Effect of Folic Acid on Alcohol Induced Congenital malformations in Mice

Thesis

Submitted for the Degree of

Doctor of Philosophy

In

(Human Anatomy)

by

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Acknowledgement

First and foremost, I express my deep sense of gratitude to revered **Prof. Mandavi Singh**, Department of Anatomy and Dean of faculty of Medicine, Institute of Medical Sciences, Banaras Hindu University for her constant inspiration, sagacious guidance, fruitful discussion, untiring help during the supervision of my research work and above all for taking care of me. Thesis completion under her guidance was a matter of pride as well as a responsibility as it was tough to match her levels of expectation from me. I am further deeply indebted to her for her critical review of the manuscript and the facilities provided to me. All the achievements of this work are her only.

I would like to express my sincere thanks to **Prof. G. L. Shah**, Head, Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University for his moral support and constant encouragement.

I am also thankful to all the teachers, **Prof. S. N. Shamal, Prof. S. K. Pandey, Dr. Royana Singh and Dr. R.S. Moore,** Department of Anatomy, of Institute of Medical Sciences, Banaras Hindu University for their direct and indirect support to complete this research work.

I would like to express my special thanks to **Dr. Anand Mishra**, Associate Professor, Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University for his help and encouragement from the beginning of this research work. During my 3 years stay in Banaras, I really enjoyed his company and had a memorable time in my life.

I would like to express my sincere thanks to **Dr. S. P. Mishra**, Assistant Professor, Dept. of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, who has constantly helped me with the biochemical work.

I am thankful to **Prof. T. B. Singh,** Department of Biostatistics for his kind help during statistical analysis. His depth of knowledge and sincere guidance throughout the pursuit of the research were really helpful.

It's my fortune to gratefully acknowledge the support of **Dr. Prof. U. P. Singh,** Prof. (retd.) Institute of Agricultural Sciences, BHU and husband of my supervisor. Words fail to express my appreciation to **Prof. U.P. Singh** for his support, generous care and the homely feeling at Banaras. He was always beside me during the happy and hard moments to push me and motivate me. He also critically corrected my manuscript before reviewing by my supervisor. His immense knowledge and valuable suggestion were always there when needed. Sir, I am really fortunate to have your blessings during my stay at Banaras.

I could not have completed my research without the technical assistance of a great number of people. I want to acknowledge all the non-teaching staff of the Department of Anatomy especially **Mr.Jagadish Shrama, Mr. Satya Pal Sharma, Mrs. Tessy Pal, Mr. Naresh , Mr. DN Vishwakarma, Mr. Gulab Chand, Mr. Mahesh, Mr. Ram Sagar, Mr. Saurabh Sameer, Mr. Munna Lal** for their constant help in my research work.

Thanks to all of my friends for your support throughout the years. I would like to extend huge, warm thanks to my senior/colleagues especially **Mr. Shamsher Shrestha, Mr. Ram Lala Mallick, Miss Sant Suman Rastogi, Dr. Amrita Singh, Dr. J.P. Pani, Dr. Amit Nayak, Dr. Vivek Parasar and Dr. Soumya Khanna** for their valuable help and love and affection which I received from them during my research work period.

I will fail in my duty if I do not thank to my experimental mice which gave their lives for my research work, without their sacrifice this work was not possible. I am also grateful to **Patan Academy of Health Sciences** for sponsoring me for the course.

Last, but not least, I would like to dedicate this thesis to my parents, my wonderful wife **Gita** and lovely daughter **Ugisha** and son **Ugesh** for their love, patience and understanding. Without support of my parents, wife and children, I would never have succeeded. Although they often had to endure my absence they seldom complained. I hope they will enjoy the effort being completed. Although hardly aware of their stimulating role a word of gratitude is also spoken to other members of my family and my friends.

May, 2014

Place: Varanasi

Mr. Uttam Shrestha

Contents

Abbreviations

ADH	:	Alcohol Dehydrogenase
ALDH	:	Aldehyde Dehydrogenase
ARBD	:	Alcohol Related Birth Defects
ARND	:	Alcohol Related Neurodevelopmental Disorders
AVOVA	:	Analysis of Variance
BACs	:	Blood Alcohol Concentrations
BECs	:	Blood Ethanol Concentrations
CA	:	Cornu Ammonis
CNS	:	Central Nervous System
CYP2E1	:	Cytochrome P450E1
DHFR	:	Dihydrofolate Reductase
DNA	:	Deoxyribonucleic Acid
DPX	:	Dibutyl Phthalate Xylene
EDC	:	Ethanol-derived Calories
EEG	:	Electro-encephalogram
EPM	:	Elevated Plus Maze
FAS	:	Fetal Alcohol Ayndrome
FASD	:	Fetal Alcohol Spectrum Disorder
GABA	:	Gamma Amino Butyric Acid
GD	:	Gestational day
GSH	:	Reduced Glutathione
H&E	:	Haematoxylin and Eosin
HCY	:	Homocysteine
HPA	:	Hypothalamic-pituitary Axis
IQ	:	*Intelligence Quotient*
IUGR	:	Intrauterine Growth Retardation
KOH	:	Potassium Hydroxide
MDA	:	Malondialdehyde
MEOS	:	Microsomal Ethanol Oxidizing System
MTHFR	:	Methylene Tetrahydrofolate Reductase
NAD^+	:	Nicotinamide Adenine Dinucleotide (oxidized)
NADH	:	Nicotinamide Adenine Dinucleotide (reduced)
NGF	:	Nerve Growth Factor

NMDA	:	N-methyl-D-aspartate
NTD	:	Neural Tube Defects
NTFs	:	Neurotrophic Factors
PAS	:	Periodic Acid - Schiff's
PCs	:	Purkinje cells
pFAS	:	Partial Fetal Alcohol Syndrome
PND	:	Postnatal Day
RBC	:	Red Blood Cell
RDA	:	Recommended Dietary Allowance
RFC	:	Reduced Folate Carrier
RNA	:	Ribonucleic Acid
ROS	:	Reactive Oxygen Species
SAM	:	S-adenosylmethionine
SD	:	Standard Deviation
SPSS	:	Statistical Package for the Social Sciences
TBA	:	Thiobarbituric Acid
THF	:	Tetrahydrofolate
THIQs	:	Tetrahydroisoquinolines
5-HT	:	5-hydroxy Tryptamine
WHO	:	World Health Organization

Chapter-1

Introduction

Introduction

Changes in environmental factors (e.g., nutrition, stress, exposure to toxicants) at critical periods during development can permanently alter the structure, physiology or metabolism of the body, resulting in a lifelong effect on the organism (Barker, 1998). This influences the risk of developing some chronic diseases later in the life (Barker, 1998; Cooper *et al*., 2002; Godfrey *et al*., 2001; Hanson *et al*., 2004; and Jones *et al*., 1999). Ethanol is one of the factors whose exposure in utero is known to have serious long-term implications for the offspring which can lead to a diverse array of health problems (Chudley *et. al*., 2005).

It is one of the most commonly consumed psychoactive drugs. It is a volatile, flammable, colorless liquid, 2-carbon alcohol-based dipole molecule. The hydroxyl group found in ethanol makes it polar and soluble in water, allowing it to be readily absorbed by the gut (Fig. 1) and distributed in body water and adipose tissue, albeit to a lesser degree (Bruckner and Warren, 2001). Due to absence of asymmetric carbon ethanol undergoes chemical interactions with other biological substrates easily. The hydroxyl group of ethanol readily forms hydrogen bonds with proteins and carbohydrates allowing it to permeate the cells easily.

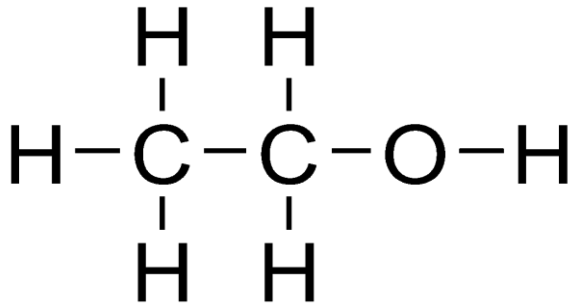


Fig 1: The Chemical Structure of Ethanol

The pharmacologic and potentially pathologic effects of ethanol depend on the concentrations of ethanol and its metabolites in the body, and on the duration of exposure to these substances. Metabolism of ethanol occurs chiefly within the liver by hepatic oxidation and is governed by the catalytic enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). There is great degree of variability in the rate of alcohol metabolism among individuals and ethnics. This may be due to difference in allelic variants of the genes encoding ADH and ALDH which give rise to isoenzymes that metabolize alcohol at different rates (Ramchandani *et al.,* 2001). However, the rate of alcohol clearance is relatively constant between individuals. It is estimated that about 0.015g/dl of alcohol is cleared from the body every hour once alcohol consumption has stopped. Although, small fractions of alcohol being excreted in the breath (0.7%), sweat (0.1%) and urine (0.3%), elimination from the body occurs predominantly through metabolism in the liver (Ramchandani *et al.,* 2001).

The rate of ethanol metabolism also varies among the species. Although, every mammalian species induces the enzymes to metabolize ethanol, the actual rate at which the enzymes function to clear alcohol from the system varies. Humans and larger animals including non-human primates have similar rates of alcohol metabolism while rodents, including rats and mice metabolize ethanol at a much faster rate than humans.

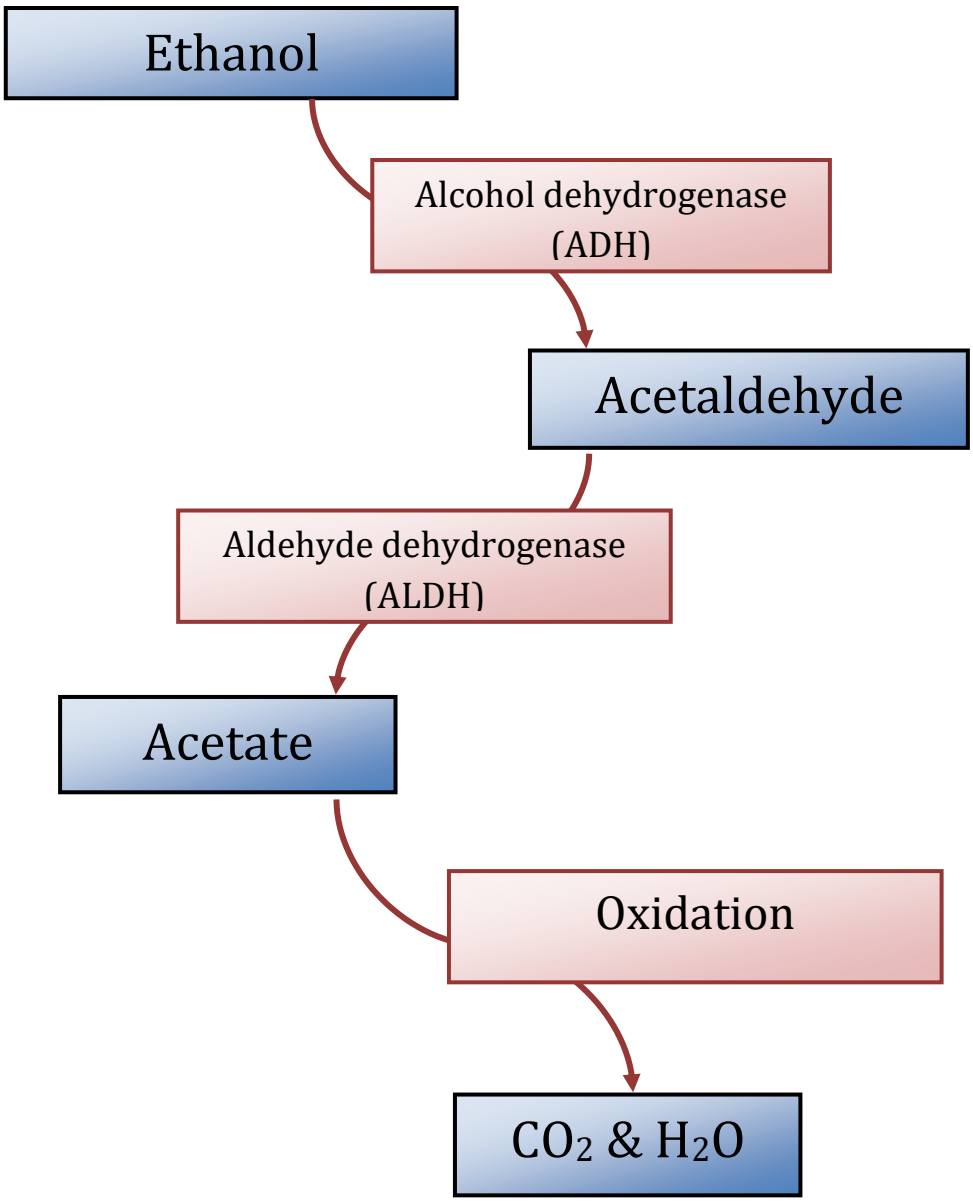


Fig. 2: Flow diagram of alcohol metabolism

Oxidation of ethanol to acetaldehyde is the first step in ethanol metabolism which is governed by the enzyme Alcohol Dehydrogenase (ADH) (Fig 2). ADH is a NAD^+ dependant cytosolic enzyme principally located in the liver but found to a lesser extent in the stomach, lungs and kidneys. It is a zinc-containing, dimeric molecule, which catalyses the oxidation of alcohol by the

removal of hydrogen from the hydroxyl group forming an aldehyde complex, acetaldehyde (Suddendorf, 1999, Lieber, 2000).

Five classes of ADH (ADHI-V) are known in human and rodents which have independent expression profiles that are time and tissue-specific (Suddendorf, 1999). While all of the ADH classes have the capacity to metabolize alcohol, it is predominantly ADH I and ADH II that are ubiquitously expressed in the liver. ADH III and ADH IV are expressed in the mucosa of the gastrointestinal tract allowing the initial metabolism of alcohol to occur in the wall of the gastrointestinal tract (Suddendorf, 1999).

When large amounts of alcohol have been consumed or in chronic alcohol exposure, ADH becomes saturated and unable to metabolize alcohol to acetaldehyde quickly. It results in the activation of an additional metabolic pathway, *i.e.,* Microsomal Ethanol Oxidizing System (MEOS). MEOS is found within the smooth endoplasmic reticulum and is a NADPH-dependant system, using oxygen as a consumable. The key enzyme involved in this pathway is the alcohol inducible cytochrome P450E1 (CYP2E1), the levels of which are increased 4- to 10-fold following chronic alcohol administration (Lieber, 2000).

Catalase can also catalyze the oxidation of ethanol using hydrogen peroxide as a co-substrate. It is often listed as a third pathway for ethanol removal, and it might act in brain where no other enzyme is known to convert appreciable amounts of ethanol to acetaldehyde (Lands, 1991). The acetaldehyde released into the brain by the metabolism of alcohol by catalase has the potential to combine with neurotransmitters to form new compounds known as tetrahydroisoquinolines (THIQs). Some researchers believe that THIQs are the cause of alcohol addiction and that the presence of THIQs distinguishes addicted drinkers from social drinkers (Lands, 1991).

Acetaldehyde is toxic substance and needs to be quickly metabolized. It is metabolized to acetate by an enzyme aldehyde dehydrogenase (Fig. 2). Aldehyde dehydrogenase has two major isoforms (ALDH1 and ALDH2) which are found throughout the body which facilitates the rapid metabolism of acetaldehyde before the cellular damage occurs (Lieber, 2000). The acetate is oxidized to carbon dioxide and water which occurs rapidly in most of the organs (Fig. 2). The clearance of acetate is dependent on age and sex, with the clearance rate slowing with age. Women metabolize acetate slower than men (Lands, 1991).

Basically there are three categories of prenatal exposures to ethanol based upon the amount of alcohol ingested. The first category, i.e., exposure to heavy drinking (over 48–60 g ethanol/day) may cause fetal alcohol syndrome, the second category, i.e., exposure to moderately high drinking (between 24–48 g ethanol/day) may result in unspecified "alcohol effects" (the differences between these two categories may not be sharp) and the third, i.e., binge drinking (occasional intakes of 4–5 drinks of ethanol) may or may not cause any alcohol effects (Kesmodel *et al.,* 2002 and Martinez-Frias *et al.,* 2004). The amount of alcohol ingested, the duration of alcohol consumption and the developmental stage of the conceptus during the exposures mediate the effects of ethanol intake on the developing fetus (Mattson *et al.,* 2001). Alcohol drinking, even in moderate amounts, is also associated with an increased risk of spontaneous abortions, especially in the first trimester of pregnancy and with infertility in both males and females (Kesmodel *et al.,* 2002).

Ethanol readily crosses the placenta and reaches concentration in the fetus which is similar to those in the maternal blood (Waltman and Iniqez, 1972). Due to low activity of hepatic dehydrogenase, the fetus is limited in its ability to metabolize alcohol. Therefore, the elimination of alcohol from the fetus is through passive diffusion of alcohol across placenta followed by

maternal elimination. In addition, the rate of diffusion of alcohol from amniotic fluid is slow, resulting in relatively high alcohol concentration in amniotic fluid when alcohol level is low or almost nil in the maternal blood. So, the amniotic fluid can act as reservoir for alcohol and the fetus can be actually exposed to it for a longer period than assumed on the basis of maternal alcohol concentration (Brien *et al.,* 1983). Thus, the consumption of alcohol during pregnancy may seriously affect the developing embryo and fetus. The severity of the malformations ranges from FAS, which is evident in 4–6% of offspring of heavy drinking mothers, to minor effects, such as low birth weight, intra uterine growth retardation (IUGR), slight reduction in IQ of the infants and increased rate of congenital anomalies (Jones and Smith, 1973). Intra uterine growth retardation as well as postnatal long-term height and weight deficits is well demonstrated among children born to ethanol using women (Ornoy *et al.,* 2010).

