fetal pancreas.

A regional variations on the morphometric effects of alcohol on the fetal exocrine parenchyma was also observed in fetal exocrine tissue in terms of the mean numbers of impaired acini clusters, the reduction in mean numerical counts of acinar cells per cluster as well as acini clusters per field from the head to the tail region. In this present study the body and the tail regions were observed to be more vulnerable to the prejudicial effects of alcohol in that the

body and the tail regions had about 30-45% of the acini clusters being disorganized and immature compared with only 20-30% in the head and neck regions. The mean numerical counts of the canalized acini in the body and tail regions were 12.6 ± 0.2 and 11.8 ± 0.9 respectively, which was 25% less compared with the proportionate mean count of acini in the head and neck regions. In addition, there was an observed 27.5% average reduction in the ranges of the acini clusters per field in the four parts namely the head, neck, body and the tail in the alcohol treated groups compared with the control. Thus, the range of acini clusters per field ranged between 15-23 in the head and neck, 10-15 in the body, and 9-15 in the tail as compared with 25-35 in the head and neck regions, 20-30 in the body and 15-20 in the tail in the controls. These regional effects on the acini clusters are however in variance with what has been reported in humans by Ammann (2001), Frenzer *et al* (2002); Chen and Nyomba (2003); Haber *et al* (2004). where the prejudicial effects of alcohol following chronic exposure were reported to be heavily manifested in the head and the neck regions.

A strong negative Pearson correlation ($\rho(r) = -0.819$, $p \leq 0.001$) between the volume densities of acini (V_{va}) and the volume densities of fibrous connective tissue deposits (V_{vCT}) was seen to exist. This relationship illustrates that the injurious effects of alcohol manifested by the reduction in the volume density of acini had a corresponding fibrous tissue replacement as would occur in a normal

scar formation following a tissue injury. Such similar relationship had been observed in studies by Rodriguez (1967), Wilson and Apte (2004), Stevens *et al* (2004), Verlaan *et al* (2004), and Wang *et al* (2006). This increase in fibrous stromal tissue can be attributed to the activation of stellate pancreatic cell in the healing process of the injured pancreatic tissue (Apte *et al* 2000). This phenomenon has been reported in human pancreas following chronic exposure to alcohol where the reduction in the exocrine tissue had a reciprocal increase in causing both liver and pancreas fibrosis (Bombi *et al* 1971; Gardner *et al* 1986; Bishr *et al* 2007). In view of this observations, it is probable that the fibrous connective tissue deposition in the interacinar spaces was a compensatory replacement mechanism for the loss in pancreatic exocrine parenchyma.

The pancreatic acini tissue per unit body weight as well as the pancreatic acini mass per unit pancreas volume was however seen not to be statistically insignificant ($p > 0.05$) by both ANOVA and the Chi-square test. The former and the latter correlations seems to imply that a change in fetal body weights linearly causes an equitable change in pancreatic weight regardless of the physiological state of the animal. This finding supports a hypothesis by Gorofano *et al* (1997), Georgia and Bhushan (2006), Green *et al* (2010) that inutero perturbation by a teratogen or by nutritional deprivation that is subsequently responsible for intrauterine growth retarded (IUGR) fetuses leads to a reduced pancreatic

parenchymal tissue mass and would be a key predisposition factor to pancreatic disorders like diabetes mellitus.

4.2.1.3 The time of alcohol exposure and the effects on the fetal exocrine pancreas

The histo-morphometric effects of alcohol on the fetal acini depicted a time dependant relationship based on the time of alcohol exposure. The first (TM₁) and second trimester (TM₂) had the worst effects on the morphology and morphometry of the fetal exocrine pancreas. Thus, the mean volume density of acini in the alcohol treated groups ranged between 39 ± 0.12 - 48 ± 0.62 when exposed at trimester one, 42.3 ± 0.66 - 55.97 ± 0.34 when exposed in trimester two and 57 ± 0.49 - 66.23 ± 0.91 when exposed in the third trimester. In addition, the mean cellular count of acini per cluster and the mean acini clusters per field were significantly lower ($p < 0.05$) when the alcohol exposures was in the first and the second trimester. It was however noted that there was no significant differences ($p > 0.05$) when the exposure were in the third trimester and particularly at low alcohol doses. A strong negative Pearson correlation [$p(r) = -0.719$; $P = 0.017$ - *two tailed*] between the mean volume density and the time of alcohol exposure was seen to exist. This relationship was also seen to be statistically significant when tested by ANOVA, students T-test, and with the chi- square test. This results on the changes of acini based on time of exposure implies that the earlier the exposure the greater are both the morphometric and morphological changes on

the acini. These results are in line with some other independent studies conducted by Gorofano *et al* 1997; Randall *et al* 2001 who evaluated the effects of alcohol on multiple doses on alcohol with different times of alcohol exposure.

Orci *et al* (1969); Orci *et al* (1970); Pictet *et al*, (1972) and Slack (1995) observed that gestational day one (GD₁) to gestational day 9.5 (GD_{9.5}) is the most critical period of pancreas morphogenesis as it is the period when the pancreatic primordia undergoes cyto-differentiation giving rise to the definitive pancreatic parenchyma. This explains why exposures to alcohol in the first (TM₁) and the second trimester (TM₂) resulted in worse deleterious effect on the fetal pancreatic acini, as alcohol would probably be interfering with the cascade of events leading to the cyto-differentiation of the pancreas primodium as well as growth of the differentiated cells as was explained by Rutter *et al* (1964) and Sadler (2005).

4.2.1.4 The effects of alcohol doses on the histo-morphometry of the fetal exocrine pancreas

The effects of alcohol on the fetal exocrine pancreas were seen to have a strong correlation with the doses of alcohol administered. In this study, a negative linear regression in the mean cellular counts per acini, the mean acini clusters per field, the mean acini mass and the mean volume densities of acini (V_{va}) were noted. Thus, the mean volume density of acini (V_{va}) ranged from 39.97 ± 0.2 -

48.9 \pm 0.34 when exposed to alcohol concentrations of 6.5gm/Kg/Bw, 41.3 \pm 0.51–56.2 \pm 0.41 when exposed to the concentrations of 5.0gms/Kg/Bw, and 59.12 \pm 0.13–70.1 \pm 0.34 when exposed to alcohol concentrations of 2.5gm/Kg/Bw. These mean ranges were seen to be 38.1% -21.7% lower when the high and the low alcohol groups were compared with the control respectively. A strong negative Pearson correlations [$p(r)=-0.673, P=0.029$; and $p(r)= -0.711, P=0.035$ - two tailed) between the mean volume densities of the acini and the alcohol doses, and between the mean acini mass and the alcohol doses was established. This correlations implied that the higher the doses of alcohol administered the higher was the histomorphometric reduction on the fetal acini parameters. These findings are in tandem with the report of a study done by Randall *et al*, (1979), Simsek and Singh (1990) who investigated the teratogenic effects of ethanol on mice following administration of multiple doses.

In overall, the comparative non parametric Mann whitenny U-test on the acini, connective tissue, blood vessels and ducts between the alcohol treated group and the control revealed that the F-values and the probabilities of occurrence of U-value on the median figures used across the study groups were statistically significant ($p<0.05$) by both ANOVA and the Chi square tests. Additionally, an exponential reduction in the volume densities of the acini, blood vessels and duct was noted in the alcohol treated groups when compared with the control. These

findings imply that pancreas histomorphogenesis is a very dynamic process with very tight control mechanisms during cyto-differentiation of its cellular lineages and therefore a change in any of the cellular components would lead to a compensatory replacement with the other. Such dynamic changes in pancreas cell differentiation was reported by Sweet *et al* (2004), and Souza *et al* (2006) following the non invasive imaging technique that they employed in the quantitative analysis of the pancreas as they studied endocrine cells and the effects of nutrient deprivations on the pancreas cellular differentiation in laboratory animals.

4.3.0 The effects of alcohol on the fetal endocrine pancreas

The observations on the endocrine parenchyma in this study show that, prenatal alcohol intoxication undoubtedly affects fetal pancreas islets development resulting to both qualitative and quantitative changes on the islets. In the alcohol treated groups, the size and shape of the islet differed significantly ($p < 0.005$) with the controls in that, the mean islet density area, the mean volume density and the mean islet mass in the alcohol treated groups were significantly ($p < 0.005$) less. In addition, the logarithmic tests on the distribution of the islet area in the alcohol treated groups also shown that they had abnormal and irregular islets while the control group conformed to the classical islet distribution reported in literature (Kaung, 1994; Wessels and Julia, 1967; Slack,

1995; Tsukamoto *et al*, 1988; Skau *et al*, 2001). The mean pancreatic weights, beta cell mass and beta-cell volume densities in the alcohol treated groups were significantly smaller compared with the control group ($P<0.014$). These observations on islets also bear resemblance with what was reported on the changes in islet morphology by Korsten *et al* 1990, Tse *et al* 1997, Gorofano *et al* 1997, Simmons *et al* 2001 following intrauterine protein deprivations or in mineral deficiencies by Partida- Hernandez *et al* (2006).