The ability of ethanol to induce malformations in the developing embryo or fetus has been described decades ago (Lemoine *et al.,* 1968; Clarren and Smith, 1978). Fetal alcohol syndrome (FAS) is the most common, serious and specific syndrome of alcohol effects in pregnancy which has been described only for regular/daily high dose alcohol users (Jones and Smith, 1973; Banerjee *et al.,* 2007). This was based on the evaluation of eight children born to chronic alcoholic mothers who present with prenatal and postnatal growth deficiency, short stature, developmental delay, microcephaly, fine-motor dysfunction and facial dysmorphism. In addition, there may be cleft palate, joint and cardiac anomalies and altered palmer creases (Jones, 2003).

The developing brain particularly hippocampus and cerebellum are the organ most vulnerable to prenatal ethanol exposure (Barnes and Walker 1981, Bonthius and West, 1991; Livy *et al*., 2003; Bauer-Moffett and Altman, 1977, Bookstein *et al.,* 2006; Parnell *et al.,* 2009). This central nervous system (CNS)

injury, which can manifest as behavioral and intellectual deficits in offspring, is the most persistent and debilitating feature of all of the observed teratogenic effects of alcohol. Learning and memory deficits are very prominent and reproducible consequences of chronic prenatal ethanol exposure (Blanchard *et al.;* 1987, Abdollah *et al.,* 1993; Gibson *et al.*, 2000; Spear-Smith *et al.*, 2000; Nash *et al*., 2007; Thomas *et al.*, 2010). Similarly, developmental delay, attention deficits, hyperactivity and diminished impulse control are also common (Coles, 1991; Sampson *et al.,* 1997).

There are a number of other physiological and morphological malformations which have been reported following in utero ethanol exposure. These include renal anomalies (Assadi *et al.,* 1991; Qazi *et al.,* 1979; Taylor *et al.,* 1994), limb and digit malformations (Jones and Smith, 1973), ventricular abnormalities in brain (Clarren *et al.,* 1978; Jones and Smith, 1973; Konovalov *et al.,* 1997) and skeletal malformations (Tredwell *et al.,* 1982; Tsukahara and kajii, 1988).

Ethanol can induce oxidative stress directly by formation of free radicals which react with different cellular compounds, or indirectly by reducing intracellular antioxidant capacity, such as reduced glutathione levels. The free radicals and reactive oxygen intermediates such as superoxide, hydrogen peroxide and other hydroxyl ions which are generated due to oxidative stress are capable of damaging proteins, lipids and DNA in a cell causing increased apoptosis which is harmful (Bergamini *et al.,* 2004, Chen & Sulik, 1996). These deleterious effects are blocked by free radical scavengers such as superoxide dismutase (SOD), peroxidase, catalase, reduced glutathione and alpha-tocopherol (Chen and Sulik, 1996) which are either in low level or total absent in fetal tissue. Ethanol increases the level of malondialdehyde which is the marker of the free radical-induced lipid peroxidation. The increased level of

malondialdehyde correlates with the suggested role of free radicals in the pathogenesis of FAS (Chen and Sulik, 1996).

Folic acid, also generically known as folate or folacin (Fig. 3), is a water soluble member of the B-complex family of vitamins that plays a vital role in one carbon-transfer reactions in purine, thymidylate and S-adenosylmethionine (SAM) biosynthesis, ultimately leading to deoxyribonucleic acid (DNA) synthesis and DNA methylation, respectively (Bailey and Gregory, 1999). It is required for production and maintenance of new cells and in DNA repair (James *et al.*, 1994; Blount *et al.*, 1997; Zeisel, 2009). Humans do not have the capacity to synthesize folic acid or folate metabolites and must obtain this vitamin from dietary sources or supplementation (Hamid *et al.*, 2007). Maternal folate requirement increases during pregnancy due to proliferation of cells in the placenta and fetus (McArdle and Ashworth, 1999). Normal gestational folate status is maintained by healthy maternal diet together with folic acid supplementation (Chanarin *et al.*, 1968; Goh and Koren, 2008). Adequate folate intake during the periconception period, the time right before and just after a woman becomes pregnant, helps protect against a number of congenital malformations, including neural tube defects (Shaw, 1995).

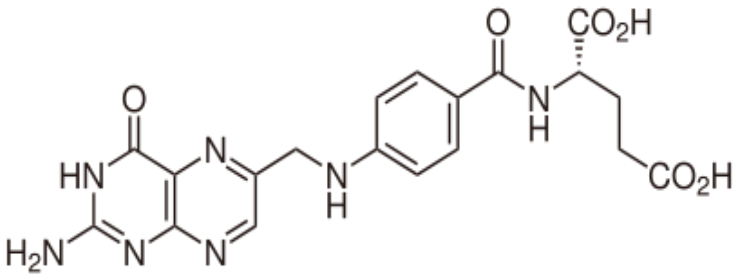


Fig. 3: Structure of folic acid (pteroylglutamic acid)

Ethanol can adversely affect folate disposition in the body by altering folate absorption, distribution and excretion (Hamid *et al.*, 2007; Tamura and Halsted, 1983; Muldoon and McMartin, 1994). Although folic acid deficiency

is a common feature in pregnancy, it becomes more severe in alcoholics (Adebisi, 2003). RBC and serum folate concentrations are decreased by 35–80% in alcoholic patients (Wu *et al.,* 1975; Halsted *et al.,* 2002), probably due to a combination of inadequate dietary folate intake and disrupted folate absorption and distribution (Hoyumpa, 1986). Ethanol ingestion induces a noted increase in urinary excretion of folic acid depleting serum and hepatic folic acid (McGuffin *et al.,* 1975). It has also been shown that ethanol reduces folic acid uptake by intestinal bacteria and its metabolism in liver which may lead to folic acid deficiency. During the pregnancy it may impair transport of folic acid across placenta by decreasing expression of folate transport proteins (Janine, 2012,) inducing its deficiency which may increase the risk of preterm delivery, infant low birth weight and fetal growth retardation. It increases homocysteine level in blood, which may lead to spontaneous abortion and pregnancy complications, such as placental abruption and pre-eclampsia (Scholl and Johnson, 2000).

Deficient maternal nutrient status before conception and throughout pregnancy can result in poor prenatal and postnatal outcomes (Doyle and Rees, 2001; Rees *et al.,* 2005a; Rees *et al.,* 2005b). In the mouse, fetal folate deficiency resulting from prenatal ethanol exposure can decrease the function of various cellular proteins that are essential for CNS development which can be reversed by maternal folic acid supplementation (Xu *et al.,* 2008). Furthermore, in the mouse, deficient maternal folate status can potentiate the teratogenic effects of ethanol (Gutierrez *et al.,* 2007). Low concentration of methanol has been found in a variety of alcoholic beverages (Sprung *et al.,* 1988). Its elevated concentration has been found in the cerebrospinal fluid of alcoholic human beings. In rat hippocampus slice culture, folic acid (1 μM) has been reported to protect against neurotoxicity produced by formic acid (Kapur *et al.,* 2007) which is a metabolite of methanol. In a recent review, it has been recommended that, in the human, an approximate maternal RBC folate

concentration of 0.9 μM is optimal for fetal development and postnatal outcomes (Goh and Koren, 2008).

In spite of sufficient evidence regarding the teratogenicity of alcohol, the incidence of FAS is on the arise. It is primarily due to a lack of adequate awareness of the harmful effects of drinking during pregnancy among the general public. Numerous preventive measures have been suggested though none is sufficiently convincing as the exact mechanism of alcohol induced damage is not known. So we propose that folic acid may reduce the deleterious effect of alcohol. The present study tested the hypothesis that maternal folic acid supplementation mitigates ethanol-induced congenital malformations, decreased body weight and crown rump length as well as histological changes observed in fetal brain, liver, kidney and placenta and altered malondialdehyde and reduced glutathione levels in fetal brain and maternal blood. The study also hypothesize that maternal folic acid supplementation improves ethanol-induced behavioral impairments and histological changes in brain of offspring. Swiss albino mice were utilized in the present study as they are well characterized as a model for prenatal ethanol exposure (Riley and Meyer, 1984). Oral route was used in the present study since it is more relevant to human ethanol exposure, produces a pharmacokinetic profile similar to that in the pregnant human. The treatment was given from gestational day 6 to gestational day 15 to reflect the human exposure during the period of organogenesis.

Chapter-2

Aims and Objectives

Aims and Objectives

The teratogenic effects of alcohol are well known but relatively little is known about the role of folic acid on alcohol induced teratogenicity and long term effect in brain and behavior in offspring of alcoholic mothers. So the present study has been undertaken with the following aims and objectives:

1. To induce and observe congenital malformations in mice by alcohol.
2. To study the protective action of folic acid on alcohol induced congenital malformations.
3. To compare maternal and fetal brain MDA level and reduced glutathione level of all experimental animals.
4. To compare the histopathological features of placenta, liver, kidney and brain of fetuses of different groups.
5. To evaluate the behavioral changes in offspring (8 weeks) of all experimental animals.
6. To observe the histopathological changes in brain of offspring (9-10 weeks) of all experimental groups.

Chapter-3

Review of Literature

Pharmacology of ethanol

Ethanol, present in alcoholic beverages, is the most widely used psychoactive drug in our society causing an anesthetic type response in the central nervous system (CNS) (Eckardt *et al.*, 1998, Weiss and Porrino, 2002). Since the effects of ethanol are non-selective, it can affect functions and properties of many cells. It disrupts membrane organization which increases the fluidity of the cellular membrane through recruitment of cholesterol to the membrane (Goldstein *et al.*, 1981). It inactivates enzyme function by binding to the active sites of enzymes causing a conformational change. It also increases the activity of Na/K-ATPase channels resulting in sodium leakage and thus altering sodium homeostasis (Blachley *et al.*, 1985).

Ethanol depresses the CNS which results in a typical behavioral phenotype observed in intoxicated individuals such as slurred speech, motor in-coordination, increased self-confidence, sedation, hypnosis and a sense of euphoria. These effects are caused by the action of ethanol on the CNS, specifically the neurotransmitters gamma amino butyric acid (GABA), N-methyl-D-aspartate (NMDA) and serotonin pathways (Eckardt *et al.*, 1998). It enhances the action of GABA which acts on the alpha receptor in a similar fashion to that of benzodiazepines. However, the magnitude of the effect produced by ethanol is smaller than that of benzodiazepines. It inhibits the

release of the neurotransmitter by causing a depolarization, inhibiting the opening of voltage-sensitive calcium channels in neurons which causes hyperpolarisation of the neuronal membrane resulting in mild sedation (Eckardt *et al.,* 1998). The NMDA subtype of the glutamate receptor is considered to be the most sensitive to the actions of ethanol. The excitatory properties of NMDA are inhibited by the actions of ethanol causing a depressant phenotype. It also enhances the excitatory effects by the activation of the atypical clozapine (nAChRs) and 5-hydroxy tryptamine (5-HT3) receptor subtypes of the serotonin receptor pathway (Eckardt *et al.,* 1998).

Ethanol and nutrition

Ethanol when consumed in excess amount can cause disease by interfering with the nutritional status of the consumer. Evidence from experimental and clinical studies suggests that alcohol consumption has the capacity to alter one's nutritional status despite an adequate balanced diet, leading to malnutrition and hepatotoxicity. It may decrease the food intake by suppressing the hunger and also effect the digestion of the nutrients. Ethanol increases the transit rate of food along the digestive tract which results in the maldigestion of the ingested food. Even a single dose of ethanol disturbs the absorption of nutrients in the gastrointestinal tract. Chronic excessive alcohol consumption eliminates the absorption of sodium and water, resulting in diarrhoea and steatorrhea due to mucosal wall bleeding and duodenal erosion (Bode *et. al.,* 2003). Absorption of proteins and vitamins are mainly affected in alcoholics among many others. Both animal and human studies showed that absorption of folic acid is decreased in chronic ethanol abuse (Halsted, 2002). Folic acid (Folate), a water soluble B-vitamin whose biologically active form is tetrahydrofolate, is essential for cell division and for the synthesis of DNA, RNA and protein.

Epidemiology of Ethanol Consumption

The worldwide per capita consumption of pure alcohol (100%) is estimated to be 6.13 liters per year (WHO, 2011). However, great variation occurs in adult per capita consumption of alcohol among countries. The highest consumption level (≥12.50 liters of pure alcohol) is found in the developed world, mostly in the Northern Hemisphere, but also in Argentina, Australia and New Zealand. Low consumption levels (<12.50 liters of pure alcohol) is found in the countries of North Africa and sub-Saharan Africa, the Eastern Mediterranean region, and southern Asia and the Indian Ocean. These regions represent large populations of the Islamic faith, which have very high rates of abstention (WHO, 2011). In India also alcohol consumption is gradually increasing with 2.6 liters per capita consumption of pure alcohol per year while that in South East Asia is only 2.2 liters (WHO, 2011). In general population studies throughout the world indicates that men are more often drinkers, consume more alcohol as compared to women (Wilsnack, 2005). However, in the US, approximately 60 percent of adult women drink alcohol, at least occasionally (Wilsnack, 1994). Rates of drinking and heavy drinking tend to be highest among young women and decline steadily with age. According to the "Gender, Alcohol and Culture, an International Study" (GENACIS) in India, 5.8% of all female respondents reported drinking alcohol at least once in the last 12 months (Benegal, 2005). In India, alcohol use is more prevalent in tribal women, tea plantation workers, women of lower socioeconomic status, commercial sex workers (women who sell sex for livelihood) and to a limited upper crust of the rich and is not favored by women from the middle or upper socioeconomic classes. In these high risk groups, the prevalence is around 28-48% (Mohan, 2001).

The worldwide per capita consumption of alcohol remained relatively stable for approximately 30 years, but in the last 5 to 10 years consumption has

increased, particularly amongst the young and more specifically amongst young women (WHO, 2004). Epidemiological studies have indicated that heavy drinking is more common in young individuals than older individuals, and it has been reported that women aged 18-39 years were significantly more likely to consume alcohol in episodic heavy drinking than those aged over 40 years (Nayak *et al.*, 2004; Strandberg-Larsen *et al.,* 2008). Furthermore, it has been identified that approximately 7% of women in Britain of childbearing age consume alcohol at levels that are classified as risky (Nayak *et al.*, 2004). In India, men are the primary consumers of alcoholic beverages. The amount of alcohol beverages consumed by people in different regions of India varies from 16.7 percent in Madras City in southern India to 49.6 percent in a Punjab village in northwest India (Isaac, 1998). Conversely, the alcohol consumption rates among women are consistently low (i.e., less than 5 percent)(Isaac, 1998). However, with the modernization of social living it is increasing progressively. With the increase in alcohol consumption amongst young women, it has been seen that there is also an increase in the percentage of pregnant women who continue drinking till pregnancy is confirmed (Nayak *et al.,* 2004; Chang *et al.,* 2006; Strandberg-Larsen *et al.,* 2008). Usually, pregnancy is not confirmed until approximately 6^{th} to 8^{th} week of gestation, leaving 6 to 8 weeks of potential fetal alcohol exposure.

Alcohol consumption during pregnancy

There is a great variation among the countries in the rate of alcohol consumption by pregnant women. Several countries recommend that abstinence is the safest option, still some women continue to consume alcohol during pregnancy (Caetano *et al.,* 2006; Chang *et al.*, 2006; Colvin *et al.*, 2007; Giglia *et al.,* 2007). In the United States, England, and Canada, 20%-32% of pregnant women drink, and in some European countries the rate is higher, exceeding 50% (May *et al.,* 2005). In a study in the Western Cape Province of

South Africa, 34% of urban women and 46%-51% of rural women drink during pregnancy. Their drinking pattern was characterized by heavy binge drinking on weekends, with no reduction of use during pregnancy (May *et al.*, 2005). Maternal drinking during pregnancy varies among and within populations throughout the world (Abel, 1998). According to animal and human studies binge drinking is more harmful to the developing brain than the regular pattern (Maier, 2001). A recent Western Australian study reported that women generally reduce their consumption of alcohol and the number of standard drinks on a typical occasion as their pregnancy progress. However, 58.7% of women still consume alcohol in at least one trimester of their pregnancy (Colvin *et al.,* 2007). In Western Australia, it was found that 35% of pregnant women consumed alcohol during pregnancy, with 82% of these women consuming up to 2 standard drinks per week (Giglia *et al.,* 2007). A small percentage (13%) of women consumed more than 2 standard drinks in the one week, while 7% consumed more than 7 standard drinks in one week (Giglia *et al.,* 2007). This pattern of alcohol consumption by pregnant women is not isolated to Australia, but also in US and British many pregnant women consume alcohol during pregnancy (Caetano *et al.,* 2006; Chang *et al.,* 2006; Tsai *et al.,* 2007). For example, Caetano *et al.* (2006) reported that 44.3% of British women drank while pregnant, and that 1.2% of these women engaged in binge drinking. Tsai *et al.* (2007) reported that 28.5% of American pregnant women consumed 5 or more standard drinks on a typical drinking occasion (Tsai *et al.,* 2007). In Russia, where 90% of non-pregnant women consume alcohol on a daily basis, the incidence of alcohol consumption by pregnant women is also common (Kristjanson *et al.,* 2007).

Ethanol teratogenicity

Maternal ethanol consumption during pregnancy can produce several birth defects in the developing fetus that manifest as congenital malformations

in postnatal life. The teratogenicity of ethanol was first described decades ago which is characterized by the ability of ethanol to induce malformations in the developing embryo or fetus (Lemoine *et al.,* 1968; Clarren and Smith, 1978). The earliest literature available on maternal alcohol consumption and adverse birth defects dates back to the period of Aristotle (Krous, 1981). It has also been mentioned in the Bible (Holy Bible, 1978). Later, it was mentioned in England in the 1700s where several physician groups described children of alcoholics as "weak, feeble, and distempered" (Royal College of Physicians, 1726) and "born weak and silly . . . shriveled and old". The first good description on adverse effects of alcohol on birth was by Sullivan in 1899 where he described the offspring of alcoholic women imprisoned in England (Calhoun, 2007). He concluded that these women produced children characterized by a pattern of birth defects of increasing severity and higher rates of miscarriage; there was a tendency for healthier infants to be born when gestation occurred in prison (thus indicating abstinence as prevention). These children were not normal, and male alcoholism was not a factor in producing the abnormalities. It was 70 years later that Lemoine of France in 1968 reintroduced the apparently ignored, unrecognized, or misunderstood concept of adverse outcomes resulting from fetal alcohol exposure. He studied more than 100 children of women who drank heavily and documented many of the physical and behavioral patterns among those children but did not present any definitive diagnostic criteria for diagnosing FAS or FASD. Later, in 1973, Jones, Smith and colleagues were the first to describe in detail the consistent pattern of malformations among children of mothers with significant prenatal alcohol intake and to provide diagnostic criteria for the condition they termed FAS (Jones & Smith, 1973).

The term Fetal Alcohol Spectrum Disorders (FASD) has been coined to describe all aspects of prenatal alcohol exposure. The National Organization on Fetal Alcohol Syndrome (NOFAS 2004) defined fetal alcohol spectrum

disorders as the range of effects that can occur in a person whose mother drank alcohol during pregnancy, including physical, mental, behavioral, and learning disabilities, with possible lifelong implications. It is an umbrella term which includes a wide range of effects of prenatal alcohol exposure that consists of Fetal Alcohol Syndrome (FAS), partial fetal alcohol syndrome (pFAS), Alcohol Related Birth Defects (ARBD), and Alcohol Related Neurodevelopmental Disorders (ARND) (Chudley *et al.,* 2005; Stratton *et al.*, 1996; Koren *et al.*, 2003; Streissguth *et al.,* 1991). The diagnostic criteria used to judge severity involve assessment of physical, cognitive, behavioral and learning abilities of the offspring.

Fetal alcohol syndrome is the most severe congenital malformation which is a consequence of alcohol exposure during pregnancy. Although the incidence of FAS varies among different countries, it is more common in indigenous communities (Harris *et al.,* 2003). The Cape Province communities of South Africa have the highest worldwide incidence of FAS, with an incidence of approximately 46 cases per 1000 live births (Peadon *et al.,* 2007). In USA, the incidence varies from 2.8 per 1,000 live births to as much as 9.8 per 1,000 live births in the Native American communities (Chiriboga, 2003; Peadon *et al.*, 2007). There is a trend towards an increase in the incidence and prevalence of FAS, according to the Birth Defects Monitoring Program of the Centers for Disease Control and Prevention, 1979-1992 (Cordero *et al.,* 1994). No prevalence data is available from Asian population.

Although the exact level of ethanol exposure required to result in FAS is not known but heavy drinking on regular basis or repeated binge drinking during gestation may result in FAS. Children born with FAS have characteristic pattern of facial dysmorphology which include flat nasal bridge, short palpebral fissure, smooth philtrum, thin upper lip and upturned nose followed by pre- and/or post-natal growth failure, and structural and/or functional abnormalities

of the CNS. Difficulties with learning and memory and other behavioral and emotional problems are the results of functional abnormalities of the CNS (Chiriboga, 2003, Stratton *et al.,* 1996). pFAS children share some of the facial features of FAS with confirmed prenatal alcohol exposure (Stratton *et al.,* 1996).

Ethanol exposure during pregnancy may result in a number of other congenital malformations without the signs of full blown fetal alcohol syndrome. Children with confirmed prenatal ethanol exposure and presence of one or more defects such as those of heart, bone, kidney, visual and auditory defects are diagnosed as ARBD (Stratton *et al.,* 1996). However, those children who are prenatally exposed to ethanol and do not display any visible malformation but have neurobehavioral disorder are diagnosed as ARND. ARND children have complex pattern of behavioral and cognitive deficits, impaired performance of complex tasks and higher level of language deficits (Peadon *et al.,* 2007; Stratton *et al.,* 1996)

Experimental Animal Models of Prenatal Ethanol Exposure

Animal models are useful in understanding the underlying mechanisms of ethanol's teratogenicity. Multiple contributing factors such as large differences in time the of exposure of ethanol during gestation and dosage of ethanol (West *et al.,* 1989; Astley *et al.,* 1999; Sulik, 2005), maternal nutritional status (Keen *et al.,* 2010), genetic background (McCarver *et al.,* 1997; Warren and Li, 2005) and simultaneous use of several drugs (Shor *et al.,* 2010) may result in large variety of clinical presentations of FASD in humans. So animal models are essential in the study of ethanol's effects on the developing fetus which allows for control over the dose, timing and duration of ethanol exposure and are not confounded by socioeconomic factors, genetic differences or multiple drug use that often complicate the interpretation of studies done in human beings.

Several animal models involving different animal species, ethanol dosage regimes and routes of administration and gestational timing of ethanol exposure have been utilized to explore ethanol teratogenicity. Many animal species have been used in assessing various effects of prenatal ethanol exposure, including mice, rats, guinea pigs, zebrafish, chick, sheep and nonhuman primates. Each species has its own strengths and limitations. Nonhuman primates, while most similar to humans in terms of development, are expensive and have a long gestation period, small number of fetuses per pregnancy and are associated with ethical concerns. So the uses of rodents are beneficial because of their low cost, short gestation period, large litters, and high fertility throughout the year under laboratory conditions. Experimental parameters can be easily controlled and manipulated and they are well characterized as a model for prenatal ethanol exposure (Riley and Meyer, 1984). Although rat fetuses are larger and thus provide a greater blood and tissue sample size with which to work but mice closely resemble humans in terms of genetic, biological and behavioral characteristics (Randall *et al.,* 1981). These species allow the study of complex behaviors and social development in offspring which proves to be advantageous when examining the developmental outcomes and behavioral consequences of chronic prenatal alcohol exposure.

Different investigators adopt different routes of administration of ethanol in rodents such as oral administration, intraperitoneal (i.p.) injection, inhalation, intravenous infusion and voluntary drinking. The ethanol dosage regimen depends on the experimental animal model due to biological differences between species. It can include ethanol administration acutely (1-2 doses) during critical periods of fetal development (Sulik *et al.,* 1981), during one particular period of gestation (Byrnes *et al.,* 2001; Simon *et al.,* 2008), throughout gestation in a binge-type model (Abdollah *et al.,* 1993; Maier *et al.,* 1996), or chronically via maternal consumption throughout gestation (Allan *et*

al., 2003; Kleiber *et al.,* 2011). Although each method has strengths and weaknesses, the ethanol dosage regimen and route of administration are dictated by the animal model, the targeted period of gestation, and the experimental questions to be addressed. The route of administration dictates the subsequent distribution of ethanol throughout maternal and fetal tissues (Clarke *et al.,* 1985). Since the oral administration of ethanol produces a pharmacokinetic profile similar to that in the pregnant human, oral route has been chosen in the present study.

Effects of ethanol on fetal development

The effects of ethanol on developing fetus are well known and depend upon time of exposure and the dose of ethanol exposed. Since each organ system has its own critical period of development, developing organ systems are affected differently at particular point of exposure.

Growth retardation

One of the characteristic and most consistent effects of prenatal ethanol exposure in humans and animal models of FASD is growth retardation, resulting in decreased body weight and length (Day *et al.,* 2002; Detering *et al.,* 1979; Hannigan *et al.,* 1993; Jones *et al.,* 1973; Keiver and Weinberg, 2004; Lee, 1987; Lochry *et al.,* 1980; Streissguth *et al.,* 1991). Growth retardation, regardless of the cause, is associated with both short-term morbidity and increased susceptibility to chronic disease in later life (Brodsky and Christou, 2004). Although significant effects on body weight tend to be associated with maternal ethanol intakes that result in BECs >100 mg/dL (Abel, 1996; Gallo and Weinberg, 1982, 1986; Savage *et al.,* 2002), the BECs required to decrease body length have not been well characterized. Studies in the rats and in mice suggest that significantly shorter body length occurs at high (Detering *et al.,* 1979; Keiver and Weinberg, 2004; Lee, 1987; Lee and Leichter, 1980; Leichter and Lee, 1979; Lochry *et al.,* 1980), but not at lower (27% ethanol-derived

calories (EDC) (Samson, 1981) levels of ethanol exposure. However, Lochry *et al.* (1980) reported a significant linear trend between body length at birth and dose of prenatal ethanol exposure (0, 12, 23 and 35% EDC), but individual group differences were not reported. In humans, prenatal ethanol exposure is also known to affect body weight and length in a dose-dependent manner, with effects being detected even at low levels of exposure (Day *et al.,* 2004).

In a retrospective analysis of the birth weights of rat litters, Hannigan *et al.* (1993) demonstrated that prenatal ethanol exposure consistently results in growth retardation in all pups in an exposed litter, as opposed to affecting only some pups in a litter. Moreover, children and animal models exposed to ethanol in utero do not experience catch up growth postnatally and so continue to remain smaller than non-exposed children (Day *et al.,* 2004; Geva *et al.,* 1993; Klug *et al.,* 2003; Leichter and Lee, 1979).

Musculoskeletal Development

There is increasing evidence that prenatal alcohol exposure contributes to the incidence of fetal bone malformations. One primary diagnostic feature of FAS is short stature, indicating defects in bone development in utero (Spohr *et al.,* 1993). Exposure to high levels of alcohol can result in permanent short stature and delayed mean bone age in children up to 14 years of age (Habbick *et al.,* 1998; Day *et al.,* 2002). Studies in rodents have identified reduced body length and length of individual bones, delayed ossification and decreased skeletal maturity in response to prenatal alcohol exposure as assessed by Alizarin red and alcian blue staining (Lee *et al.,* 1983; Keiver *et al.,* 1996, 1997). One area that has received very little research attention is the effects of alcohol on the development of muscle. Animal models of fetal alcohol exposure often report growth retardation in the form of reduced body weight, which could be a consequence of decreased muscular development. Rodent studies have shown reductions in weight and fibre content of the Tibialis

Anterior and Extensor Digitorum Longus muscles of alcohol-exposed rats (David *et al.,* 2005).

In many studies on ethanol's effects on skeletal development, ethanol was administered in the drinking water provided to the animals (Lee and Leichter 1980, 1983; Leichter and Lee, 1979; Ludeña *et al.,* 1983). As this method of administration can result in reduced maternal water and food intake, it was hypothesized that ethanol's effects on bone development might be due to dehydration and malnutrition. However, it has since been shown that although inadequate nutrition can exacerbate ethanol's effects, providing adequate maternal nutrition and hydration does not eliminate its effects on the developing skeleton. Prenatal ethanol exposure retards skeletal development in studies in which ethanol is administered to rats in a liquid diet (Weinberg *et al.,* 1990; Keiver *et al.,* 1996, 1997; Keiver and Weinberg, 2003), which does not result in dehydration and minimizes and controls for reduced maternal nutrient intake. Moreover, Weinberg and co-workers (1990) demonstrated that increasing maternal protein intake at above recommended levels could ameliorate some, but not all, of the effects of ethanol (36% EDC) on fetal skeletal ossification. The effects of prenatal ethanol exposure on postnatal skeletal development are also not likely to be due to decreased neonatal nutrition. Although maternal ethanol intake during gestation could affect neonatal nutrient intake through effects on milk quantity and quality during lactation, Lee and Leichter (1980) demonstrated no significant differences in bone size or skeletal maturity scores of pups raised in litters of three compared with litters of eight. Despite similar levels of maternal ethanol intake and methods of administration, there is variability among studies in the degree of severity of ethanol's effects on fetal skeletal development. Kelvin *et al.* (1996. 1997) found that 5 wk (2 wk before and 21 d throughout gestation) of maternal ethanol intake at 36% EDC resulted in much more severe effects on fetal skeletal development than 3 wk (21 d gestation only) at the same dose.

Respiratory Tract Development

The effects of alcohol exposure on the developing respiratory tract have not been well characterized. Children diagnosed with FAS are often prone to respiratory infections. Studies have demonstrated that macrophage activity within the lung is compromised following prenatal alcohol exposure, with the macrophages demonstrating a defect in phagocytosis of bacteria and an increased rate of apoptosis (Ping *et al.,* 2007). Wang *et al.* (2007) found that lungs of alcohol-exposed mice weighed less than control lung and histological examination showed that they were developmentally immature.

Cardiovascular Development

Cardiovascular malformations are commonly observed in children exposed to high concentrations of alcohol during fetal development (Daft *et al.,* 1986, Adickes *et al.,* 1990). The effects of alcohol on the developing cardiovascular system are dose and time (fetal stage) dependant. For example, Webster *et al.* (1984) found ventricular septal defects in the hearts of mice exposed to alcohol on days 8, 9 or 10 of gestation, but no defects were present in mice exposed to alcohol on day 7 of gestation. Turcotte *et al.* (2002) found that prenatal alcohol exposure in the rat did not result in any gross heart defects, but defects in the contractile responses of the thoracic aorta were observed. There is also evidence that the influence of alcohol on heart development is dependent on genetic background. In chicks, Cavieres *et al.* (2000) found that the pure bred strains were more prone to the effects of alcohol exposure than cross-breeds.

Urogenital Development

FAS patients often have small kidneys that have failed to rotate normally. This causes kinks in the ureter, urinary flow problems and resultant hydroureter (Qazi *et al.,* 1979; Havers *et al.,* 1980, Taylor *et al.,* 1994]. In sheep studies, prenatal alcohol exposure results in a transient reduction in urine

flow close to term (Clarke *et al.,* 1987). Rats prenatally exposed to alcohol display a urinary concentrating defect when challenged with high sodium or potassium diets and that histological examination demonstrated mitochondrial atrophy and tubular hypertrophy (Assadi *et al.,* 1991). Gallo *et al.* (1986) reported a reduction in renal protein and DNA content in alcohol-exposed rat offspring. Furthermore, a link has been made between light-to-moderate alcohol exposure during development and an increase in the risk of renal anomalies among offspring (Moore *et al.,* 1996).

Development of the Nervous System

The developing nervous system is considered to be one of the most vulnerable systems to the effects of alcohol. Because of this, the effects of alcohol on the developing nervous system have been well studied. Studies of children exposed to alcohol in utero have identified a multitude of effects ranging from neuroanatomical malformations (Clarren *et al.,* 1978, Peiffer *et al.,* 1979), to cognitive dysfunction (Coles *et al.,* 1991), to behavioral disorders (Coles *et al.,* 1985). Animal studies have found that different regions of the developing brain are differentially affected by alcohol in a time- and dose-dependent manner. Young *et al.* (2006) found that raising the maternal BAC to 50mg/dl was enough to cause neuroapoptosis in the infant mouse brain without causing a reduction in brain weight. Maier *et al.* (1998) found that a BAC of 0.2g/dl in the mouse resulted in growth differences in the different regions of the brain with the most significant reduction occurring within the cerebellum. In a follow-up study it was seen that the reduction in cerebellar weight was associated with reductions in Purkinje and granule cell numbers. Reductions in DNA and protein content have also been observed in those brain regions most affected by fetal alcohol exposure (Miller, 1996).

Placental development

The placenta is a unique, autonomous and transient organ which is the place of exchange of molecules between fetal and maternal blood (Akay and Kockaya, 2005). For normal fetal growth and development, an adequate supply of essential nutrients such as amino acids, glucose, trace elements, vitamins, and oxygen is necessary. The placenta is responsible for the transfer of these nutrients to the fetus. Thus, any factor that disrupts placental structure and function could adversely affect the nutritional status of the fetus. Toxic and/or foreign compounds may interfere with placental function at many levels, (Foster *et al.,* 2008) including signaling, production and release of hormones and enzymes, transport of nutrients and waste products, implantation, cellular growth and maturation and finally in the delivery and any deviation from normal development may constitute a potential threat to placental function, resulting in preterm delivery, congenital malformation or abortion (Myllynen *et al.,* 2005).

It has been demonstrated that ethanol exposure induces oxidative stress in the human placental villi (Kay *et al.,* 2000). This may account for the decreased nitric oxide (NO) release because NO may be shunted towards scavenging free radicals (Dotsch *et al.,* 2001). Decreased NO availability could adversely affect placental blood flow regulation, which could, in turn, account for the restriction of growth observed in ethanol-exposed fetuses. Gundogan *et al.* (2010) have demonstrated the impact of ethanol-mediated oxidative stress on placental trophoblast function and its potential impact on pregnancy loss. They found that rat chronically exposed to ethanol during gestation showed increased placental apoptosis/necrosis. Inhibition of prolactin family of hormones by ethanol was found to be the mediator for adverse pregnancy outcome (Soares, 2004).