4.3.1 The qualitative effects of alcohol on the fetal endocrine pancreas

In rats, two types of islet are distinguishable based on the cell composition; pp-rich islets that are predominantly found in the head and the neck region and alpha rich islets that are predominant in the body and the tail region. In the alcohol treated groups, the two types of islets were disrupted by the alcohol exposure. The pp-rich islets were seen to have a highly reduced number of the PP-cells and at high concentrations the pp-cells very scarce. In the glucagon rich (i.e. the Alpha (α) rich islets) they shown a high reduction in alpha cells alongside the β , δ , pp and the C-cells similar to findings by Chem *et al*, (2003); Bhushan and Georgia, (2004). This regional effect on the cellular composition of the islet in the different parts of the pancreas can probably be attributed to the embryological origin of the pancreas where it develops from a dorsal and a ventral bud that give rise to different cellular islet primodium.

Alcohol was also seen to cause disaggregation of the islet clusters with the resultant disaggregated cells appearing as single unit cells or small group of islet cells in the interacini spaces. It also altered the zonal organization of cellular components in an islet cluster. In the alcohol treated groups the cells were seen to be haphazardly organized unlike in the control where a distinct zonal cellular arrangement was observed and the B-cells were seen forming the mantle and the core of the islet. This observation is in agreement with previous reports by Schweisthal *et al* (1963), Slack *et al* (1985), Vondorshe and Hahn (1988). This defect in the cellular composition and in the arrangement of the cells in the islet was seen to have a linear correlation with the proportionate numbers of the cell types in the islets. In the alcohol treated groups the mean totals of alpha cells and beta cells ranged between 14-25% and 35-55% for the alpha cells and the beta cells respectively. Again, this was a 17.5% and 30% reduction in both the alpha cells and beta cells respectively when compared with the control.

Further, the reduction in the number and the volumes of islet cells, the B-cell inter-linkages was noted to be greatly affected. In the alcohol treated groups, only 20-25% of the B-cells were seen to be linked to each other unlike what

was seen in the control where 60 to 65% of the beta-cells were linked side by side. It can therefore be seen that, alcohol interferes with this physiological adaptation in the beta cells that seems to play an important role in influencing the cell to cell "communication" This interlinkage in the B-cells in rats is however in variance with the human pancreas in that only 25% of the beta cells are reported to be linked to each other side by side (Clark 1983). This could probably explain the reason why humans are more predisposed to diabetes mellitus than rats.

The alpha cells were observed to be distributed between the beta cells and more to the periphery of an islet cluster. Such similar findings in the distribution of that alpha cells in rats had been reported by (McEvoy *et al*, 1981; Gundersen *et al*, 1988; Kaung *et al*, 1994; Finegood *et al*, 1995; Tse *et al*, 1997). This distribution of the alpha cells in rats is however in variance with the human pancreas where they are evenly distributed in an islet and relativey more in numbers than in the rats (Conkolin, 1962; Bauwns *et al*, 1997; Beringue *et al*, 2002; Edlund, 2002; Gupta *et al*, 2002). Comparisons in the number of alpha cells in the alcohol treated groups showed that they were significantly ($p=0.01$) fewer in the alcohol groups than in the control. It was also notable that the A-cells reduction was greatest when alcohol was administered in the first and second trimester and was more severe at moderate and high alcohol doses.

The δ -cells were ovoid in shape and their numerical numbers was highly reduced compared to both the β -cells and the alpha cells in the experimental and the control groups. They were also seen to be unevenly spread in an islet cluster. In the alcohol treated groups the delta cells per islet were fewer in numbers compared with the control. However, the distribution of the delta cells in the islets did not show variance between the low dose alcohol treated groups in the third trimester compared with the control. This distribution of the delta cells is in line with what has been reported in rodents (Bock *et al*,1999), in Cats (Syed-Alis *et al*, 1984), and in mice (Tomita *et al*, 1992).

The PP-cells were observed to be variable in sizes and in numbers between the control and the experimental groups. They were less numerous and were found adjacent to the D-cells in an islet cluster. These cells were noted to be highly vulnerable to alcohol since at high alcohol concentrations they were very scarce. The reason for this vulnerability is not very clear but may be attributed to the biochemical functions of the PP- cells or as a result of embryological interference during cellular differentiation. According to some authors, PP-cells differentiate much later after the beta, alpha and the delta cells (Pitctet *et al* 1972, Piper *et al*, 2004). It is then probable that alcohol interferes with the essential molecular regulation factors like the epithelium signaling factor IPF1/PDx1 in the early

stages of pancreas development. These factors were noted by to play a key role in the cyto-differentiation of the pancreatic islet cells and once interfered with by a teratogen like alcohol would lead to disappearance of specific cell types (Ackermann and Mourine, 2007).

The C-cells were observed to be variable in size and numbers like in the PP-cells. They also shown high vulnerability to alcohol like the PP-cells. They were fewer in number in the alcohol treated groups compared with the control and at times they could hardly be seen at high concentrations and following early exposures in the first and second trimesters. This phenomenon could be attributed to the embryological interference with the delicate molecular regulating factors during pancreas cytodifferentiation as reported by many authors in pancreas development (Apte *et al*, 1998; Ahlgren *et al*, 1996; Amart and Saunders, 1996; Ashery-padan, 2004; Amada *et al*, 2007; Ackermann and Maureen, 2007).

The reduction in endocrine tissue and B-cells mass bears resemblance to the morphological changes in endocrine cells observed during protein energy malnutrition as well as in deprivation of other important nutrients like zinc necessary during pancreas cell development as reported by Lopez *et al* (1996), Gorofano *et al* (1997), Wilson *et al* (1988) and Regnault *et al* (2002). The mechanism behind the observed effects of alcohol on the endocrine pancreas is not very clear though, it could be due to interference with the absorption of

important nutrients required in islet development or due to hormonal changes as reported in literature by Parsons *et al* (1995), Nussery whitehead (2001).

4.3.3 The effects of alcohol concentrations and morphology of the islets

The mean numerical count of the various types of islet cells was seen to have a strong Pearson correlation with the alcohol concentrations used in this study. Thus, the mean cellular counts of the B-cells ranged from 71.27 ± 1.09 - 88.97 ± 0.9 when exposed to the dose of 6.5gms/Kg/Bw , 84.93 ± 0.63 to 99.03 ± 0.64 when exposed to an alcohol dose of 5.0gms/Kg/Bw , and 93.41 ± 0.93 to 103.09 ± 0.7 when exposed to an alcohol dose of 2.5gms/Kg/Bw . These ranges in mean cellular counts were also found to be statistically significant ($p < 0.05$). These finding clearly demonstrates that alcohol interferes with islets cyto-differentiation observations that is in line with what has been reported in literature by (Brown *et al*, 1979; Amart and Saunder, 1996; Apte *et al*, 1998; Endlund, 2002; Chen and Nyomba, 2003). In view of this reduction in the mean numerical count of the cellular components in an islet, it can then be inferred that alcohol could probably be interfering with the gene coding or interfering with both DNA and RNA component in the cell hindering the cytogenesis of islet cells that are required in normal development as reported by Chao *et al* (1997), Morsy *et al* (1995), Bock *et al* (2005), and Amada *et al* (2007).

Though this study did not explore the mechanism behind the observed effects on the fetal endocrine pancreas, it is known that alcohol can exert its prejudicial effects on fetal organs development via several mechanisms (Kronick, 1976; Lieber, 1979; Kotch *et al*, 1995; Chao *et al*, 1997; Kono *et al*, 2001). Alcohol and its principal metabolites acetaldehyde, rapidly crosses the placental barrier (Ho *et al*, 1972) and that the two substances can be teratogenic through inhibition of the synthesis of nucleic acid proteins (Brown *et al*, 1979).

The three linear correlations between the volume densities of islets (V_{vis}) and the volume densities of beta cells (V_{β})- [$p(r)=0.819;p=0.013$] , the one between the islet mass and beta cell mass [$p(r)=0.603;p=0.03$] and lastly the one between islet mass and body weight [$p(r)=0.567;p=0.045$] have also been reported in a study by Bock *et al* (1999). These correlations could be explained by the fact that the total islet volume is a factor of proportional growth in the total pancreas mass as well as increase in body weights supporting the growth models by Lieber (1979), and Barker (1992) that the normal physiological growth in organs like the pancreas are proportionately related to the animals' body weights.