IUGR is a key feature of FAS and recently Gundogan *et al.* (2008, 2010) have demonstrated in the rat that chronic gestational exposure to ethanol causes

increased fetal resorptions as well as impairment in placental development and placentation. Since ethanol in maternal blood reaches the fetus and/or the placenta, it directly or indirectly mediates the toxic effects on the fetus. The direct effect of fetal exposure to ethanol was demonstrated by Chu *et al.* (2007) on rat fetal brain development and the indirect effect is related to placental pathology, especially within the rat placental barrier. The ischemia or infarction observed in ethanol-exposed placentas reduced the thickness of the organ by an increase in cellular necrosis. Since the exchange of nutrients between the mother and the fetus occurs within the placental barrier, ethanol-induced reductions in the mass of this layer could impair the delivery of nutrients to the rat fetus and thereby resulting in IUGR.

According to Gundogan *et al.* (2010) a second major placental abnormality associated with chronic gestational exposure to ethanol was failure of maternal uterine spiral arteries to remodeling by extra villous tree. This compromises both placental blood flow as well as the nutrients exchange (Pijnenborg *et al.,* 1981). Therefore, the motile and invasive properties of extra villous tree are critical for the establishment and maintenance of pregnancy, and ensuring adequate blood and nutrient delivery to the fetus in order to support growth and development (Gundogan *et al.,* 2008).

Liver development

The liver is also affected in FAS and the characteristic deformities observed are similar to those evidenced in alcoholic liver disease in adult. The more commonly seen features include hepatomegaly and raised levels of serum transaminases. Light microscopy revealed increased parenchymal fat with portal and perisinusoidal spaces containing deposits of intermediated and large size collagen fibres, myo-fibroblast and occasional Ito cells, as well as subendothelial basement membrane-like material (Lefkowitch *et al.,* 1983). The presence of thick sclerotic central veins in the hepatic lobule has also been

evidenced, in conjunction with the occurrence of extrahepatic biliary atresia (Daft *et al.,* 1986). Furthermore, Renaul-Piqueras *et al.* (1997) demonstrated that prenatal exposure to ethanol affects the morphological, structural and functional features of the Golgi apparatus, thus altering the glycosylation process in foetal hepatocytes, causing finally an accumulation of hepatic proteins. Additionally, Fofana *et al.* (2010) demonstrated that prenatal alcohol exposure alters protein phosphorylation in rat off-spring liver and that the principal pathway affected by these protein alterations includes cell signalling, cellular stress, and protein synthesis, as well as glucose, aminoacids, adenosine and energy metabolism.

Neurobehavioral impairments

Children who are prenatally exposed to ethanol are known to have developmental disabilities that manifest in the form of delayed growth, deficits in memory and learning, and deficits in motor coordination. These disabilities have long-term consequences in the form of limiting education and influencing employment prospects. Prenatal exposure to ethanol can alter brain structure and organization on a variety of levels due to which it impairs cognitive and behavioral functions. The ethanol neurobehavioral teratogenic effects can be present without any sign of craniofacial dysmorphology (Girard *et al.,* 2000).

The behavior data generated with rats are consistent with clinical observations of behavioral disturbances in children born to women who consumed alcohol during pregnancy (e.g., Streissguth *et al.,* 1984). However, the rat appears to be less sensitive to the "classic" teratologic effects of alcohol, except at doses that interfere with adequate nutrition (Fernandez *et al.,* 1983). The mouse, on the other hand, clearly demonstrates a dose-related sensitivity to the teratogenic actions of ethanol on major organ systems (Randall *et al.,* 1981). Moreover, the pattern of morphological defects observed in mice resembles those seen in clinical cases of the Fetal Alcohol Syndrome.

Unfortunately, only a paucity of literature exists on the behavioral teratology of ethanol in this species (Wainwright *et al.,* 1985; Randall *et al.,* 1986; Gilliam *et al.,* 1987). If deleterious behavioral effects similar to those reported in humans can be demonstrated after prenatal ethanol exposure in mice, the mouse model has the potential of becoming an invaluable tool for alcohol-induced teratology research. So the mice model has been chosen in the present study.

In a variety of animal models of ethanol teratogenicity, including the mice, learning and memory deficits are very robust and reproducible consequences of chronic prenatal ethanol exposure (Blanchard *et al.,* 1987; Abdollah *et al.,* 1993; Gibson *et al.,* 2000; Spear-Smith *et al.,* 2000; Nash *et al.,* 2007; Thomas *et al.,* 2010). The effects of ethanol on learning and memory on the exposed offspring can be measured using a variety of testing paradigms such as the Morris water maze, delay fear conditioning, trace fear conditioning and the radial arm maze task. Among these the Morris water maze test (Morris, 1981) is the most popular one. The Morris water maze task involves placing the animal into a pool of opaque water containing an escape platform hidden below the water surface (Morris, 1981). The animal uses visual cues, such as colored shapes placed on the testing-room walls around the pool, to orient itself and find the escape platform. The time taken to locate the escape platform is recorded, and a decrease in escape latency is interpreted as an improvement in learning and memory. The task appears to require spatial mapping based upon localization of extra-maze cues (Morris, 1981). Certain "probe" measures taken with the platform removed (e.g., percent time spent near the platform location, angle of first approach) help discriminate spatial learning and memory performance (i.e., knowing where the platform is) from some motor responses (e.g., learning a swim pattern that leads to escape). Hippocampal damage substantially impairs rodent's ability to learn platform location (Morris *et al.,* 1982). Studies identifying spatial learning deficits in a Morris maze by young rats (PND20–PND30) after perinatal (i.e., prenatal or neonatal) alcohol

exposure, and thereby suggesting hippocampal dysfunction, were first published in 1987 (Blanchard *et al.,* 1987; Goodlett *et al.,* 1987). In one study, young Long-Evans rats born to dams fed 35% EDC liquid diets took longer paths to reach the escape platform in the Morris maze than controls. The prenatal alcohol exposed females tended to perform more poorly than the males over the 4 days of acquisition training. Probe trials revealed specific spatial rather than general response deficits, and here males tended to be more affected on spatial learning than females (Blanchard *et al.*, 1987). Goodlett *et al*. (1987) tested juvenile Sprague-Dawley rats after early neonatal alcohol exposure on postnatal days 4–10 (PND4–10). The ‘‘pup-in-the-cup’’ artificial rearing procedure was used to generate high peak BACs (i.e., binge exposures) during the period of postnatal brain growth spurt. In a detailed analysis of spatial learning and memory over 12 days of testing, both male and female alcohol-exposed pups had significantly longer escape latencies and traveled more circuitous paths in the Morris maze than controls. Analyses of probe trials without a platform confirmed specific deficits in spatial learning (Goodlett *et al.,* 1987). Similar spatial deficits in juvenile rats were reported following direct intubations of alcohol to neonates from PND7–9, without the complications of the artificial rearing method (Goodlett and Johnson, 1997). Matthews and Simon (1998) modified the Morris task by increasing the delay between training and testing from 1 to 3 days. They found that the offspring of Long-Evans rats intubated with 3 g/kg/day ethanol during gestation showed significantly increased escape latencies as adults. Kim *et al.* (1997) compared the effects of prenatal alcohol exposure via a 36% EDC diet on non spatial and spatial learning in male Sprague-Dawley rats. There were no deficits on a non spatial delayed non matching to-sample object recognition task. However, the same rats showed increased latencies in Morris maze acquisition, demonstrating the specificity of spatial deficits. Several studies have demonstrated the effects of chronic prenatal ethanol exposure on learning and

memory, which show impaired task acquisition or inability to learn the task. chronic prenatal ethanol exposure delays development of spontaneous alternation behavior in adult rat offspring (Thomas *et al.,* 2010), which has been associated with performance deficits in the radial arm maze task (Omoto *et al.,* 1993; Reyes *et al.,* 1989), T-maze task (Lochry *et al.,* 1985; Nagahara and Handa, 1997; Zimmerberg *et al.,* 1991), and spatial lever response task (Zimmerberg *et al.,* 1989). Chronic prenatal ethanol exposed offspring show deficits in stationary-platform and moving-platform versions of the Morris water maze task (Blanchard *et al.,* 1987; Gianoulakis, 1990; Kim *et al.,* 1997; Richardson *et al.,* 2002; Christie *et al.,* 2005;Nash *et al.,* 2007; Wang *et al.,* 2009; Thomas *et al.,* 2010).

In children with FASD and animal models of ethanol teratogenicity, prenatal exposure to ethanol can increase anxiety-like behavior in postnatal life (Ogilvie and Rivier, 1997; Dursun *et al.,* 2006). In animal models, the behavioral manifestations of this increased anxiety have been shown as less time spent in the open arm of the elevated plus maze (Vaglenova *et al.,* 2008; Brocardo *et al.,* 2012), and less exploratory behavior in an open field test novel environment (Zhou *et al.,* 2010). The elevated plus maze (EPM) is used to measure anxiety and consists of two open arms (consisting of floor only) and two enclosed arms (consisting of a floor and side walls), with an open roof, arranged in such a way that the two open arms were opposite to each other (Pellow *et al.,* 1985). The maze is elevated off the ground. Chronic prenatal ethanol exposed offspring exhibit an altered HPA set point (Rivier, 1996), hyperresponsive HPA axis and altered corticosterone response following a stressful stimulus (Lee and Rivier, 1996; Taylor *et al.,* 1982, 1986, 1984; Weinberg, 1988, 1992; Weinberg *et al.,* 1996; Glavas *et al.,* 2007), which may explain, in part, the increased anxiety-like behaviors. The EPM is a reliable and validated task used to measure anxiety/fear in rodents, as indicated by behavioral, physiological and pharmacological responses (Lister, 1987; Pellow

et al., 1985; Pellow and File, 1986). Animals confined to the open arms of the EPM show elevated corticosterone levels (Pellow *et al.*, 1985), and measures such as number of open arm entries and time spent in the open arms are inversely related to anxiety (Cruz *et al.*, 1994), as rodents have an innate fear of open spaces. Locomotor activity can also be quantified by total distance traveled and total number of arm entries. This latter measure is independent of anxiety as sedative treatments reduce total arm entries without changing time spent on the open arms (Pellow and File, 1986).

Both in humans and in rodents, one of the most characteristic effects of perinatal alcohol intoxication is locomotor activity. Locomotor hyperactivity has been consistently reported in children and in preweaning and juvenile rats after perinatal exposure to ethanol (Abel, 1982; Abel and Reddy, 1997; Bondand Di Giusto, 1977;Leonard, 1988; Mattson *et al.*, 2001; Tran *et al.*, 2000). However, studies on adult rats (2 months and older), brought controversial results. Some authors reported increased locomotor activity in prenatally alcohol-exposed rats at ages varying between 2 and 18 months (Osborn *et al.*, 1980; Abel and Berman, 1994). In many other studies, however, hyperactivity has not been observed in adult fetal-alcohol rats (Abel and Berman, 1994; Bond and Di Gusto, 1977; Randall and Hannigan, 1999; Westergren *et al.*, 1996).The open field (OF) is utilized as a test of exploratory locomotor behavior and emotionality (Archer, 1973; Prut and Belzung, 2003; Walsh and Cummins, 1976). Animals are placed in a novel arena for 5–15 min/day for 1 day or several consecutive days and time in the center/periphery, as well as overall locomotor activity, are recorded. Ambulation (squares crossed) is typically used as a measure of exploratory behavior, although locomotor hyperactivity is thought to reflect neophobia. By contrast, increased time in periphery or avoidance of the center of the field reflects anxiety/emotionality. Importantly, anxious or neophobic behavior is reversible with most anxiolytics that decrease anxiety in humans (Prut and Belzung,

2003). In the present study, animals were exposed to a small, dimly lit open field for 5 min over three consecutive days.

Depression is a highly prevalent, chronic, recurring, and potentially life-threatening mental illness (Nestler *et al.,* 2002, Berton and Nestler 2006), and prenatal ethanol exposure is associated with an increased risk of depression in offspring (Forrest *et al.,* 1992, Larkby and Day 1997, Mancinelli *et al.,* 2007, Hellemans *et al.,* 2010). Both mouse and rat offspring, whose mothers had consumed moderate quantities of ethanol throughout gestation, demonstrated dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis which is common in depression and is primarily reflected by increased HPA tone and activity (Bale and Vale 2004, Hellemans *et al.,* 2010). Experimental paradigms, such as learned helplessness, the forced swim test or behavior despair test and tail suspension test induced behaviors modeling depression in laboratory animals. The forced swim test (FST) is the most widely used preclinical test of antidepressant action (Cryan *et al.,* 2005; Porsolt *et al.,* 1978; Porsolt, 1979). Animals are placed in a cylindrical tank of water (deep enough so the tail cannot touch the bottom) for 15 min on day 1 and re-exposed for 5 min on day 2, and the time spent 'immobile' (minimal movements to keep the head above water) is analyzed. As antidepressants reverse immobility (Cryan *et al.,* 2005), time immobile is interpreted as reflecting depressive-like behavior, or 'behavioral despair'; i.e., animals ''give up'' and stop swimming or trying to escape. The tail suspension test is a derivative of the forced swimming test and is based on the fact that a mouse suspended by the tail alternates periods of agitation and immobility similar (but not identical) to that observed in the FST (Steru L, 1985). Mice are suspended by the tail, using an adhesive scotch tape, to a hook connected to a strain gauge that picks up all movements of the mouse and transmits them to a central unit which calculates the total duration of immobility during a 6 min test (Porsolt *et al.,* 1978). The TST presents some

advantages over the FST in allowing an objective measure of immobility and does not induce hypothermia by immersion in water (Ripoll *et al.,* 2003).

Maternal consumption of ethanol is known to be teratogenic, and the mechanism of ethanol neurobehavioural teratogenicity is multifaceted and remains to be fully elucidated (Abel and Hannigan, 1995; Goodlett *et al.*, 2005; Guerri *et al.,* 2009; Kimura *et al.,* 2000; Riley and McGee, 2005; West *et al.*, 1994). There is substantive evidence suggesting that the HPA axis plays a major role in some cellular and behavioural consequences of chronic prenatal ethanol exposure (Weinberg *et al.,* 2008). Other researchers suggest that oxidative stress plays a major role in the cellular demise and neuronal loss that contributes, in part, to ethanol neurobehavioural teratogenicity (Brocardo *et al.,* 2012) and that maternal supplementation with folic acid and other antioxidants may mitigate some of these effects of oxidative stress (Cohen-Kerem and Koren, 2003). The effects of low-to-moderate-dose ethanol exposure on the developing fetus and offspring have similar but, arguably, more subtle neurobehavioural effects compared with high-dose chronic prenatal ethanol exposure (Savage *et al.,* 2002). The present study uses mice model to elucidate the role of folic acid in ethanol neurobehavioral teratogenicity during the organogenesis.

Ethanol metabolism and oxidative stress

Ethanol consumption is associated with an increase in the abundance of oxygen free radicals, resulting in increased oxidative stress within the cell. Alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) are two enzymes that are primarily responsible for the oxidative metabolism of ethanol, resulting in the production of acetaldehyde (Lieber, 2004; Wu *et al.,* 2006). Oxidative stress, resulting from an imbalance between the formation and degradation of ROS, appears to play a role in ethanol-induced toxicity in a number of organ systems, including the liver and brain (Morimoto *et al.,* 1994;

Montoliu *et al.*, 1995; Mari and Cederbaum, 2001). Ethanol can increase ROS by various mechanisms, including changes in intracellular redox state (decrease in the NAD+/NADH ratio during ADH-catalyzed oxidation of ethanol), production of acetaldehyde, damage to mitochondria, direct effects on membranes caused by hydrophobic ethanol interactions, induction of CYP2E1, mobilization of iron, effects on antioxidant enzymes and biochemicals, particularly mitochondrial and cytosolic glutathione (GSH),and one-electron oxidation of ethanol to the reactive,1-hydroxyethyl radical (Cederbaum, 2001). There is interplay of these different mechanisms, and it is likely that several of them contribute to the ability of ethanol to induce a state of oxidative stress (Jimenez-Lopez and Cederbaum, 2005). Direct mechanisms of ethanol-induced oxidative stress involve mitochondrial-respiration-dependent production of free radicals, including hydroxyl and hydroxyethyl radicals, which react with various cellular components, and the indirect mechanisms include decreased intracellular antioxidant capacity, such as GSH peroxidase (Cohen-Kerem and Koren, 2003). Chronic ethanol exposure can induce CYP2E1 activity in microsomal and mitochondrial subcellular fractions of adult liver, which also increases ROS production (Morimoto *et al.*, 1994; Mari and Cederbaum, 2001; Robin *et al.*, 2005; Bai and Cederbaum, 2006). The hydroxyl and hydroxyethyl radicals react with lipids, DNA and proteins resulting in the formation of peroxyl radicals. The degradation of these macromolecules due to increased oxidative stress results in the apoptosis of cell (Green *et al.*, 2005; Jacobson, 1996; Ramachandran *et al.*,, 2001; Rathinam *et al.*, 2006; Watts *et al.*, 2005).

The developing fetus is particularly susceptible to the effects of oxidative stress. This is because the fetus's ability to eliminate reactive oxygen species through the action of antioxidants is lower than the adult. Oxidative stress, resulting from the oxidative metabolism of ethanol and formation of ROS, is a potential mechanism of chronic ethanol exposure induced injury in the developing fetus (Ferreira and Willoughby, 2008; Koop, 2006; Cohen-

Kerem and Koren, 2003). The fetus lacks the ability, both enzymatic (catalase, superoxide dismutase, GSH peroxidase, and GSH-S-transferase) and non-enzymatic (GSH, vitamins A, C and E) antioxidants, to efficiently detoxify ROS (Henderson *et al.,* 1999), thereby making the fetus more vulnerable to oxidative damage than the adult (Gerdin *et al.,* 1985; Mariucci *et al.,* 1990, Devi *et al.,* 1996). In the human, hepatic CYP2E1 and other CYPs are expressed in the liver and placenta (Hakkola *et al.,* 1998) during early fetal development, which indicates that fetal biotransformation of maternally ingested xenobiotics that distribute across the placenta, including ethanol, may play a role in their teratogenicity via the formation of toxicologically active metabolites (Hakkola *et al.,* 1998). Addolorato *et al.* (1997) reported that offspring of rats fed low levels of alcohol during pregnancy had reduced growth and antioxidant activity in their livers while their mothers were unaffected. It has also been reported that rats exposed to alcohol during gestation and the lactation period had higher activity levels of oxidative damage in their livers and pancreas. (Cano *et al.,* 2001). It has been proposed that an increased cellular oxidative state causes an increase in DNA damage in highly proliferative embryonic tissue leading to programmed cell death, thereby reducing the number of cells within susceptible embryonic tissues. This proposal was supported by the findings of Mitchell *et al.* (1998) who cultured rat hippocampal cells in the presence of alcohol. This resulted in increased neuronal apoptosis which was ameliorated by the addition of the antioxidant vitamin E or β-carotene (Mitchell *et al.,* 1998). Based on these and similar findings it has been hypothesized that the reduction in brain weight and growth of children diagnosed with FAS, may in part be the result of increased neuronal apoptosis due to the highly oxidative state. Similarly, mice embryos co-treated with ethanol and the antioxidant enzyme, superoxide dismutase, showed diminished superoxide radical anion generation, lipid peroxidation, cell death, and dysmorphogenesis than embryos treated with ethanol alone (Kotch *et al.,*

1995). These data support the role of ROS in ethanol teratogenicity (Kotch *et al.,* 1995). Dysfunction of mitochondria has long been implicated in cellular demise, as these organelles are responsible for the generation and release of ROS in the cell (Kroemer *et al.*, 1997; Hirsch *et al.*, 1997). In addition to the direct effects of ROS on macromolecules, the electron-transport-chain complexes are quite sensitive, and their function can be compromised following exposure to ROS, leading to decreased mitochondrial energy production, further increased ROS and, ultimately, ROS-induced cell death (Devi *et al.*, 1994; Lemasters and Nieminen, 1997).

Folic acid

Folic acid or vitamin B9 is a synthetic form of folate (biologically active). It is essential for human growth and development (Beaudin and Stover, 2007). It is an essential coenzyme in one-carbon transfer reactions which is required for the synthesis of thymidylate and purines (Ulrich *et al.,* 2010; Zeisel, 2009). It is necessary for the production and maintenance of new cells, for DNA synthesis and RNA synthesis, and for preventing DNA mutation (Kamen, 1997).

Dihydrofolate reductase (DHFR) is responsible for reducing folic acid to folate and folate to dihydrofolate (DHF) which is subsequently reduced to tetrahydrofolate (THF), the active form of folic acid. THF is needed for the synthesis of both purines and pyrimidines, which are the building blocks for nucleic acids. Methylene-THF is formed from THF by serine hydroxymethyltransferase, which adds a methylene group from one-carbon donors (e.g., formaldehyde, serine, or glycine). Methyl-THF (MTHF) can be formed following the reduction of methylene-THF by methylene tetrahydrofolate reductase (MTHFR). In red blood cells, MTHF accounts for 40-50% of the total folate polyglutamates, which are transported across cellular membranes and are used in one-carbon metabolism (Shane, 1989; Stanger and

Wonisch, 2012). Methyl-THF is responsible for donating a methyl group to homocysteine, thereby forming methionine. Methionine is an important amino acid that can be converted to S-adenosylmethionine (SAM) which is a methylating agent involved in the methylation of DNA, RNA, proteins, phospholipids, and neurotransmitters (Kruschwitz *et al.*, 1994; Locksmith and Duff, 1998; Hall and Solehdin, 1998; Clarke and Banfield, 2001). Appropriate control of methylation status of DNA is required for normal embryonic development (Li *et al.*, 1992).

Folic acid and pregnancy

Pregnancy is a period of rapid growth due to which the requirements for many micronutrients, including folate, are increased (Baker *et al.,* 1981). Folate deficiency in pregnancy has been associated with increased adverse reproductive outcomes, including neural tube defects (NTD), preterm delivery, low birth weight and restricted growth (Whiteside *et al.,* 1968; Martin *et al.,* 1967; Tchernia *et al.,* 1982; Malinow *et al.,* 1998). Maternal intake of folic acid during the periconceptual period has been shown to decrease the occurrence of NTD (Czeizel and Dudas, 1992; Medical Research Council, 1991). Folic acid has been added to a variety of foods, which has decreased the occurrence of NTD and other complications of folate deficiency in pregnancy (Centers for Disease Control and Prevention, 2004; Berry *et al.,* 2000). Supplementation with folic acid has also been shown to reduce the risk of congenital heart defects, cleft lips (Wilcox *et al.,* 2007), limb defects and urinary tract anomalies (Goh and Koren, 2008).

The recommended daily dose of folic acid varies with age and pregnancy status, but the folate recommended dietary allowance (RDA) for the non-pregnant adult is 400 µg/day (Institute of Medicine and Committee, 1998). The RDA increases to 600 µg/day for a pregnant woman due to the increased demands of the fetus. During pregnancy, the nutritional demands of the

developing fetus take priority at the expense of the mother. Folate is actively transported by folate receptor α and reduced folate carrier (RFC) across the placenta from the mother to the fetus, as is seen by higher folate concentration in cord blood compared with maternal blood (Ek, 1980; Yasuda *et al.*, 2008). However, higher folate concentration in cord blood does not occur when maternal folate stores (primarily in the liver) are already decreased (Blocker *et al.*, 1989). Due to increased demand of folate by the fetus, maternal folate stores tend to decrease throughout pregnancy. Other factors that decrease maternal folate stores during pregnancy are increased blood volume, increased folate catabolism and renal clearance, decreased folate absorption and, in some cases, inadequate folate intake might also be the cause (Chanarin, 1969; Tamura and Picciano, 2006).

Several investigations reported that folic acid supplementation is needed to prevent detrimental effects, including NTD, in the developing fetus, as most pregnant women are not obtaining sufficient amount of folate from their diet. While some investigators suggested that daily intake of 4-5 mg folic acid would provide greater benefit (Centers for Disease Control and Prevention, 1991; Bar-Oz *et al.*, 2008; Goh and Koren, 2008), other researchers suggested that there is no added benefit to the fetus with the higher folic acid daily dose (Daly *et al.*, 1995). Furthermore, rigorous study has yet to prove that the increased plasma folate concentration is safe. A recent study in mice has shown that a 10-fold increase in maternal plasma folate concentration can produce toxicity in the developing mouse embryo, including embryonic developmental delay and growth restriction (Pickell *et al.*, 2011). In the human, maternal red blood cell (RBC) folate concentration of 0.9 μM is associated with decreased incidence of NTD (Daly *et al.*, 1995) and has been proposed for optimal fetal development and postnatal outcomes (Goh and Koren, 2008).

Effects of ethanol on folate level

Chronic alcohol exposure decreases RBC and serum folate level, as ethanol has a negative impact on folate absorption, distribution and metabolism in the body and increased urinary excretion (Wu *et al.,* 1975; Glória *et al.,* 1997; Halsted *et al.,* 2002; Hamid *et al.,* 2007b; Tamura and Halsted, 1983; Muldoon and McMartin, 1994). The decrease in folate status observed in the alcoholics is likely due to a combination of inadequate nutrition, decreased folate absorption, via the RFC (reduced folate carrier) in the intestine, and altered distribution primarily involving the liver (Hoyumpa, 1986; Villanueva *et al.,* 2001; Hamid *et al.*, 2007a). Animal and in vitro studies have shown that ethanol may impair transport of folic acid across placenta by decreasing expression of folate transport proteins (Hutson *et al.,* 2012) which may alter the folate status in the maternal-fetal unit.

Maternal health is crucial for normal fetal development and micronutrient status in the maternal-fetal unit is of particular importance, as micronutrient deficiencies prior to and throughout pregnancy are associated with poor prenatal and postnatal outcomes (Doyle and Rees, 2001; Rees *et al.,* 2005a; Rees *et al.,* 2005b). In the mouse, ethanol-induced folate deficiency in the fetus decreased the function of various cellular proteins such as serine/threonine protein phosphatase, COP9 signalosome complex and nucleoside diphosphate kinase B, which are essential for CNS development; this effect was reversed by maternal folic acid supplementation (Xu *et al.,* 2008). Furthermore, folic acid deficiency enhanced the teratogenic effects of ethanol in pregnant mice (Gutierrez *et al.,* 2007) and also impairs the motor and cognitive behaviors in the litters (Ferguson *et al.,* 2005).

Consumption of ethanol during pregnancy does not affect only the disposition of folate in the maternal-fetal unit by decreasing the intestinal absorption of folic acid in the mother, but it can also induce oxidative stress as discussed previously. Ethanol increases oxidative damage in several maternal

and fetal tissues, including the fetal brain (Davis *et al.*, 1990; Henderson *et al.*, 1995; Kotch *et al.*, 1995; Heaton *et al.*, 2002; Shirpoor *et al.*, 2009). In the rat, ethanol increases GSH reductase in the mother and offspring, as well as lipid peroxidation and amount of carbonyl groups of proteins in the liver and pancreas of the offspring (Cano *et al.*, 2001). This ethanol-induced oxidative stress was mitigated by concurrent folic acid supplementation throughout pregnancy, potentially due to the ability of folate to scavenge free radicals (Cano *et al.*, 2001; Joshi *et al.*, 2001).

The effects of ROS and oxidative stress on placental, embryonic and fetal development may adversely alter the development by causing oxidative damage on the cellular lipids, proteins and DNA, and/or by altering signal transduction which can adversely alter the cellular function or trigger apoptotic or necrotic cellular death (Wells *et al.*, 2009). Folic acid is required for methylation reactions involving DNA, RNA, proteins and neurotransmitters (Urlich *et al.*, 2010; Balion and Kapur, 2011; Zhu, 2002; Selhub, 2002). Ethanol ingestion during pregnancy could decrease folate status in the maternal-fetal unit which may contribute, at least in part, to ethanol teratogenicity. Maternal supplementation with folic acid could mitigate ethanol induced folate depletion in the maternal-fetal unit and may act as an antioxidant and counteract oxidative stress induced by chronic ethanol exposure.

Chapter-4

Material and Methods

The present study was done in the Department of Anatomy in collaboration with the Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi. Prior approval of Animal Ethical Committee, Banaras Hindu University was taken before the start of the study.

Animals

In the present study female Swiss albino mice weighing approximately 27g (±2g) at the age of 3 months were used. The mice were housed in animal house of the Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University in a temperature-controlled environment with a 12h light/12h dark cycle. They were permitted access to standard animal feed and tap water *ad libitum*.

Determination of pregnancy

The male and female mice were mated in the ratio of 1:2. The next day, presence of a vaginal plug was considered as gestation day 0 (GD 0). Plug positive dams were housed individually in polypropylene cages in the same laboratory conditions. In case of doubt, the plug was examined microscopically for the presence of sperms. Each sperm positive mouse was housed individually in separate cages under similar laboratory conditions with access to water and food *ad libitum*.

Experimental design and drug treatment

On GD 6 the pregnant mice were randomly assigned to Control group (Group I, n=20), Alcohol group (Group II, n=20), Alcohol and Folic acid group (Group III, n=20) and Folic acid group (Group IV, n=20).

a. Preparation of alcohol solution (25% v/v)

Twenty five milliliter of absolute alcohol (Merck) was taken in a measuring cylinder and final volume was made to 100 ml with normal saline. It was stored in screw capped bottle till use.

b. Preparation of folic acid solution (0.02% w/v)

Hundred milligram of folic acid (Hi-Media) was taken and mixed in 25 ml of distilled water. After the folic acid was completely dissolved, the final volume was made to 50 ml with distilled water. The folic acid solution was kept in a dark screw capped bottle. It was stored in refrigerator at 4° C till use. The folic acid solution was freshly prepared every week.

c. Calculation of dose of the drug

A pilot study was carried out for the determination of teratogenic dose of alcohol in Swiss albino mice. Four groups of plug positive female mice with two mice in each group were given alcohol at the dose of 3 gm/kg, 5 gm/kg, 6 gm/kg and 7 gm/kg body weight of mice, respectively from GD 6 to GD 15 to cover organogenesis. The teratogenic dose of alcohol in the present strain of mice was found to be 6 gm/kg as 3 gm/kg and 5 mg/kg didn't produce any congenital malformation in the progeny while 7 gm/kg dose resulted in maternal death.

Similarly, to determine the protective dose of folic acid, four groups of plug- positive female mice with two mice in each group were given alcohol 6 gm/kg body weight of mice plus folic acid at the doses of 5 mg/kg, 30 mg/kg, 60 mg/kg and 80 mg/kg body weight of mice, respectively from GD 6 to GD

15. The lowest protective dose of folic acid was found to be 60 mg/kg and this dose of folic acid was chosen for further study.

d. Drug treatment

Group II mice were given alcohol 6 gm/kg (0.03 ml of 25% alcohol/gm) body weight of mice from GD 6 to GD 15 through oral gavage needle at 9 a.m. Mice in group III received alcohol 6 gm/kg and folic acid 60 mg/kg body weight on the same gestational days. The folic acid was administered 2 h after alcohol treatment. Group IV mice received folic acid 60 mg/kg body weight only. Group I mice were termed as control and were given equal volume of normal saline. The weight of the pregnant dams was measured daily. Any mouse whose weight didn't increase till GD 10 was sacrificed on the same day to observe for pseudo pregnancy or total resorptions of the fetuses and those mice were excluded from the study.

Maternal and fetal assessments

On GD 18, 12 pregnant dams of each group were weighed, blood was collected from retro-orbital sinus for biochemical investigations and at last sacrificed by cervical dislocation. The abdomen of the mouse was dissected. The uterine horns were cut open and carefully inspected for all implantations. The number of live, dead and resorbed fetuses was counted. The dead and resorbed fetuses were excluded from further study. Live fetuses were extracted from the uterus, examined for the presence of external malformations and their weight, crown-rump length and tail length was recorded. Two fetuses from each litter were kept in 70% alcohol for bone staining, heads of two fetuses were kept in 0.02M phosphate buffer saline (pH:7.4) for biochemical investigations and remaining fetuses were preserved in 10% neutral buffered formalin for soft tissue examination. The placenta was washed with tap water, blotted dried, weighed and thickness & diameter were measured with the help

of a Vernier Caliper and preserved in 10% neutral buffered formalin for histological study.

The brain, liver and kidney of the fetuses were dissected out, weighed and kept in 10% neutral formalin for histological study. The relative brain weight was calculated as :

Relative brain weight = (Weight of brain/Weight of fetus) x 100

a. Preparation of 0.02M phosphate buffer saline (pH 7.4)

Stock solution (1) was made by dissolving 28.40 gm of sodium phosphate dibasic (Na_2HPO_4) anhydrous and 85 gm of sodium chloride (NaCl) in 500 ml of distilled water and final volume was made to 1000 ml with distilled water.

Stock solution (2) was made by dissolving 27.60 gm of sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$) and 85 gm of sodium chloride (NaCl) in 500 ml distilled water and final volume was made to 1000 ml with distilled water.

Five hundred milliliter of 10 times diluted solution (1) and 100 ml of 10 times diluted solution (2) was taken in separate beakers. Using pH meter, diluted solution (1) was titrated to pH 7.3-7.4 by adding about 65 ml of diluted solution (2). The resulting 0.02M phosphate buffer saline solution (pH 7.4) was divided into aliquots, filter sterilized and stored at room temperature.

b. Preparation of 10% neutral buffered formalin

3.25 gm Sodium Phosphate, dibasic (Na2HPO4) and 2 gm Sodium Phosphate, monobasic (NaH2PO4) was dissolved in 450 ml distilled water and 50 ml of 37 % formaldehyde was added to it. It was stored in a capped bottle till use.

Alizarin red staining

Alizarin red staining was done to observe the gross morphology of skeletal system in the fetus by slight modification of method as described by Dawson (1926). Two fetuses from each dam were preserved in 70 % alcohol for bone staining.

a. Solutions and reagents

I. 0.1% aqueous alizarin red solution

100 mg of alizarin red powder was dissolved in 100 ml of distilled water and was filtered before use.

II. 1% KOH solution

1 gm of KOH was dissolved in 100 ml of distilled water. The solution was freshly prepared every day.