In view of the islet morphology observed in the current study, alcohol would be interfering with the fetal intrauterine environment required in the uptake and utilization of nutrients hence resulting in 'compromised' fetal growth and

impaired islets development. These suggestions are in agreement with the findings by Kaung *et al* 1994, Apte *et al* (2003), Green *et al* (2008), who found discernible reduction in fetal islet mass following intrauterine perturbations following in-utero combinations of alcohol exposure and nutrients deficiencies.

The percentages proportions of the beta cell mass was seen to linearly reduce with increasing alcohol concentrations among the alcohol treated groups. In the alcohol groups the percentage proportion of beta cells ranged between 51 and 63% as compared with 65-75% in the control. These drop in the percentage of beta cell mass in the alcohol exposed groups seemed to show similarities to a normal cellular composition reported in humans where the beta cells contributes about 55% of total islet cells with only 25% of the beta cells found lying side by side (Piper *et al* 2004). It however differs slightly with that of the GK rats where the beta cells make up to 63% of the total cells and found forming the core and the mantle of the islets. In this type of rats it is also reported that 65% of their islet beta-cells are linked to each other in a cord like structure of between 8-16 cells similar to what was seen in this study.

In this study, a significant reduction in the mean islet volume (V_{visl}) and the mean beta cell volume ($V_{\beta-c}$) per islet in all the alcohol treated groups was observed particularly when alcohol treatment was instituted in the first and

second trimester ($P < 0.05$). It was also observed that there was no significant difference ($P > 0.005$) in the islet composition when alcohol was instituted in the third trimester (TM₃) and the control. This findings demonstrate that the worst deleterious effects of alcohol on the fetal endocrine pancreas occur in the early period of pancreas organogenesis during the cytodifferentiation of the islet cells as reported by Pictet *et al* (1972), Rutter *et al* (1980), Slack (1995).

Further, a significant difference ($p < 0.05$) in the regional distribution of the mean volume density of the islets, mean islet mass, mean islet volume, mean number of cells per islet and mean numerical densities of α , β and δ cells per islet was noted between the head/neck region and body/tail regions. These parameters in the head and neck regions were significantly higher than the body and tail regions. This is in variance with human pancreas where the tail and the body records a higher volume density of islets that the neck and the head regions (Pelletier, 1977; Gupta *et al*, 2002).

4.3.4. The ultrastructural effects of alcohol on the morphology of fetal endocrine cells

The ultrastructural changes in islet cells including the δ , β , α , and pp-cells indicate that alcohol has a profound effects on the architectural integrity of the cytoplasmic organelles in the islet cells. The specific changes included reduction in the number of secretory granules, disintegration of the membranes binding the

sub-cellular organelles, increase in lipid vacuolations and an increase in myeloid inclusion bodies. These observed morphological changes on sub cellular structural organization in islets bear a striking resemblance to those observed to occur in the islet cells of laboratory animals experimented in states of zinc deficiency, protein synthesis inhibition or disturbed protein metabolism (Regula *et al*, 2003; Chen *et al*, 2003; Partida-Hernandez *et al*, 2006).

The specific changes on the organelles observed in this present study included increased lysosomal vacuolations similar to findings from Wilson *et al* (1990) who reported that a combination of alcohol consumption with protein deficiency increased lysosomal activity leading to autolysis of pancreatic acinar and islet cells. These observations are also in tandem with findings by Wang *et al* (2006) who reported that alcohol consumption alters the pancreatic cellular morphology signaling death to the pancreatic cells following chronic alcohol intake in human subjects.

Further, the findings of this study show that alcohol also alters the structural assembly of the membranes that bind the subcellular organelles responsible for the metabolic functions and sequestering of secreted products. Among the worst affected membrane bound organelles were the rough endoplasmic reticulum, mitochondria, and the Golgi apparatus whose alterations included,

mitochondrial membrane fragmentation as well as interference with inner folding of the mitochondrial membrane in the formation of cristae, disorganization of the golgi complex apparatus and the endoplasmic reticulum, leading to increased presence of free ribosomes in the cell cytoplasm. In view of the fragments of granular endoplasmic reticulum and free cytoplasmic ribosomes, it is then probable that defective lipid and proteins components that make up the membranes of this organelles could have lead to leakage of autolytic enzymes causing the observed membrane degradation of these organelles and the presence of myeloid inclusion bodies as was observed by Lehninger (1962). This presumptions can be justified by the experimental evidence that any substance causing degradation of lipids and protein that interferes with essential minerals like zinc absorption would results in disintegration of molecular conformation and increased catabolism of proteins that are critical in maintenance of membrane integrity (Hackenbrock 1968, Haber *et al* 1993, Partida-Hernandez *et al*, 2006; Wang *et al*, 2006).

Alcohol also impaired the secretory functions of the islet cells as was seen by the notable increase of the fragmented secretory granules and those that looked unfragmented depicted impaired development "*immature form*" with irregular binding membrane contrary to the "*mature*" form that had a well developed membrane seen in the control groups. It was notable that, the numbers of

"immature" granules increased linearly with increasing alcohol doses and with early exposure in the first trimester. Hence the dose of 6.5gms/Kg/Bw and when exposure was in the first trimester (TM₁) resulted to the worst histomorphometric effects. It is probable that alcohol interfered with the protein synthesis cycle in the islet cells responsible for the production of important hormones like insulin and glucagon among others. It is also probable that, the weakened cell membrane could be responsible for the free passage of alcohol molecules reaching the subcellular structures leading to direct or indirect injury to these organelles as observed in this study as was also reported by other authors (Hajnal *et al* 1990, Werner *et al*, 2002; Wilson and Apte, 2003; Wang *et al*, 2006).

Alcohol was also seen to increase the cytoplasmic lipid vacuolations, and rupturing of the membranes binding the secretory granules in all the islet cells. This increase in lipid vacuolations would be as a result of interference with the smooth endoplasmic reticulum that are responsible for lipid biosynthesis as well as alterations in the biochemical processes of fat metabolism in these cell. The cause of the ruptured membranes in the secretory granules was not very clear but it may be attributable to the decreased stability of the membrane of these secretory granule. This suggestion is supported by studies conducted by Korsten *et al* (1992), Heber *et al* (1995), Frenzer *et al* (2002), Criddle *et al* (2006), who

reported that alcohol is very soluble in water and lipids, and it easily crosses any biological membranes and rapidly equilibrates the total water volume content in the cell destabilizing the integrity of any biological membranes.

The rupture of lysosomes and secretory granules, however, may not be solely responsible for the cytoplasmic degradation observed in this study. In view of the fragments of granular endoplasmic reticulum and the presence of free ribosomes included within the focal degenerative bodies, it seems probable that defective ribonucleic acids may directly contribute to the origination of the focal cytoplasmic degradation. This assumption is justified by the experimental evidence that any substance interfering or causing protein deficiency or interferes with zinc absorption results in disintegration of molecular conformation and increased catabolism of nucleic acids and proteins (Packer 1963; Ghischan *et al*, 1982). It can also be considered that focal cytoplasmic degeneration represents a process by which cells sequester and dispose of defective proteins.

The zonation in the accumulation of lipid droplets and vacuolations in the apical, middle and basal regions in islet cells observed in this study can be interpreted to be as a result of impaired fat metabolism, secondary to the inhibitory effect of alcohol to the uptake or through integral interference in the cycles of lipid bio-synthesis. Dreiling and Borado (1977), Wilson *et al* (1982) and

Sisek and Singh (1990) observed that the frequent occurrence as basal clusters of lipid droplets in the pancreatic injury to islet or acinar cells of the rat inhibited protein and lipid synthesis and promoted cellular degradation. A similar phenomenon occurred in the rat endocrine pancreas during protein deprivation (Wilson *et al*, 1990). A study by Pikkarainen and Riaha, (1967) observed a fatty liver syndrome in rats analogous to what was observed in pancreas islets in this study following exposure to alcohol and reasoned that the lipid accumulation was secondary to defective lipoprotein synthesis.