III. Glycerol (40%, 80% and absolute)

Glycerol (40%) was prepared by mixing 40 ml of absolute glycerol and 60 ml of distilled water and glycerol (80%) was prepared by mixing 80 ml of absolute glycerol and 20 ml of distilled water. Both the solutions of glycerol were freshly prepared.

b. Procedure

The fetus was skinned out and eviscerated and transferred to 70 % alcohol for 24 hours. Next day it was transferred to 90% alcohol and kept in it for at least 5 days for proper fixation. After that it was placed in acetone for 3 days to remove the fat. It was transferred to 1% KOH solution in the volume of approximately 10 times of the volume of fetus. Solution was changed daily till all the soft tissues got dissolved and bones became clearly visible. The specimen was then transferred in 1% KOH solution containing a few drops of 0.1 % alizarin red. The fluid was changed daily till the bones were properly

stained. The solution was replaced by 40% glycerol solution for 24 hours which was further replaced by 80% glycerol. Finally, 80% glycerol was replaced by absolute glycerol after 24 hours. The specimen was kept in the same solution till analysis.

Biochemical study

Estimation of Malondialdehyde (MDA) level

a. Principle

Malondialdehyde is one of the products of lipid peroxidation. It can be estimated by the thiobarbituric acid (TBA) test. MDA reacts in the TBA test to generate a pink colored product. In acid solution, the product absorbs light at 530 nm, and is readily extractable into organic solvents such as n-butanol (Devasagayam *et al*., 2003; Satoh 1978).

b. Solutions and reagents

I. **Reagent (1):** Trichloroacetic acid 0.3gm/ml in distilled water
II. **Reagent (2):** Thiobarbituric acid 7.5 mg in 50 ml 0.25N HCl
III. **Reagent (3):** 0.25N HCl

c. Preparation of sample

I. Serum from maternal blood

Maternal blood from orbital sinus was kept in an eppendorf tube at 4°C for 2 h. It was centrifuged at 10,000 g for 10 minutes and the clear supernatant or serum was transferred to another tube and stored at -20°C till use.

II. Homogenate of fetal brain

Fresh fetal brain was dissected out from the fetus, weighed and kept in phosphate buffer saline (0.02M) at pH 7.4 in the ratio of 1:9. It

was homogenized with the help of Polytron PT3100 homogenizer and stored at -20°C till use.

d. Procedure

1. 0.25 ml of sample and 0.25 of distilled water was taken in two test tubes labeled test and blank, respectively.
2. 0.25 ml of reagent (1) and 0.25 ml of reagent (2) was added to each test tube.
3. Samples were thoroughly mixed in each tube and heated in a water bath at 80°C for 15-20 minutes.
4. The test tubes were cooled and 2 ml of butanol was added to each.
5. Tubes were shaken vigorously and centrifuged at 10, 000 g for 15 minutes.
6. Supernatant was collected and optical density was measured at 530 nm against blank using a ELICO SL 164 Double beam UV-VIS Spectrophotometer.

e. Calculation of MDA

Serum MDA level= (O.D. of test – O.D. of blank)/0.156 µg/litre

Estimation of reduced glutathione (GSH) level

a. Principle

The glutathione determination was performed by the method described by Beutler *et al.* (1963) using 5, 5'- dithiobis-(2 nitro benzoic acid). This method is based on the development of relatively stable yellow colour when 5, 5'- dithiobis-(2 nitro benzoic acid) reagent is added to sulfahydryl compounds. The color thus developed was fairly stable for about 10 minutes. The reaction was read at 412 nm.

b. Solutions and reagents

I. **Reagent (1):** Precipitating solution; 1.67 gm glacial metaphosphoric acid in 100 ml D/W

II. **Reagent (2):** Phosphate solution; 4.26 gm Na_2HPO_4 in 100 ml distilled water (0.3M)

III. **Reagent (3):** DTNB reagent; 40 mg 5'5' bis (2 nitro) benzoic acid in 100 ml of 1% sodium citrate (1% sodium citrate; 1gm sodium citrate in 100 ml distilled water)

IV. **Reagent (4):** Standard Glutathione; 10 mg GSH in 100 ml distilled water.

c. Preparation of hemolysate

0.2 ml of whole blood from orbital sinus was added to 1.8 ml distilled water.

d. Procedure:

1. 2 ml of blood hemolysate or 2 ml of brain homogenate was taken in a test tube.
2. To it 3 ml of reagent (1) was added.
3. Waited for 5 minutes and the solution was filtered.
4. 2 ml of filtrate was taken in a test tube and 8 ml of reagent (2) was added.
5. Then 1 ml of reagent (3) was added
6. Blank was prepared by taking 0.8 ml distilled water, 1.2 ml reagent (1), 8 ml reagent (2) and 1 ml reagent (3).
7. Absorbance was taken within 30 seconds at 412 nm using ELICO SL 164 Double beam UV-VIS Spectrophotometer.

e. Calculation of reduced glutathione

The concentration of maternal blood GSH level and fetal brain GSH level was calculated from the standard curve of GSH (Fig. 4).

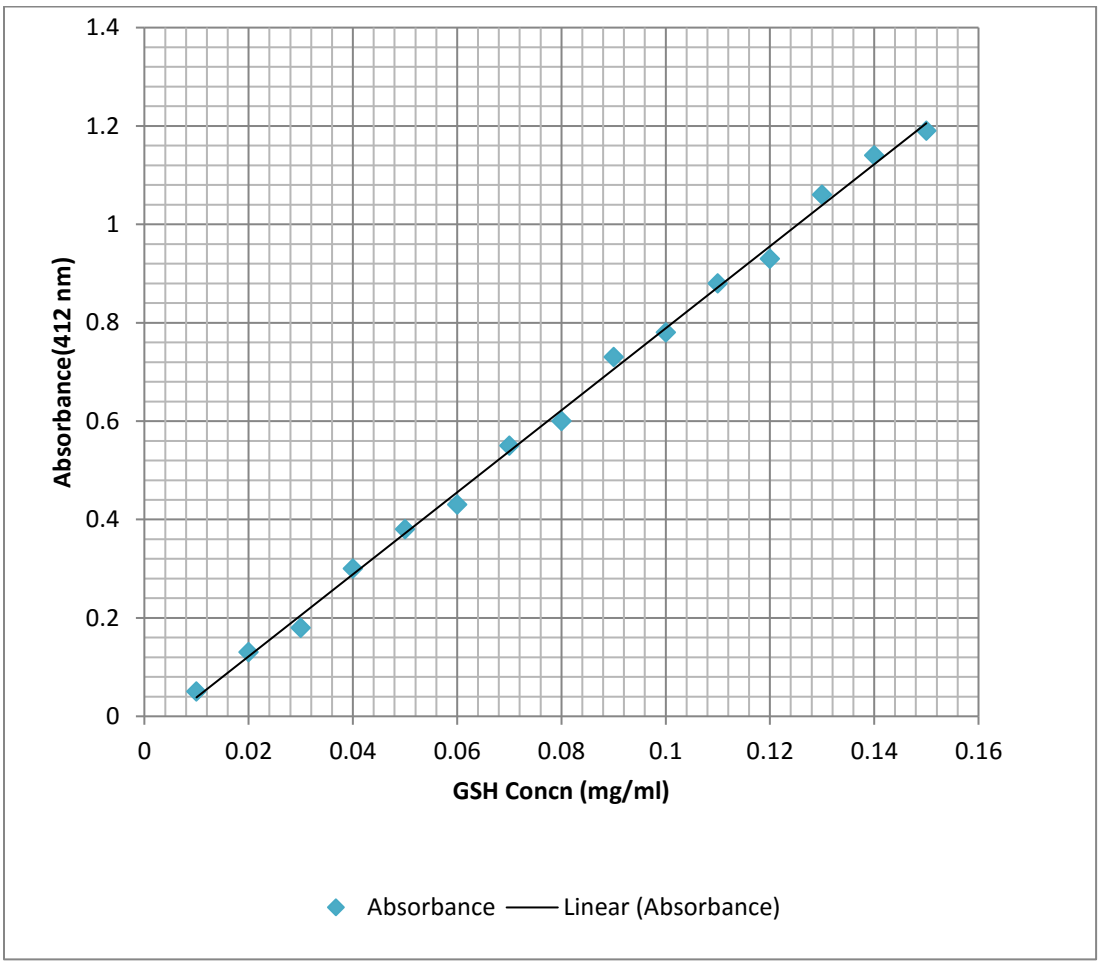


Fig. 4: Standard curve of reduced glutathione (GSH)

Behavioral study

Eight pregnant dams from each group were allowed to deliver their offspring normally. The day of birth was considered as postnatal day 0 (PND 0). At birth, pups from each group were culled to four pups (2♀,2♂) per litter on the basis of body weight. The culled pups were weaned with their natural mothers till 6th week. Eight male pups (n=8) and eight female pups (n=8) were selected randomly from each group (n =16 / group) for the behavioral study.

The selected mice were kept in separate cages. The males and females were also kept separately in each group. The remaining pups were discarded. The weight of pups selected for behavioral study was taken every week from the time of birth to PND 70 (11^{th} week) to compare their postnatal growth.

The selected male and female pups from each group were tested for locomotor activity, anxiety, exploration, learning, memory and depression from 8 weeks (PND 52) onwards in a series of behavioral tests which included open field (first 3 days), elevated plus maze (1 day), Morris water maze test (12 days), behavior despair test (2 days) and tail suspension test (1 day) (Fig. 5).

Open field test

The open field test was done to access exploratory and locomotor activity in the pups as described by Walsh and Cummins (1976). In this test, the mouse was placed on one of the corners of open field (60 cm X 60 cm X 60 cm) the floor of which was divided into 16 squares (15 cm X 15 cm) by white painted lines. The field was lit by a 100 watt bulb which was kept 2 m above. The test lasted for 5 minutes. The observed parameters were the number of squares crossed, i.e., ambulation, the number of times mice stood on its hind limb, i.e., rearing, the duration for which mice remains still without any movement, i.e, freezing time, the number of responses of scratching, licking and washing made, i.e., grooming and number of faecal boli exuded by each individual mice. Before each trial, the floor and the walls were cleaned with cotton soaked in 70% ethanol. The test was done for 3 consecutive days between 9.00 am to 11.00 am. The scores of three days were averaged and the average value of each parameter was used for further calculations.

Elevated plus maze test

The elevated plus maze was done to access anxiety and exploration behavior in the experimental animals as described by Pellow *et al.*(1985). The apparatus consist of two open arms (50 cm X 10 cm) and two closed arms (50

cm X 10 cm X 40 cm) which are connected through central platform (10 cm X 10 cm). The arms are arranged in a crossed- shape with the two open arms facing each other and two closed arms facing each other. The maze was kept 45 cm above the floor. The mouse was placed at the centre of the plus maze with its face directed towards one of the closed arms and observed for 5 minutes. The number of entries into open arm, closed arm and central square as well as period of permanence in those areas were observed. The floor and the walls of the open and closed arms were cleaned with 70% alcohol before each trial. All the trials were made between 9.00am to 11.00 am.

Morris water maze test

The Morris water maze test was done to access learning and memory in the rodents according to the method of Morris (1981). The maze consisted of a black circular pool (Diameter- 1.44 m, Height-80 cm) filled with water (25°c ± 1°c) to a depth of 44 cm. Four points were marked on the wall of the pool which divided the pool into four different quadrants. Initially all the pups were exposed to water maze for 1 minute and trained to climb a visible circular (Diameter-9cm) platform. Next day place acquisition test was performed in which the platform was kept hidden 1 cm below water level in the centre of one of the quadrants. The platform remained in the same position during 5 training days. At the beginning of each session, a random five sequences of four starting points along the perimeter of the pool was generated. All animals followed this sequence for that session. Each mouse was placed in the water facing the wall at the starting point and was allowed 90 seconds to find the hidden platform. The animal was allowed for 20s rest on the platform. The latency to reach the platform was recorded. If the mouse didn't locate the hidden platform within the given time it was lifted out and placed again on the same platform for 20s. The procedure was repeated for all the four start locations. Five sessions of four trials each were conducted. After that, the

platform was removed and a probe trial (w/o platform) was conducted. In probe trial each mouse was placed in the pool at the same randomly selected starting point and time spent in the quadrant of pool that initially contained platform, was measured. On completion of the probe trial, a reversed acquisition was conducted in which the hidden platform was placed in the opposite quadrant and five sessions of trials were performed as that was in acquisition sessions. Finally, the mice were tested with a visible platform that extended 1 cm above the surface of water, placed in a quadrant other than that chosen for the submerged platform. Each mouse was then given a single session of four trials to locate the platform. The latency to reach the platform was recorded (working memory procedure).

Behavior despair test

To evaluate the depression status the individual mouse was placed in a circular glass chamber, 45 cm in diameter containing 25 cm deep water, so that mice could not touch the bottom of the cylinder with their hind limbs or climb over the edge of the chamber (Porsolt *et al.,* 1978). Two swim sessions were conducted, an initial 15 minute pretest, followed by a 5 min test 24 h later. The period of immobility (remain floating in water without struggling) during 5 minute test period was noted and evaluated.

Tail suspension test

In tail suspension test, the animal was suspended by the tail, using an adhesive scotch tape, to a hook connected to a strain gauge that picks up all movements of the mouse and transmits them to a central unit (Porsolt *et al.,* 1978). Each mouse was given 1 trial that lasted 6 minutes. The total duration of immobility was recorded.

After completion of the behavioral tests, the pups were sacrificed by cervical dislocation on PND 70. Their brains were dissected out, weighed and preserved in 10% neutral formalin for neurohistological study.

Open field apparatus

Morris water maze tank

Elevated plus maze apparatus

Tail suspension test

Behavior despair test

Fig. 5: **Photograph of apparatus for behavioral tests**

Histological study

The histological study was done in placenta, fetal liver, fetal kidney, fetal brain and also the brain of pups at postnatal day (PND) 70 after completing the behavioral experiments. The tissues were preserved in 10 % neutral formalin and processed under following steps:

a. Dehydration

After fixation in 10% buffered formalin for about 48-72 hours the tissue was washed in tap water. Dehydration was done by passing the tissue serially in 70% alcohol (Overnight), 90% alcohol (2-3 hours), rectified spirit (1 hour) and finally in absolute alcohol I and II (1 hour in each).

b. Paraffin wax embedding:

I. **Clearing:** Tissue was cleared by giving two changes of xylene with 10 minutes in each.

II. **Infiltration:** The specimen was placed in a container containing fresh molten paraffin wax which was maintained at a temperature of 58-60° C in an incubator for 1 h. The process was repeated once.

III. **Embedding:** Molten paraffin wax was poured on a rectangle formed by two Leuckhart "L" pieces, placed on a glass slab. The tissue was placed into it with its cutting surface directed inferiorly and allowed to harden at room temperature. Precautions were taken to remove the air bubbles and to orient the specimen correctly.

c. Sectioning

Serial sections of tissue block were cut (6-8 µm thick) with the help of a Spencer's rotary microtome.

d. Mounting of sections on microscopic slides

The serial sections were put on albuminized slides, flooded with distilled water and kept on hot plate maintained at 45°C for proper spreading. After the water had vaporized, the slides were dried overnight in a storage box.

The egg albumin solution was prepared by mixing equal volume of glycerine and egg white. The mixture was filtered and a few crystals of thymol were added which prevent the growth of moulds.

e. Staining

The following staining methods were employed in the study:

1. Haematoxylin and Eosin staining
2. Periodic acid - Schiff's staining
3. Mallory's staining
4. Golgi staining
5. Nissl's staining

Haematoxylin and eosin staining

a. Reagents and solutions

I. Preparation of Harris's haematoxylin

2.5 gm of Harris's haematoxylin was dissolved in 25 ml of absolute ethyl alcohol and 50 gm of potassium or ammonium alum was dissolved in 500 ml of distilled water with the aid of heat. Each solution was removed from the heat and mixed. The solution was boiled for one minute with continuous stirring. The solution was removed from the heat and 1.25 gm of mercuric chloride was added slowly. The solution was reheated to simmer until it becomes dark purple. After that the solution was cooled quickly in the basin under the tap water. 15-20 ml of glacial acetic acid was added to it which increases the precision for nuclear staining. The solution was filtered before use.

II. Preparation of Eosin

1 gm of eosin was dissolved in 100 ml of distilled water and filtered after 24 h.

III. Preparation of 1 % acid alcohol solution (for differentiation)

1ml concentrated hydrochloric acid was added to 100 ml of 70% alcohol, mixed and stored in a reagent bottle.

IV. Preparation of 0.2% ammonia water solution (bluing)

2 ml of conc. ammonium hydroxide solution was mixed with 1000 ml of distilled water and stored in a bottle.

b. Procedure

I. Deparaffinization and rehydration

The sections were dipped in xylene; 2 changes at 10 minutes in each to remove the wax. It was dried and passed through descending grades of alcohol (absolute, rectified, 90% and 70%) by putting them for 2-3 minutes in each grade of alcohol. Finally the sections were rinsed in tap water.

II. Staining

Hydrated sections were kept in Harris Haematoxylin solution for 5 minutes and washed in tap water. Thereafter, sections were quickly rinsed in 1 % acid alcohol and washed with tap water and again quickly rinsed with 0.2% ammonia water and washed with tap water. Finally, the sections were kept in Eosin solution for 2 minutes and washed with tap water.

III. Dehydration and mounting

The sections were passed through ascending grades of alcohol (70%, 90%, rectified and Absolute) for about 30 seconds in each. The

sections were dried and cleared in xylene for 5-10 minutes. Finally the sections were mounted with DPX.