The myeloid inclusion bodies seen in the cytoplasm contained fragments of the endoplasmic reticulum, free ribosomes, cytoplasmic and nucleoplasmic vacuolations, myeloid structures and other amorphous, intensely osmiophilic material. These focal cytoplasmic lesions demonstrated a progressive structural transition from one containing recognizable structures like endoplasmic reticulum and free ribosomes into another containing amorphous dense material. These degenerative changes seen in the both the islets and acinar cells were also reported by Herman and Fitzgerald 1962, Laposa and Lange (1986), and Dumortier *et al* (2007) following interference with the cellular growth and development in situations of zinc deficiency. It is then probable that the morphological differences seen among the cytoplasmic lesions in the alcohol treated groups may be due to lysosomal digestion which produces a spectrum of

changes from recognizable cytoplasmic components to various myeloid structures and finally to homogeneous dense bodies observed in this study confirming what had been hypothesized by Hales and Baker 1992.

It is also probable that the ruptured secretory granules in beta, alpha and delta cells and the accumulated cytoplasmic lipids could have acted as noxious stimuli, contributing to the degradation of different organelles in the cell cytoplasm. This hypothesis is supported by the biochemical data authored by Kotch *et al* (1995), Hebel *et al* (1995), Gukovskaya *et al* (2002), and Haber *et al* (2004). In their reports, these authors suggested that the release of amylase and ribonuclease from the zymogen granules into the cytoplasm is responsible for the early manifestations of pancreatic autolysis. They reasoned that pancreatic lipase and other proteolytic enzymes may also be released from zymogen granules during pancreatic autolysis. Following this reasoning by these authors, and the observations in the present study, thus, it is possible that alcohol intoxication in both the endocrine and the exocrine pancreas could have caused release of amylase and ribonuclease from the acinar cells zymogen granules into the cytoplasm and caused the membrane degradations and increase in the numbers of ruptured secretory granules.

The abnormal change in the endoplasmic reticulum and mitochondria in alcohol treated rats was important from the standpoint of their role in enzyme synthesis in the islet cells. This study indicated degenerative changes in the endoplasmic reticulum that may relate to alcohol perturbation in the most important site of protein synthesis in the pancreatic acinar cells.

4.4.0. Effects of prenatal alcohol exposure on the fetal pancreas duct system

The prenatal alcohol exposure caused disorganization of the normal patterns of the fetal duct system as well as reduction in luminal diameters of the ducts. The ranges in luminal diameters (LuD) of the interlobular ducts varied between 4-5.5mm in the control and 2-3.5mm in the alcohol treated groups. A strong positive linear correlation [$\rho(r)=0.617$; $P=0.031$ -two tailed] was established between the luminal diameters of the duct and their volume densities implying that changes in luminal diameters in the ducts had a direct correlational reduction in the mean volume densities of the duct system (V_{vds}).

The changes in duct system that manifested itself with the reduction in luminal diameters was observed to be as a result of increased deposition of the periductular fibrous connective tissues on the wall of the ducts. This is likely to have resulted from the activation of the pancreatic stellate cells along the wall of the duct system that lead to the increased deposition of the fibrocystic stroma.

The current observations are also in support of the findings on the pancreatic fibro-genesis data by Githens (1988), Apte *et al* (1999) and Apte *et al* (2000). These authors observed that the pancreatic stellate cells are responsible for the deposition of fibrous connective tissues responsible for pancreas fibrosis. They suggested that direct ethanol intoxication induces the release of a subfamily of cytochrome P-450 which is an important components to the biochemical activation of stellate cells (Apte *et al*, 2000).

4.5.0 Effects of alcohol on the fetal pancreas connective tissues

Prenatal exposures to alcohol lead to increased fibrosis in between the parenchymal tissues and around the duct walls. In the alcohol treated groups, the volume densities of the fibrous stroma increased from 17.1 to 38.9% between those exposed to low dose of 2.5gms/Kg/Bw and those exposed to a high dose of 6.5gms/Kg/Bw respectively. The fibrous stroma that contributes the bulk of connective tissues with a volume density (V_{vf}) of between 15 to 20% of the total pancreas mass in a normal pancreas was seen to contribute between 30-45% of the total pancreas mass in the alcohol treated groups while the volume densities of blood vessels, nerves and ducts (V_{vbd}) ranges decreased by 14-22%. This proportionate decrease in volume densities of the various components of the connective tissues is in line with reports by Bonner-weir *et al* (1989) and Apte *et al* (2000). They also support a hypothesis by Scaglia *et al* (1997) who suggested

that changes in connective tissues in the pancreas is an important indicator of pathophysiological state of the gland. This further supports the report by Lifson *et al* (1985) who noted that the proportionate volumes densities of connective tissues including the fibrous connective tissue, blood vessels and ducts is usually as a result of compensatory remodeling of pancreatic structural architecture based on the physiological and pathological states.

There was a positive linear correlation ($\rho(r)=0.671;p=0.001$) between volume densities of blood vessels (V_{vBv}), and total islet mass. This relationship illustrates that a physiological or “*symbiotic*” coexistence between vascularization and growth of islets do exist. The importance of this linear correlations agrees with the findings of Lifson *et al*, (1985) who reported that vascularization of the endocrine tissue is a more important consideration in determining the islet functions than the numbers or the volumes densities of islet cells.

There was a positive linear correlations between the volume density of connective tissues and the fetal body weight [$\rho(r)=0.593;p=0.0031$], as well as between the volume densities of connective tissues and pancreatic weight [$\rho(r)=0.692;p=0.002$]. These correlations illustrates that the disturbances that would result to impaired fetal growth would contribute to the reduction in fetal pancreas weight as well the volume densities of various pancreatic parenchymal

and stromal components. This inference is supported by reports from other authors who observed that intrauterine nutrients deprivations actually do result in reduced pancreas exocrine and the endocrine tissues (Abel and Greizestein 1979, Armat and Saunder 1996, Apte *et al* 2006).

4.6.0 The postnatal effects of prenatal alcohol exposure on the pups pancreas

The structural alterations observed in the pups pancreatic cells demonstrate that *in utero* exposure to alcohol leads to a number of complex morphological changes within the fetal pancreases that were seen to be sustained in the offsprings. These observed alterations could be as a result of direct or indirect alcohol intoxication mechanisms that caused perturbations to the overall fetal growth with subsequent morphological alterations to the pups pancreas (Bock *et al*, 2003).

In this study, it was noted that the pancreatic structural defects induced by prenatal exposure to alcohol did not show signs of recovery in the pups, an indication of arrested development or permanent damage to the pups pancreatic tissues. This observation echoes the findings by Persaud (1985), Georgia and Bhushan (2004), Lichen and Nyomba (2003), Chen and Nyomba (2004), Inuwa and Mardi (2005), who reported that, the prenatal events that hinder the

pancreatic development are a key determinant to the postnatal quantitative morphology of the islets. Further observations in this study show that there is a strong Pearson correlation ($P(r) > 0.500$) between pups body weight and the pancreas weight. In view of this relationship it can be inferred that there exists a strong linear relationship between pancreas weight and pups weight an indicator that the effects on one would equally affect the other. This inference is supported by the observations made on the pups whose mothers had been exposed to alcohol where they were small for age and had significantly smaller pancreas and reduced beta cell masses.

The specific structural alterations observed in pup pancreases included changes in size, shape, cellular composition and the alteration in the patterning of both the exocrine and endocrine parenchyma. These observations were seen to be a prototypic carryover of the structural defects observed in the fetal pancreas. It was notable that the structural and morphologic alterations in the pups pancreas were dependant on the time of alcohol exposure as well as with doses of alcohol administered to the mother during pregnancy.

4.6.1 The postnatal effects of the prenatal alcohol exposure on the pups exocrine pancreas

The pancreatic exocrine tissue in pups whose mothers were exposed to alcohol were significantly reduced compared with the controls. The size, shape and

numbers of the acinar cells for the pups whose mothers were exposed to alcohol were significantly different ($p < 0.05$) compared with the controls. The mean numerical counts of acinar cells per cluster, and the acini clusters per field, the mean volume densities and acini (V_{va}) and the acini masses were significantly lower compared with the controls. Traces of undifferentiated cells masses in between the acini clusters were also observed illustrating remnants of acinar cell with arrested or delayed development from the fetus.

The acini clusters were distributed evenly in the rat pancreas, but with distinct regional differences in their volume densities following alcohol exposure. The volume density of the acini in the head and neck region ranged between 53.13 ± 0.76 to 62.91 ± 0.63 , and between 47.23 ± 0.56 to 58.91 ± 0.45 in for the body and the tail regions. When this regional variations in the mean volume densities of acini was compared with the control it was found that in the alcohol treated groups, it was 25.6% less in the head, 22.3% less in the neck, 32.9 % less in the body and 30.3 % less in the tail. This implied that the recuperation process of acini was still slow in the body and the tail as compared with the head and the neck regions. These observations are in line with observation made by Lopez *et al* (1996); Marriam *et al* (2001); Berney *et al* (2003); Bhushan and Georgia, (2004) that prenatal cellular differentiation process

is a key determinant to the postnatal morphological feature of the cells and the overall structure of the organs.