Periodic acid Schiff staining

a. Principle

The tissue is first oxidized by periodic acid. It breaks C-C bonds in various structures where these are present as 1:2 glycol groups (CHOH-CHOH) and converts them into dialdehydes (CHO.CHO). The equivalent amino or alkylamino derivatives of 1:2 glycol or its oxidation product (CHOH.CO) are also converted into dialdehydes. It doesn't oxidize further the resulting aldehydes, if kept for a limited period (i.e. not more than 10 min.) at room temperature. This aldehyde is localized by combining with Schiff's reagent and given a substituted dye which is red in colour.

Sulphite washings were given afterwards to remove uncombined leucofuschin before washing.

b. Solutions and Reagents

I. Preparation of Schiff's reagent

1 gm basic fuschin and 1.9 gm sodium metabisulphite were dissolved in 100 ml of 0.1N hydrochloric acid. The mixture was shaken and agitated with a mechanical shaker for 2 h. 500 mg of activated charcoal was added to it and shaken for 1-2 minutes. The mixture was filtered and made up to 100 ml with distilled water. The filtrate should be clear and colorless. It was stored at 4^{o}C in the dark.

II. Preparation of aqueous periodic acid (0.5 %)

0.5 gm of periodic acid was dissolved in 100 ml of distilled water and stored at 4^{o}C.

III. Preparation of sulphurous acid solution

36 ml of 10% potassium metabisulphite and 30 ml of 1N HCL were taken in a beaker and final volume was made to 600 ml with distilled water.

c. Procedure

1. The sections were deparaffinized and hydrated through the descending grades of alcohol as described above.
2. Staining: The sections were oxidized in 0.5% periodic acid solution for 5 minutes. It was rinsed in distilled water and placed in Schiff reagent for 15 minutes. Three changes of sulphurous acid solution were given, 2 minutes in each. It was washed in tap water for 5-10 minutes and counterstained with Harris haematoxylin for 1 minute. Finally washed in tap water and differentiated in 1 % acid alcohol.
3. The sections were dehydrated, cleared in xylene and mounted with DPX.

Mallory's stain

a. Principle

Mallory's stain is a stain utilized in histology to aid in revealing different macromolecules that make up the cell. It includes three stains: aniline blue, acid fuchsin, and orange G. As a result this staining technique can reveal collagen fibres, muscle fibres, cytoplasm, and red blood cells. It is helpful, therefore, in examining the collagen of connective tissue.

For tissues that are not directly acidic or basic, it can be difficult to use only one stain to reveal the necessary structures of interest. A combination of the three different stains in precise amounts applied in the correct order reveals the details selectively. This is the result of more than just electrostatic interactions of stain with the tissue and the stain not being washed out after each step. Collectively the stains complement one another.

b. Solutions and reagents

I. 1 % acid fuschin

Prepared by dissolving 1 gm of acid fuschin in 100 ml distilled water and filtered.

II. 1% phosphotungstic acid

Prepared by dissolving 1 gm phosphotungstic acid in 100 ml distilled water and filtered before use.

III. Staining solution

0.5 gm of aniline blue was dissolved in 100 ml of distilled water. 2 gm of orange g was added to it and stirred until it was dissolved in it. Finally, 2 gm oxalic acid was added and dissolved. The solution was filtered before use.

c. Procedure

1. The sections were deparaffinized and hydrated as described above.
2. Staining:The sections were placed in 1% acid fuschin for 2-3 minutes and rinsed with distilled water. Then the sections were immersed in 1% phosphotungstic acid for 2-3 minutes. It was rinsed quickly with distilled water and kept in staining solution for 15 minutes.
3. The sections were dehydrated and differentiated with ethanol, cleared with xylene and mounted with DPX.

d. Result: Cytoplasm - Pinkish or pale red; Red blood cells - Orange; Collagen fibres - Deep blue; Muscle fibres-Red

Golgi Staining

a. Principle:

Golgi's method stains a limited number of cells at random in their entirety. Dendrites, as well as the cell soma, are clearly stained in brown and black and can be followed in their entire length, which allowes to track connections between neurons and to make visible the complex networking structure of many parts of the brain and spinal cord.

Golgi's staining is achieved by impregnating fixed nervous tissue with potassium dichromate and silver nitrate. Cells thus stained are filled by microcrystallization of silver chromate.

b. Solutions and Reagents

I. Preparation of fixative solution

60 ml of 3% potassium bichromate was mixed with 20 ml of 10% formalin and stored in dark bottle.

II. Preparation of 2% silver nitrate solution:

2 gm was silver nitrate was dissolved in 100 ml distilled water and stored in dark bottle in a dark space.

c. Procedure

1. The tissue blocks (less than 1 cm thick) were fixed in 10 % neutral formalin for 24 hours. The blocks were transferred to fixative solution for 3-7 days and kept in dark. The solution was changed each day.
2. After that, the tissue was transferred into 2% silver nitrate solution and kept in dark for 3 days at room temperature. Before transferring the tissue into 2% silver nitrate solution precaution was taken to absorb excess fixative solution. The silver nitrate solution was changed several times until brown precipitate didn’t appear.
3. The tissue was dehydrated and embedded in paraffin wax as described above.

4. Thick sections (40-50 µm) were cut using Spencer's rotary microtome and the sections were transferred to glass slides and dried.
5. The sections were deparaffinized by giving 3 changes of xylene, 10 minutes in each and mounted with DPX.

d. Result: Neurons and processes stained black

Nissl's (Cresyl violet) Staining

a. Principle

The Cresyl violet method needs basic aniline dye to stain RNA blue, and is used to highlight important structural features of neurons. The Nissl substance (rough endoplasmic reticulum) appears dark blue due to the staining of ribosomal RNA, giving the cytoplasm a mottled appearance. Individual granules of extranuclear RNA are named Nissl's granules (ribosomes). DNA present in the nucleus gives a similar color.

b. Solutions and reagents

I. 0.1% cresyl violet solution

0.1 gm Cresyl violet was dissolved in 100 ml distilled water and filtered. 10 drops (or 0.3 ml) of glacial acetic acid was added just before use.

c. Procedure

1. The sections were deparaffinized and hydrated to distilled water as described above.
2. It was stained in 0.1% cresyl violet solution for 3-10 minutes in an oven at 37°C and rinsed quickly with distilled water.
3. It was differentiated in rectified alcohol for 2-5 minutes.
4. It was dehydrated in absolute alcohol and mounted with DPX.

d. Result: Neurons (Nissl granules) will be stained as pinkish violet.

Photography

In the present study, the photography of gross specimens was carried out by using Nikon Coolpix S8200 digital compact camera. The histological slides were examined with a light microscope (Nikon Eclipse E200, Japan), which was connected to a computer (Lenovo) via a 5 megapixel digital camera (Nikon DS-Fi2, Japan). Through these connections, images from the microscope could be visualized on the color monitor with high resolution (2560 x 1920 pixels, 300 Dots Per Inch). The computer was equipped with *NIS* Elements *D* microscope imaging software (Nikon) for quantitative analysis of the images.

Histomorphometry of fetal kidney

The study included ten left kidney of the fetuses of each group. In haematoxylin and eosin stained slides, the nephrogenic zone was identified as an area containing nephrogenic mesenchyme which was situated immediately beneath the renal capsule. Cortical, medullary and nephrogenic zone thickness were measured at five different points perpendicular to the cortex from capsule to arcuate arteries, by using the linear measuring tool provided in the *NIS* Elements *D* microscope imaging software and 10x objective; the points were selected on going around the circumference of the cortex from one pole of the kidney section to the other. The glomeruli were counted irrespective of their size and shape in a square grid (50 x 50 μm^2) by using dissector method at five random locations at 40 x objective. The glomeruli were identified by their tuft of capillaries and surrounding Bowman's capsule. Diameter of ten renal corpuscles was measured at juxtamedullary region of the cortex in 100 x objective. The line connecting the vascular pole to the urinary pole was taken as the vertical axis, and the widest distance at a right angle to the vertical axis was regarded as the diameter of the renal corpuscle, regardless of the size and

was measured between the inner edges of the thin parietal layer of the cells forming Bowman's capsule.

Histomorphometry of fetal cerebrum

Sagittal sections of fetal cerebrum were taken. Haematoxylin and eosin stained matched sections from all the groups were used for morphometric study. Ten left hemispheres of fetal brain were included in each group. The parameters which were measured were total thickness of fetal cerebrum, thickness of marginal zone, thickness of cortical plate and the thickness of ventricular zone. They were measured by using the linear measuring tool provided in the *NIS* Elements *D* microscope imaging software. The number of cells in cortical plate was counted in a square grid (50 x 50 μm^2) by using dissector method at five random locations in each section and then the density of cells (cells / mm^2) was calculated.

Histomorphometry of cerebrum of pups (PND 70)

The study was performed in 5 left cerebral hemispheres of pups from each group. Three random sagittal sections of the hemisphere which lay between 40 μm to 100 μm were selected and stained with Nissl's staining. The quantitative analysis was done using stereological principles and includes neocortex from the frontal lobe. At first the thickness of grey matter was measured in three different locations in each of the selected areas and the average length was recorded. The diameter of randomly selected 20 pyramidal cells of layer III and V was measured using linear measuring tool at a known magnification of 1000 x, both at horizontal and vertical axis. Mean neuronal diameters were calculated. The number of neurons per unit area was counted using a square grid (100 x 100 μm^2) of the different layers of frontal cortex. No distinction was possible between neurons of layers II and III, hence they were considered together for this study. In each section, counting was done in 5 fields at random each in layers II/III, IV and V of frontal cortex. The average

number of neurons per unit area was calculated and compared among the groups.

Histomorphometry of hippocampus of pups (PND 70)

The study included 5 brains. Anatomically matched sets of Nissl stained sections from left hemisphere of each brain, approximately 300 μm from midline, were selected and used for quantitative histological analysis of the CA1 and CA3 regions of pyramidal layer of the hippocampus . Based on the section thickness (6 μm), the sections calculated to be 300 μm from midline were identified for each brain. These selected sections were then compared with regard to intra- and extra-hippocampal landmarks and the matching thus further refined. Three sections were counted from each brain; the matched section , the sections 40 μm medial and 40 μm lateral to the matched section. Determination of the mean number of CA1 and CA3 hippocampal pyramidal cells per section was made by counting (magnification, 400 x) the total number of soma in a square grid (50 x 50 μm^2) by dissector method using *NIS* Elements *D* microscope imaging software. Cytoarchitectonic regions of the hippocampus were defined according to the criteria and nomenclature of Lorente de N621.

Histomorphometry of cerebellum of pups (PND 70)

For this study 5 left hemisphere of cerebellum of pups of each group were included. Three random vermial sections which lay between 40 μm to 100 μm from the median plane were selected and stained with Nissl's staining. Under the magnification of 100 x, the thickness of the molecular layer, the granular layer, the total cortex and the folia were measured at the midpoint of the total height of the folia 1,2,3, 8 and 9 using the linear measuring tool provided in the *NIS* Elements *D* microscope imaging software, performed perpendicular to the surface of the folia. For each folium, 3 measurements were taken in each of the selected areas and the average length was recorded. Under

the magnification of 400 x, the number of Purkinje Cells (PCs) was counted in those folia and their linear density was then calculated (cell number / mm), whereas the number of neurons in the molecular layer and granular layer were counted in a square grid (50 x 50 μm^2) by using dissector method at three random locations in each folium and then the density of cells (cells / mm^2) was calculated. The diameter of PC soma was measured only from the cells with clear cell body and distinct nucleus at magnification of 1000 x. A total of eight randomly selected PCs were measured from each folium.

Statistical analysis

The experimental results were expressed as Mean±SD. Data were analyzed by one way AVOVA and Kruskal-Wallis test using SPSS (Version 16) system to determine their significance. If the comparison between the groups was significant, SNK test and Mann-Whitney U test were used for *post hoc* analysis. $p \leq 0.05$ were considered as significant.

Chapter-5

Observation and Results

Maternal weight gain

Maternal weight gain from GD 0 to GD 18 was accessed in 12 pregnant dams in each experimental group. Their weight gain from GD 0 to GD 18 significantly differed between the groups ($p<0.01$) when analyzed by one way ANOVA (Table 1 and Fig. 6). On post hoc analysis using SNK test the dam's weight gain in group II was significantly lower than that in groups I, III and IV ($p<0.01$). There was no significant difference in dam's weight gain between groups I, III and IV ($p>0.05$), although mean maternal weight gain was highest in group IV (Table 2, Fig. 1 & Fig. 6). The data showed that alcohol consumption during pregnancy has severe deleterious effect on maternal weight gain while these effects were reduced by folic acid administration.

Table 1 : Maternal weight gain throughout gestation in different groups of mice

Groups	**Weight gain (g) (Mean±SD)**
I (n=12)	13.75±3.05
II (n=12)	8.75±4.22
III (n=12)	14.67±2.81
IV (n=12)	15.75±3.12
F-value	10.299
p-value	<0.01

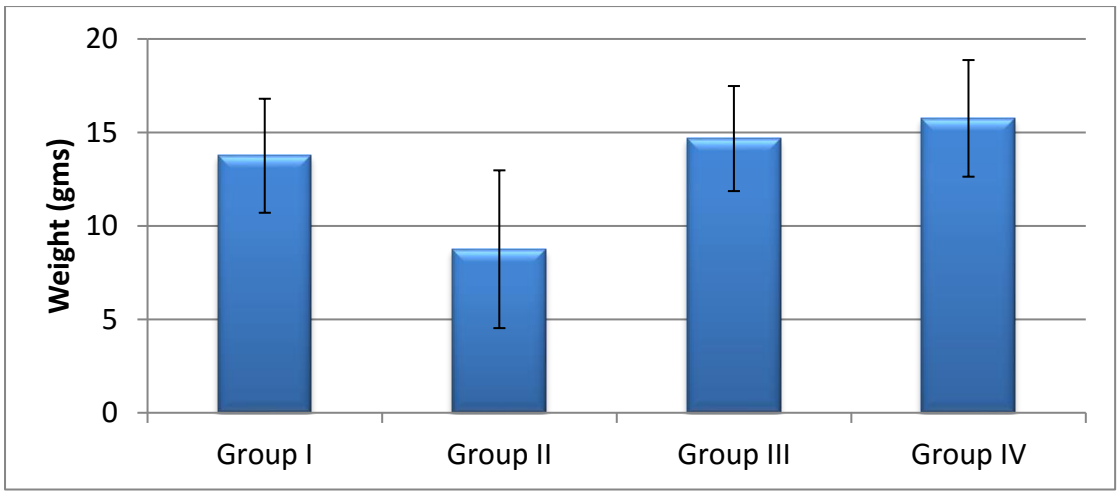


Fig. 6: Maternal weight gain throughout gestation in different groups of mice

Table 2 : Groupwise comparison of maternal weight gain

Groups compared	p-value
I Vs II	<0.01
I Vs III	>0.05
I Vs IV	>0.05
II Vs III	<0.01
II Vs IV	<0.01
III Vs IV	>0.05

Maternal malondialdehyde (MDA) and reduced glutathione (GSH) levels

Maternal MDA level and GSH level was measured in the blood of all experimental groups of pregnant dams. The results showed significant effect of the treatments ($p<0.001$) in both parameters. The maximum MDA level was observed in group II and minimum in group IV. Inversely, the minimum GSH level was observed in group II and maximum in group IV (Table 3, Fig. 7 & Fig. 8).