The sub-cellular structure in the pups acini cells from the alcohol treated groups also depicted poor development with sparsely distributed subcellular organelles, degraded secretory granules as well as the membrane bound organelles including the mitochondria, lysosomes and golgi complex apparatus. The findings on the altered pups pancreas in the alcohol exposed groups were seen as a carry over to what had been observed in the fetus and supports the observations of Conklin, (1962); Wilson *et al*, (1990); Morsey *et al*, (1995) and Amada *et al*, (2007) who reported that the size of the pancreas and its cytostructures are determined by all factors controlling its cytogenesis and cytodifferentiation. It also supports other observations by Barker (1992) and Herrera (2000) who reported that effects of prenatal cytodifferentiation to the fetal pancreatic organ is a strong indicator of the related pancreatic adult diseases.

The pancreas of the alcohol treated pups had smaller and irregularly shaped acini clusters as compared with the controls. 95% of the pancreases showed morphological alterations particularly those exposed to alcohol in the first and second trimester (TM₁, & TM₂). These alterations were observed as changes on the volume densities, total cell masses and volume weighted means of the acini

cells components. These observations on the postnatal effects on the pups exocrine pancreas are in line with the observations by Morsey *et al* 1995, Wilson and Apte 2003, Wu *et al* 2008. It was also notable that, the reduction in the volume densities of acini between the control and the alcohol treated groups was statistically significant ($p < 0.005$) by both the Chi-square test and ANOVA. In all the alcohol treated groups the intra and inter-groups means on the acini volume densities shown significant F-values ($P < 0.001$ and $p < 0.005$). When this was further tested with Scheff's multiple comparison procedures, it was established that the intergroup and intragroup significant changes in the mean volume densities of acini between the alcohol treated groups and the controls exist. This implies that alcohol is a strong teratogenic agent capable of inducing irreversible changes in the volume densities of acini.

The comparative mean numerical counts of the pups acinar cells per acini cluster for the alcohol treated group from the head to tail region ranged between 30.1 ± 0.3 in the head, 28.3 ± 0.9 in the neck, 25.9 ± 0.1 in the body and 18.6 ± 0.4 in the tail. This was significantly ($P < 0.005$) lower as compared with the regional distribution of the mean acini clusters in the control where means ranged between 28.1 ± 0.9 in head, 24.1 ± 0.9 in the neck, 19.6 ± 0.2 in the body, and 14.1 ± 0.2 in the tail regions. This variation in the regional effects of alcohol on the distribution of acini is in variance with what is reported in human pancreas

and in animal experimental models for chronic alcoholic pancreatitis where the head and the neck are reportedly seen to suffer the greatest effects (Apte and Wilson, 2003, Apte *et al*, 2006)

The volume density of acini and the calculated acini masses were seen to be significantly lower in all the alcohol treated groups. The tail and the body was the most susceptible areas of alcohol perturbations in terms of the mean areas occupied by the acini clusters while the head and the neck region showed some relative stability.

High deposits of fibrous stromal deposits were also seen in the pups pancreas whose mothers were prenatally exposed to alcohol in the prenatal life. The volume densities of connective tissue stroma in the alcohol exposed groups ranged 28.12 ± 0.97 to 49.36 ± 0.45 as compared with 25.91 ± 0.07 in the control. This implies that the connective tissue stroma induced prenatally following prenatal alcohol exposure was maintained postnatally.

4.6.1.1 Ultrastructural effects of prenatal alcohol exposure on the pups acini cells

The ultrastructural changes in the pups acini cells in the present study bear resemblance to the ultramicrostructural changes in the fetus including reduced zymogenic granules, traces of myeloid inclusion bodies, degraded organelles and

increased presence of lipid vacuolations. This ultrastructural changes indicate that alcohol is able to cause long term changes in the cell organelles that was perpetuated at postnatal life. This observations are similar to the prenatal changes in pancreatic cells was seen to be carried over to postnatal life (McEvoy, 1981; Movassat *et al*, 1997; Verlaan *et al*, 2004; Wang *et al*, 2006).

4.6.2 Effects of prenatal alcohol exposure on the morphology of the pups islets

The endocrine pancreas in the pups showed morphological effects of prenatal alcohol exposure on the fetal pancreas that are sustained postnatally. It was seen that the numerical counts, size and shape of the pups islets whose mothers were given alcohol during pregnancy differed significantly ($P < 0.05$) when compared with the controls. The mean volume densities of islets, total islet mass, beta cell mass and volume densities of beta cells as well as the mean areas occupied by islets were significantly ($p < 0.005$) reduced in the alcohol exposed groups. These findings are similar to those of McEvory (1981) and Kaung *et al* (1994) who found that the size, volume and beta cells mass at postnatal life is determined by the integrity of the islet inherited at prenatal life.

The pups pancreatic islets though better developed than the fetal pancreas, depicted same morphological patterning in the zonal localization of the beta, alpha, delta and other islet cells. The alpha cells were seen localized around the

periphery or cortex of the islets, while the beta cells were predominantly found at the centre or medulla of the islets. This organization of the islet cells in pups is in line with what had been observed in the fetus and congruent with what was reported by McEvoy, (1981) and Kaung *et al*, (1994). This cellular arrangement is also comparable to what is reported in rodents by Hellman 1959, Bork *et al*, (1999). They however still depict divergent morphological appearance to the human islets (Gerssell *et al* 1979, Redecker *et al* 1992, Piper *et al* 2004). When the shapes of each islet clusters was assessed against the ratio of each islet area to its perimeters, it was established that the pups islets in the alcohol group had more irregular shapes and significantly smaller ratios ($p < 0.05$) compared with the controls. Subsequently, the numerical counts of the islet clusters as well as volume densities of islets in the pups that were prenatally exposed to alcohol were fewer compared with the control group ($P < 0.01$). This shows that the control pups islets were strikingly different from the experimental groups, an indication that *in utero* exposure to alcohol leads to a number of changes within the fetal pancreases sustained in the offsprings. This is suggestive that alcohol could have either affected the pups pancreas development either directly or indirectly by interfering with normal pancreas morphogenesis or by impairing processes responsible for recuperation of the affected cells.

Further, an assessment on the morphological restoration of the effected pancreatic cells in the alcohol exposed pups show incomplete or absolutely no recovery four weeks after birth. These finding reflect on the irreversible teratogenic effects of alcohol on fetal organs like the liver, brain and other organs when exposed to alcohol. (Chernoff 1977, Frenzer *et al* 2002, Chen and Nyomba 2003, Criddle *et al* 2006, Jennifer *et al* 2006). These authors have reported that prenatal exposure to alcohol causes irreversible congenital anomalies to a wide range of organs including pancreas of the offsprings. The broad range effects of alcohol on the pancreas alongside other organs can be attributed to its molecular nature of a simple organic molecule (MW 46) composed of a single hydroxyl group and a short, two-carbon aliphatic chain $\text{CH}_3\text{CH}_2\text{OH}$ whose hydroxyl and ethyl moieties confer both hydrophilic and hydrophobic properties on the molecule. By nature of its molecular structure, ethanol is a very amphophile, a property important for toxicological effects to the fetus as it gets absorbed in the gastro intestinal tract of the mother (Kaufman and Woolam 1981; Deitrich and palmer, 1994; Hunt, 1994; Lee and Becker, 1995).

In the pup whose mothers were exposed to alcohol in trimester one (TM_1) and two (TM_2) during pregnancy, the cellular arrangement and composition of islets looked disorganized an indication that alcohol at early exposure alters the progenitor endocrine mesenchymal tissue that differentiates into the different

cellular lineages that gives rise to all endocrine cells. This supports the evidence that islet cytogenesis follows a default path when both mesenchyme and basement membrane are absent (Apte *et al* 1998, Deprins and VanAssche, 1982; Lee and Becker, 1995) This findings means that the reduced and disorganized islets in the alcohol treated pups could have been due to inability of the pancreas to regenerate beta cells by neotransformation from progenitor cells that were probably diminished following prenatal exposure to alcohol at pregnancy. This could as well have contributed to inhibition of beta cell regeneration by the replication of existing mature beta cells in the pups as occasionally reported in some studies during normal circumstances (Clark and Grant 1983, Zhayield *et al* 2001, Edlund 2002). It is then probable that, any factor affecting pancreatic development in early life will lead to permanent changes in islet morphology postnatally.

The shape and size of the islets differed significantly between the low, medium and the high alcohol groups. The medium and the high alcohol groups had significantly smaller islets ($p < 0.005$) that were more irregular in shape than in the low and the control groups. This tendency to irregularity in the shapes of the islets was not corrected by postnatal recuperation, since all the experimental groups had maintained significantly ($p < 0.005$) smaller islets than the controls. This differential growth in sizes of islets in the various study groups could have

been due to alterations on stimulants for new islet formation, such as transforming growth factor α (TGF α) shown by (Amada *et al* , 2007) when the protein and other nutrient supply to the fetus were interfered with by alcohol.

Thus, it can be inferred that, the structural changes that occurred within the pancreas islets in the fetus following prenatal exposure to alcohol were not repairable after birth. This may be due to decreased beta-cell replication. This inference was confirmed by a significant Pearson correlation observed to exist between the reduced mean pancreatic weight and the mean beta cell mass ($p(r)=0.798$; $p=0.001$) in all alcohol exposed pups. Additionally, the Student's *t*-test comparisons on the calculated mean islet weights tissue per pancreas with the beta cell mass and volumes for the alcohol treated groups against the control was also observed to be significant [$P<0.05$; $P<0.001$]. These observations compares well with observations made in studies done with protein deprived rats. In these studies it is reported that the fetal islets weré significantly smaller, and had less beta cells counts compared with those on balanced diets (Garofano *et al* 1998, Beringue *et al* 2002).

The cellular compositions of the islets were also significantly different in pups whose mothers had been exposed to alcohol during pregnancy. The ratios of alpha cells to the beta-cell in the alcohol treated groups was 1: 2 as compared to

the ratio of 1: 3 in the control. These changes in islets were predominantly seen in the medium and the high alcohol groups when exposure to alcohol was in the first (TM₁) and the second trimester (TM₂). This implies that the prenatal changes in islet morphology induced at prenatal life could not entirely be rectified after birth, implying that these structural changes could be permanent and may lead to a predisposition risk factor in developing insulin and non insulin independent diabetes mellitus. This bears resemblance with what was reported by Nicholas *et al* (1998); Bock *et al*, (2003); Chen and Nyomba, (2003); Bhushan and Georgia, (2004) that the morphological feature of islets reflected concentration of different toxicants that caused perturbation to fetal islets development.

The vascularity in the Islets of Langerhans in the pups pancreas depicted a complex blood supply; the afferent blood in an islet were seen to first encounter the beta cells, before establishing contacts with the alpha cells. This vascular arrangement suggest that changes in islet shape, size, and cell distribution would definitely affect this pattern of blood supply and subsequently alter the morphology of the islets. These observations are intardem with some morphological studies that have suggested that this may be the case (Kaung, 1994). It is possible, however, that the reduced volume densities in blood vessels

in the alcohol exposed groups compromised the blood supply to islets and could be the one primarily responsible for the observed changes in islet morphology.

The percentage of islet tissue per unit body weight as well as the volume weighted mean islet volume in all the study groups was seen to be identical. Additionally, the percentage of beta cell tissue per pancreas and body weight was similar in all the study groups. Thus, despite the striking morphological changes in the pancreas that occurred in the maternal exposure to alcohol, there appear to be homeostatic mechanisms which maintain a constant amount of endocrine tissue per unit of body weight. It is a well known fact that, the weight changes in organs are not always proportionate to body weight and asymmetric growth retardation like what was reported in the sparing of the brain tissue in babies that are small for gestational age (Kaung, 1994). The importance of pancreatic islet tissue to the development of the rats is confirmed by the equitable relationship observed in volume weighted mean islet volume and the insignificant difference in the percentage of the islet tissue per unit body weight between the controls and the alcohol treated groups. However this preservation in the islet tissue in the alcohol treated groups seemed to be at the expense of fundamental changes in pancreatic structure and cellular composition. This observations are in suggestions made by Newsome *et al* 2003 that neonatal weights has a strong relationship to the glucose and insulin metabolism later in life.

4.6.2.1 Beta cells and alpha cells in each islet

The percentage volume of beta cells per islet revealed that all the three alcohol treated groups had a smaller percentage of beta cells than the control group ($P < 0.05$). The differences were most marked in the high alcohol groups and when the alcohol was instituted in the first and second trimester. The reduced percentage of the beta cells counts per islet, beta cell mass and total beta cell volumes when exposed early in trimester one and two in all the alcohol doses suggest a tendency of suppressed development or cytodifferentiation of the endocrine pancreas in the alcohol treated groups compared with the control. This findings on the reduction in beta cell histomorphometry resembles the finding by Bonner-Weir *et al* (1989); Finegood *et al*, (1995); Garofano *et al*, (1997). This percentage reduction in all islet cells in an islet cluster suggests a tendency for the maternal alcohol exposed off-springs to have a smaller percentage of islet cells per islet than the control group, and this is confirmed by the significance levels ($P < 0.005$) when the ratio of beta cells to alpha cells within an islet is calculated where the control group has a ratio of over 9:1 while the ratio in the other three groups are in the region of 5:1.

4.6.2.2 Differences in total pancreatic morphology

The mean pancreatic weights, the volume density of islets (V_{islet}), islet mass and the beta cell mass in the study groups show that the medium and the high

alcohol treated groups have significantly smaller pancreases than the control group ($P < 0.001$). The low alcohol group had an intermediate weight between the control and the two other alcohol treated groups (i.e. the Medium and the High alcohol groups). The calculated mean weights of the total islet tissue, the beta cell mass and the alpha cell counts per pancreas showed that there was significantly less islet tissue, beta cell mass and less alpha cell counts per pancreas in the alcohol treated groups ($P = 0.001$). The reduced islet tissue as well as the altered cells composition per pancreas across the experimental groups show a tendency for the prenatal alcohol exposure to cause reduction in islet tissue in both the fetus and the offspring's.

The pancreatic weight expressed as a percentage of the total body weight shows that, while the low alcohol groups do not differ significantly from the control group, the medium and the high alcohol groups have significantly smaller pancreases per total body weight ($P < 0.01$). When the total islet cell mass, beta-cell mass, and alpha-cell mass are compared as a fraction of the body weight, there is a remarkably major difference between the control and the alcohol groups, especially for the total islet tissue per body weight. Though the beta-cell volume densities ($V_{v\beta}$) for the low alcohol groups did fall below the significant levels ($p > 0.05$) when compared with the controls, the medium and the high alcohol groups had a significantly smaller ($p < 0.005$) amount of beta cells compared with the controls.

The correlation between the number of islet clusters with total islet mass across the alcohol groups shows that a weak Pearson correlation ($\rho(r)=0.317$) does exist while there was no statistical significant difference between them ($p>0.05$) by both ANOVA and the Chi-square test. Additionally, there existed strong correlation ($\rho(r)=0.917$) between volume densities of islets and the total islet vascularity. This relationship suggests that, the architectural arrangements of the intra-islet vascular structure of the islets is possibly such a complex arrangement to a degree that only new islets are added during formation, growth, or regeneration of the pancreas as occurs during postnatal life as was observed by (Bock *et al*, (2003) following partial pancreatectomy. The number of pancreatic islets therefore seem to be under tight genetic control (Bock *et al*, 2005) and that alcohol would be evoking selective deleterious effects on beta cells and hence could not cause complete disappearance of the entire islets even on high dose exposures.

The observed decrease in total islet mass can be attributed to decrease in size of cells, decrease in mean cellular volume or a combination of both. These changes can be attributed to interference with intra islet beta cell mitosis, as observed in the presence of many cells showing mitotic figures in the intra islet cell clusters in the fetus and in the pups. It can then be inferred that, alcohol must be hindering cellular regeneration of beta cells postnatally since the volume

weighted mean islet volume in both the alcohol treated groups and the control did not show any statistical significance difference ($p > 0.05$). This was confirmed following the observations in the reduction of volume densities, and masses of islet and beta cell population in the alcohol treated groups compared with the control.

In this study there was no statistical significant difference ($P > 0.05$) between the volume weighted mean of the mean islet volume between the control and the alcohol exposed pups. This suggests that the differences in mean islet volume seen between the alcohol treated groups and the controls was as a result of islet disaggregation and not the actual reduction in numerical numbers of islet cells, and that the decrease in the total islet volume during physiological growth is attributable to islet cells atrophy and delayed maturation with no contribution to islet hypoplasia. This is also in line with a study by Skau *et al*, (2001) who reported that there is no linear correlation between the volume weighted mean of the mean islet volume in protein deprivations.

The changes seen between the fetal and the pups pancreas show that the pancreatic tissue is dynamic and can be modified to maintain normal-glycemia in response to changes in metabolic demands. Islets mass depend on among other factors, the change in beta and alpha cells formation, individual cell size and rate of cell death and the balance between these elements determine whether

islet mass increased, remains stable or is reduced as reported by Bhushan and Georgia (2004). With exposure to teratogenic agents like alcohol that interferes with growth and regeneration of β -cells would probably explain why children born of alcoholic mothers would be highly susceptible and at risk of developing pancreatic related diseases like diabetes mellitus.

4.6.3. The postnatal effects of alcohol exposure on the duct system.

The duct systems in the alcohol treated pups were poorly developed and disorganized an observation that was concomitant with the doses of alcohol administered during pregnancy. This finding confirms the report by Sonoda *et al* (2005) who observed that intragastric ethanol feeding altered the duct system and sphincter in-activities of the main pancreatic ducts in rats.

The ductular epithelium also looked poorly developed with some ductular cells looking pronounced and at some points the ducts lumens looked clogged up. These changes in the ductular epithelium of the alcohol treated pups bared similarity to the pronouncement of ductular cells observed in the pancreatic acinar cells of the fetus. These changes could be explained by the functional relationship between the ductular propulsive epithelium and the pancreatic acinar cell; the former is not primarily involved with the synthesis of exportable proteins but transportation of acinar secretions (Lugea *et al*, 2003) . It was however notable that some ductular cells were not greatly affected like was the

case in the acinar cells that depicted cytoplasmic degeneration in the alcohol treated groups. This could be due to the short cycle of differentiation, maturity and death, of the ductular cells that are renewed constantly as reported by Mayhew, (1999).

4.6.4 Effects of postnatal alcohol effects on the pups stromal tissue deposits

The pups pancreas whose mothers had been exposed to alcohol show high presence of stromal tissue deposits in the inter-acinar spaces. The increase in the deposition of stromal tissues is in line with findings of some previous study by Apte *et al*, 2000; Apte and Willson, (2003) and Mccarroll *et al* (2006), who found that alcohol induces astrogenic cell to produce more collagenous tissue hence influencing deposition of stroma. Another study by Ali and Persaud (1998) reported that high doses of enteral feeding of alcohol to mature rats resulted in acinar cell atrophy, fatty infiltration in the acinar and islet, inflammatory cell infiltration and focal necrosis and associated increase in collagen $\alpha 1(I)$ mRNA expression. In addition Randall and Tylor (1979) reported that feeding on high dose alcohol caused atrophy and apoptosis in pancreatic acinar cells.

A strong pearson correlation between volume densities of stromal tissue deposition and the alcohol doses was also seen to exist in all the alcohol treated groups as follows {[LAG $\rho(r)= 0.687$, MAG $\rho(r)= 0.819$,HAG $\rho(r)= 0.913$];

[Pups LAG $\rho(r) = 0.713$, MAG $\rho(r) = 0.842$, HAG $\rho(r) = 0.987$]. This shows that the higher the dose the higher was the deposition of stromal tissues. These findings seem to support the hypothesis by Barker (1992), Montonya *et al* (2002), Edlund (2002), Bhushan and Georgia (2004), that there is a very strong relationship between prenatal organ developments and associated adult diseases particularly in conditions relating to increase in fibrogenesis.

4.7.0 CONCLUSION

Prenatal alcohol exposure impairs the histomorphogenesis of exocrine and endocrine pancreas of fetal and pups pancreas with a net reduction in the cell mass especially beta cells and increase in stromal tissues and these effects are sustained postnatally. These outcomes correlates with the dose of alcohol, with most effects recorded at high dose during the first and second trimester. Though there were no physiological studies done, the disturbances on the beta cells may explain the association of prenatal exposure and diabetes mellitus.

4.8.0 RECOMMENDATIONS

The current study relied on fixed specimens harvested from fetal and pups pancreas following experimentation to inutero exposure to varied doses of alcohol. However, it was noted that growth of the pancreas is highly dynamic with many cellular lineages. *In vivo* and *invitro* time lapse imaging studies will need to be conducted to visualize such dynamic processes, for instance, real time

tracking of cell movement and tissue morphogenesis as they interact with the teratogenic agents like alcohol. Such live studies would document effects of alcohol teratogenesis on the dynamic B-cell formation and migration alongside other pancreatic cells.

Further studies with tissue cultures may elucidate the genetic effects of alcohol to developing fetal pancreas. This would shed light on the specific genetic codes that alcohol interferes with during development. Studies with Mice genetic markers can also be conducted to confirm morphometric changes in fetal and pups pancreas for better understanding the association of these changes to diabetes mellitus. Physiological studies will also need to be conducted to confirm the association between prenatal alcohol exposure and diabetes mellitus.

CHAPTER FIVE

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APPENDIX 1

Histology: Slides check list

1. Slide number: _____ code: _____
2. Staining: H&E[____] (b) masson trichrome[____] c) MAF{____}
3. Slide category (a)control [____] (b)experimental[____]
4. Study period(a) prenatal[____] (b) postnatal[____]
5. Alcohol dosage _____
6. Time for a alcohol administration: (a) trimester 1 TM1[____] (b)trimester 2 TM2[____] (c)trimester 3 TM3[____]

a) Quantitative description

1. Number of islet clusters per section [____]
2. Number of islet per cluster[____]
3. Location of islet cell clusters (a) central [____] b)peripheral[____]
4. Number of clusters located centrally[____]
5. Number of clusters located peripherally[____]
6. Number of islets located at the a)tail[____] b)body(middle)[____] c) tail[____]
7. Number of acinar cells per cluster surrounding one lumen[____]
8. Number of aciner clusters per field(5 fields)[a]____(b)____ c)____d)____e)____
9. Number of acinars canalized (a)[____].
10. Number not canalized per field[____]

b) Qualitative description

Magnification (a)x400[____] (b) x1000[____]

a) Acinar cells

1. Organization of acinar cells (a) organized [____] (b) disorganized [____]
2. Level of maturity of the acinar cells(a)some immature[____] b) all immature[____] (c) mixed[____]
3. Shapes of the acinar cells (a) pyramidal[____] (b)columnar[____] c) columnar and pyramidal[____] (d) some irregular shapes[____]
4. Cytoplasm (a) acidophilic [____] (b) aecinophilic[____]

5. Secretory granules (a) numerous and conspicuous[___] (b)less numerous [___]
6. Cell nuclei (a) normal shapes and basally located[___] (b) pyknotic and large[___] (c) pyknotic and small[___]
7. Positions of central acinar cells (a) centrally placed[___] b) basally with acinar cells[___]
b) Along the duct [___]
8. Any other observations: _____

b) Duct system

1. Organization of the duct system a) regular[___] (b)irregular[___]
2. Shape of the duct cells epithelium: a) columnar[___] b)cuboidal[___] d) irregular cells
3. Presence of goblet cells and brush borders in large ducts: a) present [___] b) absent[___]
4. Any other observations: _____

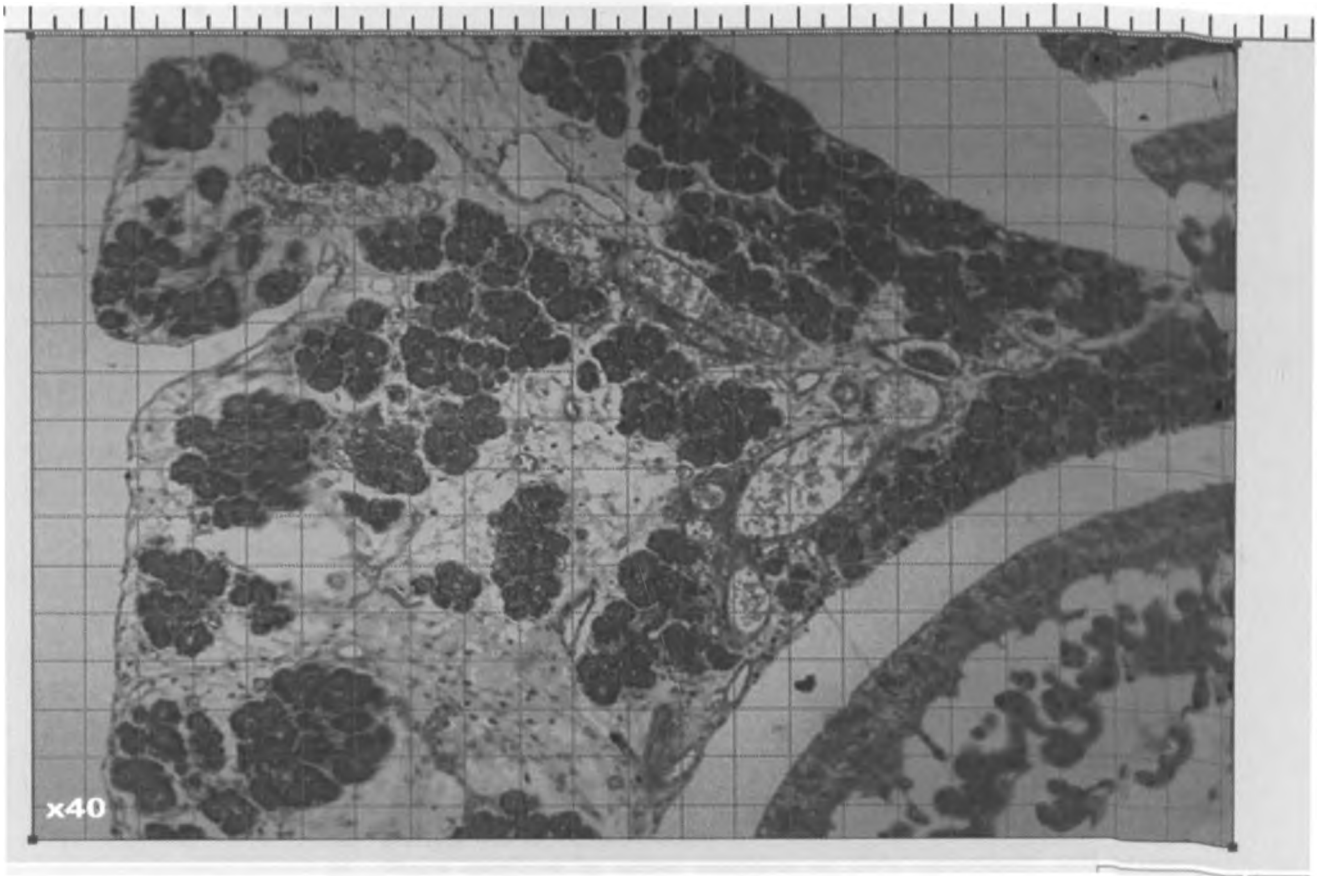
c. Islet cells clusters(x100)

1. Distribution of clusters (a) regular [___] (b) irregular[___]
2. shapes of the clusters (a) round {___} b) ovals[___](c) irregular{___}

Magnification (x1000)

3. Arrangement of cells(Beta, alpha D, PP Cells) in a cluster (a) regular[___] (b) irregular[___]
4. maturity of cells (a) mature[___] (b) immature[___]
5. Numbers of each type in a cluster
(a)beta(B cells) _____
(b) alpha (A&A1 cells) _____
(c)delta (D orA1 cells) _____
(d) somatostatin(SS) _____
(e) PP cells _____
6. relation with islet cells : separated from islets[___] (b) not separated[___]
7. any other observations _____

APPENDIX 2: A photo micrograph showing the grid used for morphometry



Appendix 3: PROCEDURE FOR PREPARATION OF STAINS

a) Preparation of staining solutions

b) *staining to demonstrate D (α_1) Cells of Pancreas*

Alcoholic surver Nitrate method for argyrophil cells, especially D (α_1) cells of the pancreas. (Hellerstrom & Helman 1960)

Fixation: Bouins or Formaldehyde followed by secondary fixation in Bonin for 2 hours at 37 ° c. or Zenkers solution (fixative) preparation

Sections: -Paraffin wax

Preparation of staining solutions

a) Silver solution

Silver Nitrate.....10gms
Distilled Nitrate.....10 cm³
95% alcohol.....90 cm³

Before use the PH of the solution is adjusted to 5.0 with conc. Ammonium hydroxide

b) Developing solution

Pyrogallic acid.....5gms
95% alcohol.....95cm³
Formaldehyde (40%).....5 cm³

Method for staining:

- i. Take sections down to running tap water, 1 hour.
- ii. Dehydrate to 95% alcohol.
- iii. Place sections in silver solution at 37°C overnight and protect from light.
- iv. Rinse rapidly in 95% alcohol- no longer than 10 seconds.
- v. Transfer sections to developing solution for 1 minute.
- vi. Rinse in 3 changes of 95% alcohol for 1 minute.
- vii. Rinse in absolute alcohol for 1 minute each.
- viii. Mount in DPX

*Expected results: D cells granules for the pancreas (α_1) – positive (Brown black)
A & B cells – Negative Some other argyrophil cells – positive*

(b) Modified aldehyde fuchsin stain (Demonstration of islet cells) Halami, 1952)

Fixation: Formal saline or Bonin's Fluid

Sections: Thin paraffin sections

Preparation of solutions

- Aldehyde Fuchsin solution
- Basic fuchsin.....1gm
 - Paraldehyde.....2 cm³

- Concentrated hydrochloric acid.....1 cm³
- Ethanol.....60 cm³
- Distilled water40 cm³

Dissolve the basic fuchsin in the alcohol-distilled water. Add the hydrochloric acid and the paraldehyde. Allow to "ripen" 2-7 days at room temperature then filter. Store at 4°C.

b) Counter stain

- Light green.....200mg
- Orange G.....1g
- Phosphoturystic acid.....500gms
- Glacial acetic acid.....1 cm³
- Distilled water.....100 cm³

Methods for staining

- 1) Take sections to water
- 2) Oxidize with lug's iodine 10 minutes.
- 3) Rinse in tap water and bleach with 2.5 percent sodium thiosulphate.
- 4) Wash in tap water followed by 70% alcohol.
- 5) Stain in aldehyde fuchsin stain for 15-30 minutes.
- 6) Wash in 95% alcohol followed by water.
- 7) Stain Nuclei with Celestine blue and haemalum.
- 8) Wash in water, differentiate briefly in alcohol and wash well in tap water.
- 9) Rinse with distilled water and counter stain with orange G-light green solution 45 seconds.
- 10) Rinse briefly with 0.2 percent acetic acid followed by 95% alcohol.
- 11) Dehydrate in absolute alcohol, clear in Xylene and mount in DPα.

Expected Results

B - Cells - purple - violet

A - Cells - yellow

D - Cells - green

Nuclei - blue - black

c) **Gomori's method for Reticular Stroma (pancrease). (Gomori, 1937).**

Preparation of silver solution

To 10 cm³ of 10 percent potassium hydroxide solution, add 40 cm³ of 10% Silver Nitrate solution, allow the precipitate to settle and decant the supernatant. Wash the precipitate several times with distilled water. Add ammonia drop by drop until the precipitate has just dissolved. Add further to 10 percent Silver Nitrate solution until a little precipitate remains. Dilute to 100 cm³ and filter. Store in a dark bottle.

Method for staining



- i) Dewax sections and bring to water.
- ii) Treat with 10% potassium permanganate solution 2 minutes.
- iii) Rinse in tap water.
- iv) Bleach in 2 percent potassium metabi-sulphate solution.
- v) rinse in tap water
- vi) treat with 2 percent iron alum- 2 minutes
- vii) Wash in several changes of distilled water.
- viii) Place in coplin jar of silver solution – 1 minute.
- ix) Wash in several changes of distilled water.
- x) reduce in 4% aqueous Formalin solution 3 minutes.
- xi) rinse in tap water.
- xii) tone in 0.2 percent gold chloride solution 10 minutes.
- xiii) rinse in tap water.
- xiv) Treat with 2 percent potassium metabi-sulphite solution 1 minute.
- xv) rinse in tap water.
- xvi) treat with 2% sodium thiosulphate solution 1 minute.
- xvii) rinse in tap water.
- xviii) counterstain as desire (Van Gieson or eosin are suitable).
- xix) Dehydrate through alcohols.
- xx) clear in Xylene and mount in DPα

Expected results:

Reticular fibres – black

Nuclei – grey

Other tissues – according to counterstain employed.

>stain one set of 60 slides with modified haematoxyline and eosin, aldehyde fuchsin stain **Hamali (1952)** to demonstrate acinar and islet cells,
 >Stain the other set of 60 slides with **Gomori stain (1937)** to demonstrate reticular stroma.

5.1.0 Procedure for studying the cellular and stromal components

1. Select the good slides well stained from head to tail section of the pancreas per rat/fetus, (total of 60 slide).
2. Photograph each section of the head, body and tail regions on systematically selected random fields at a magnification x100.
3. Count clusters of islet cells and find the mean of the four fields recorded as the count for each region.

NB> exclude all clusters not well displayed or disaggregated. The pancreatic islet cells clusters with modified aldehyde fuchsin stain will be displayed as follows: β – Cells:- purple – violet; α:- Cells – yellow, D - Cells – purplish green, Nuclei – blue – black.