Table 3 : Maternal serum MDA and Blood GSH levels on GD 18 in different groups of mice

Parameters	Group I	Group II	Group III	Group IV	F-value	p-value
Serum MDA (nM/ml)	0.38 ± 0.05	1.35 ± 0.38	0.67 ± 0.08	0.35 ± 0.04	38.349	<0.01
Blood GSH (mM/ml)	0.39 ± 0.01	0.33 ± 0.03	0.36 ± 0.03	0.42 ± 0.01	23.004	<0.01

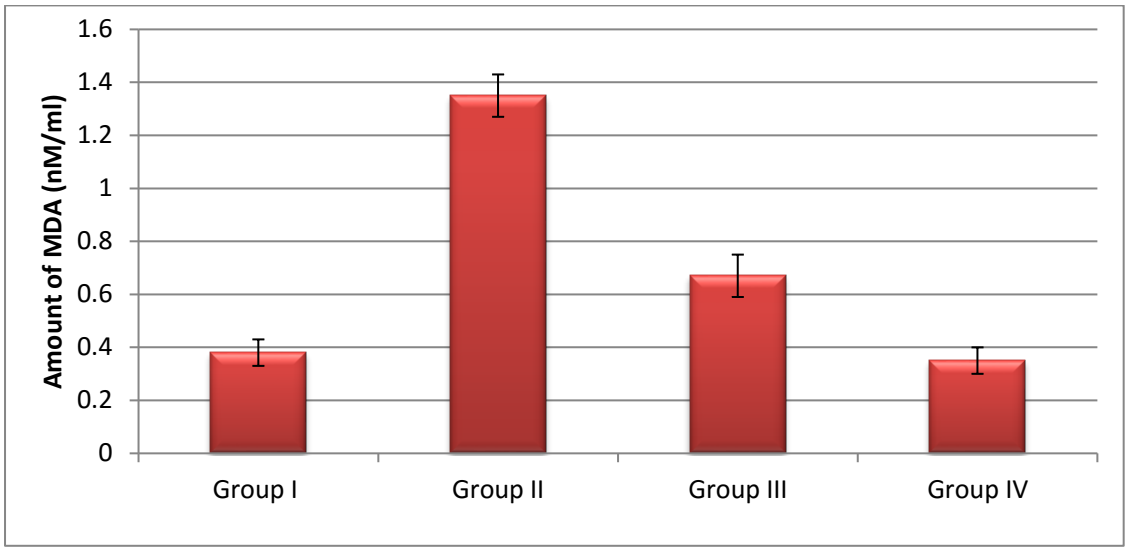


Fig. 7 : Maternal serum MDA level in different groups of mice

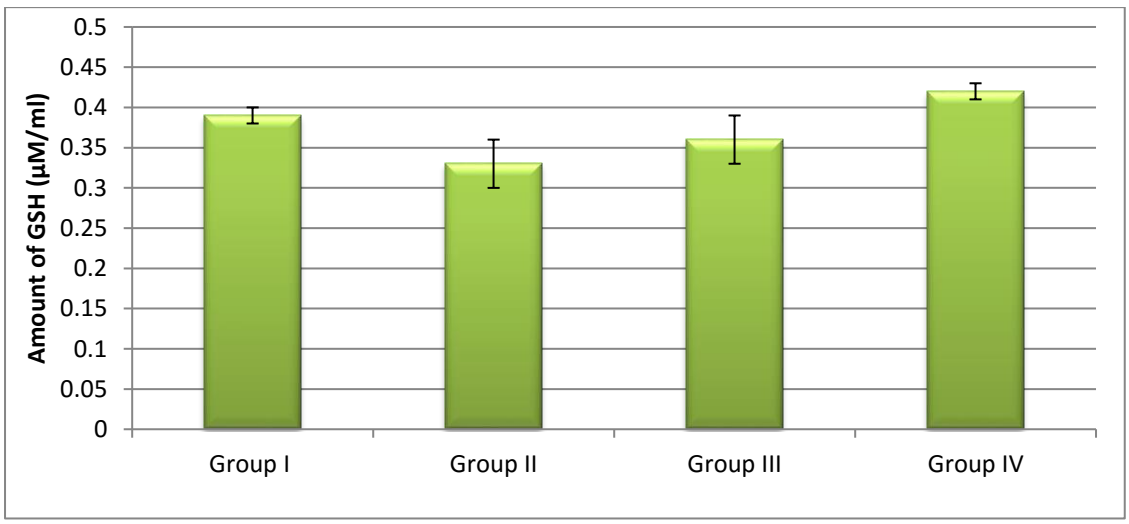


Fig. 8 : Maternal blood GSH level in different groups of mice

Table 4 : Groupwise comparison of maternal serum MDA and blood GSH levels

Groups	p-value	
	Serum MDA	Blood GSH
I Vs II	<0.01	<0.01
I Vs III	>0.05	<0.05
I Vs IV	>0.05	>0.05
II Vs III	<0.01	<0.05
II Vs IV	<0.01	<0.01
III Vs IV	<0.05	<0.01

Groupwise comparison showed that ethanol consumption by pregnant dams of group II significantly increased serum MDA level ($p<0.01$) and decreased blood GSH level ($p<0.01$) in comparison to those in dams of groups I, III and IV. Folic acid supplementation along with alcohol significantly decreased the MDA level and increased the GSH level in dams of group III as compared to alcohol fed dams of group I. No significant difference in MDA level was observed between dams of group I and III ($p>0.05$), however, the GSH level was significantly lower in group III as compared to that in dams of group I ($p<0.05$). No significant difference in MDA and GSH level was observed between dams of groups I and IV ($p>0.05$). The MDA level was significantly high ($p<0.05$) and GSH level was significantly low ($p<0.01$) in dams of group III as compared to those in dams of group IV. The result showed that alcohol intoxication for 10 days (GD6 to GD15) increased the serum MDA level and decreased blood GSH level in pregnant dams which were reversed by folic acid supplementation (Table 4, Fig. 7 & Fig. 8).

Pregnancy outcome

There was no significant difference among the groups in terms of number of implantation per dam ($p>0.05$) although highest mean implant per

dam was observed in group II and lowest in group I. The number of live fetuses per dam significantly differed between groups ($p<0.01$) (Table 5, Fig. 9 & Fig. 46-49).

Table 5 : Pregnancy outcome in different groups of mice

Groups	Implants per dam (Mean±SD)	Live fetuses per dam (Mean±SD)	Resorptions/dead fetuses per dam (Mean±SD)
I	7.25±1.91	7.00±2.04	0.17±0.12
II	8.58±1.31	5.00±1.35	3.33±1.23
III	7.92±0.99	6.83±1.27	1.08±0.79
IV	8.42±0.79	7.58±1.16	0.83±0.63
F-value	2.460	6.679	19.139
p-value	>0.05	<0.01	<0.01

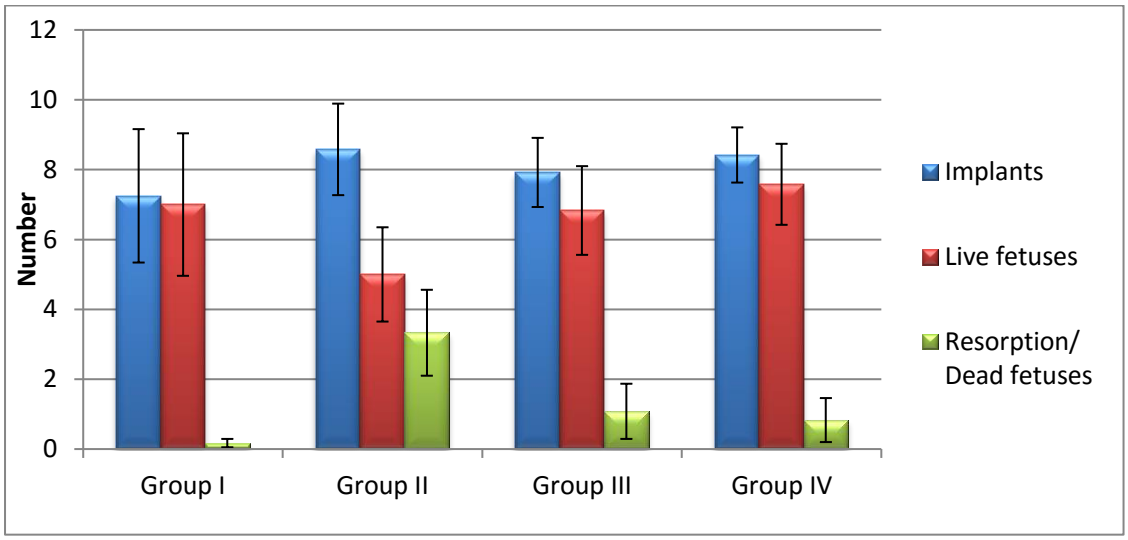


Fig. 9 : Pregnancy outcome in different groups of mice

Post hoc analysis showed significantly lower number of live fetuses in group II as compared to groups I($p<0.05$), III($p<0.05$) and IV ($p<0.01$) but such difference was not observed among groups I, III and IV($p>0.05$) although the number of live fetuses per dam was highest in group IV (Tables 5, 6 & Fig. 9).

The number of resorptions and dead fetuses per dam also significantly differed between the groups ($p<0.01$). Highest numbers were observed in group II and lowest in group I. On post hoc analysis resorptions and dead fetuses per dam were significantly higher in group II ($p<0.01$) as compared to groups I, III and IV, while no such difference was observed between groups I, III and IV ($p>0.05$) (Table 5, Table 6, Fig. 9 & Fig. 46-49). The data showed that ethanol exposure to pregnant dams (6g/kg/day) from GD6 to GD 15 significantly decreased the number of live fetuses and increased the number of resorptions and death of fetuses. Such findings were reversed after folic acid administration along with alcohol.

Table 6 : Groupwise comparison of pregnancy outcome

Groups compared	p-value	
	Live fetuses per dam	Resorptions/dead fetuses per dam
I Vs II	<0.05	<0.01
I Vs III	>0.05	>0.05
I Vs IV	>0.05	>0.05
II Vs III	<0.05	<0.01
II Vs IV	<0.01	<0.01
III Vs IV	>0.05	>0.05

Congenital malformations

Maximum number of fetuses with congenital malformations was observed in group II which received ethanol only, followed by group III while such malformations were not observed in other groups (Table 7). In group III there were only two fetuses with hemorrhagic patches. In group II congenital malformations were seen in 31.81% of the fetuses, there were 8 fetuses with hemorrhagic patches on different parts of body, limbs and tail, 5 fetuses with amelia, 3 with bilateral anophthalmia, 4 with kyphosis and 1 fetus with defect in face development with elongated snout (Fig. 51).

Table 7 : Congenital malformations in different groups

Groups	Total number fetus observed	Number of fetus with congenital malformations
I	82	0
II	66	21
III	77	2
IV	91	0

Skeletal malformation

Alizarin red staining of whole fetus was done to reveal the gross malformations of different bones in all the groups. In group II, the developing maxillary bones were less developed and separated as compared to group I. The ossifications of parietal as well as occipital bones were also reduced in comparison to group I. The ossifying occipital bones were widely separated in some fetuses. In one fetus, the bony orbit was bigger. The phalyngeal ossification was deficient in both fore limbs as well as hind limbs. Similarly, the ossifying humerus, radius and ulna in the fore limbs were comparable, while the ossifying femur, tibia and fibula were shorter that group I indicating delayed ossification of these structures. Only four sternebrae were visible ossifying while in group I there were five. The two halves of xyphoid process hadn't fused yet resulting into divided xiphoid process. In some fetuses the vertebral column was curved laterally (scoliosis) as compared to group I. The number of ossifying coccygeal vertebrae was reduced in some specimens (Fig. 52 A-C). In some fetuses, the bones were faintly stained with alizarin red indicating deficient calcium deposition in developing bones (Fig. 53). In group III, no such skeletal malformation and faulty ossification was observed indicating improvement in skeletal development. In group IV also, the developing bones were comparable with group I (Fig. 52 A-C).

Fetal growth

The fetal weight was compared between different groups on GD 18. There was significant difference in mean fetal weight among the groups ($p<0.01$). The highest fetal weight was observed for group I and least for group II (Table 8 and Fig. 50). Post hoc analysis revealed that the mean fetal weight in group II was significantly ($p<0.01$) lower as compared to groups I, III and IV. The increase in fetal weight after folic acid treatment in group III was significant as compared to that of group II ($p<0.001$) but was still significantly lower than that of group I ($p<0.01$) while it was comparable with that of group IV ($p>0.05$). There was no significant difference in fetal weight between groups I and IV ($p>0.05$) (Table 9, Fig. 10, Fig. 11 & Fig. 50).

Similarly, the highest mean crown rump length and tail length was observed in group I and lowest in group II. Post hoc analysis showed that the crown rump length was significantly decreased in prenatally alcohol exposed fetuses in group II as compared to remaining groups. The crown rump length and tail length of fetus was significantly increased after folic acid supplementation in group III in comparison to group II ($p<0.01$), but it was statistically not different from that of group I and IV ($p>0.05$) (Tables 8, 9, Fig. 10 & Fig. 11).

Table 8 : Fetal weight, crown rump length and tail length on gestational day 18

Groups	Fetal Weight (g) (Mean±SD)	Crown rump length (mm) (Mean±SD)	Tail length (mm) (Mean±SD)
I	1.17±0.15	24.90±2.27	11.97±0.90
II	0.91±0.14	21.37±2.70	10.26±1.59
III	1.09±0.10	24.13±1.50	11.33±0.84
IV	1.12±0.14	24.25±1.46	11.54±0.86
F-value	49.463	42.504	19.223
p-value	<0.01	<0.01	<0.01

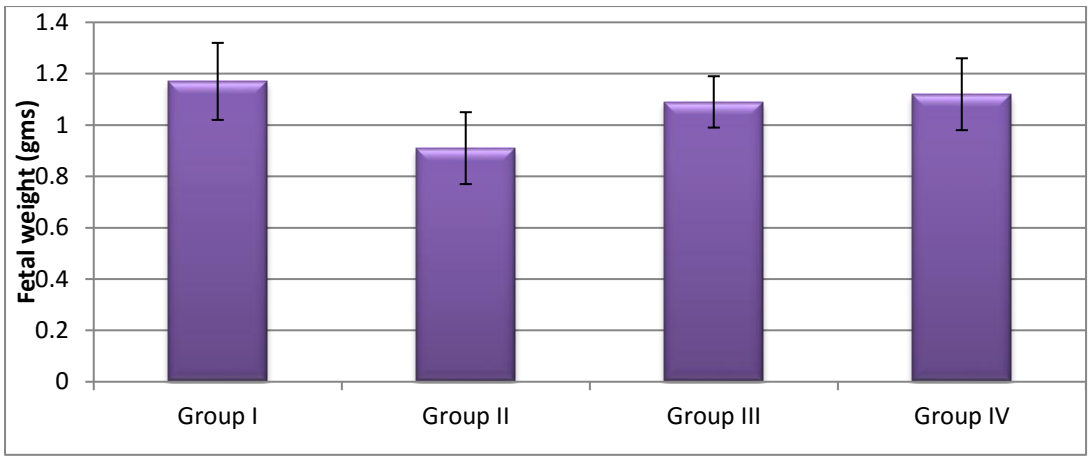


Fig. 10 : Fetal weight in different groups of mice

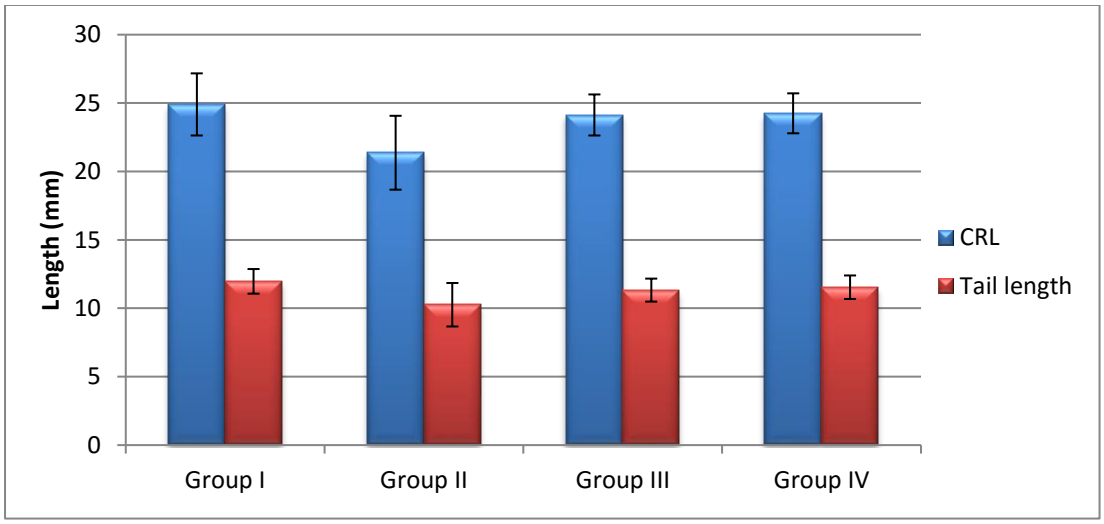


Fig. 11: Fetal crown-rump length (CRL) and tail length in different groups of mice

Table 9 : Group wise comparison of fetal weight, crown rump length and tail length

Groups compared	p-value		
	Fetal Weight	Crown rump length	Tail length
I Vs II	<0.01	<0.01	<0.01
I Vs III	<0.01	>0.05	>0.05
I Vs IV	>0.05	>0.05	>0.05
II Vs III	<0.01	<0.01	<0.01
II Vs IV	<0.01	<0.01	<0.01
III Vs IV	>0.05	>0.05	>0.05

Placenta

Significant difference of the treatments was observed in placental weight, diameter and thickness in between different groups (Table 10, Fig. 12 Fig. 54 & Fig. 55). Prenatal exposure to alcohol in group II not only decreased the placental weight but also decreased the placental diameter and thickness significantly ($p<0.01$). When folic acid was given along with alcohol the placental weight ($p<0.05$), diameter ($p<0.01$) and thickness ($p<0.01$) was significantly increased in group III as compared to those in alcohol-treated group II. The placental weight was still significantly low ($p<0.01$) in group III as compared to that in group I, however, the difference in placental diameter and thickness was not significant ($p>0.05$). No significant difference in these parameters was observed between groups I and IV. Although the placental weight was significantly higher ($p<0.01$) in group IV as compared to that in group III but again no difference in placental diameter and thickness was observed between them (Tables 10, 11 & Fig. 12). The result showed that decrease in placental weight, diameter and thickness due to exposure to alcohol was recovered by concomitant folic acid supplementation.

Table 10 : Placental weight, diameter and thickness in different groups

Groups	Weight (mg) (Mean ± SD)	Diameter (mm) (Mean ± SD)	Thickness (mm) (Mean ± SD)
I	13.42±1.93	8.44±0.47	3.01±0.32
II	11.24±2.00	7.43±0.48	2.60±0.26
III	12.02±1.16	8.40±0.40	2.94±0.35
IV	12.92±1.68	8.69±0.46	3.09±0.21
F-value	23.539	42.909	15.845
p-value	<0.01	<0.01	<0.01

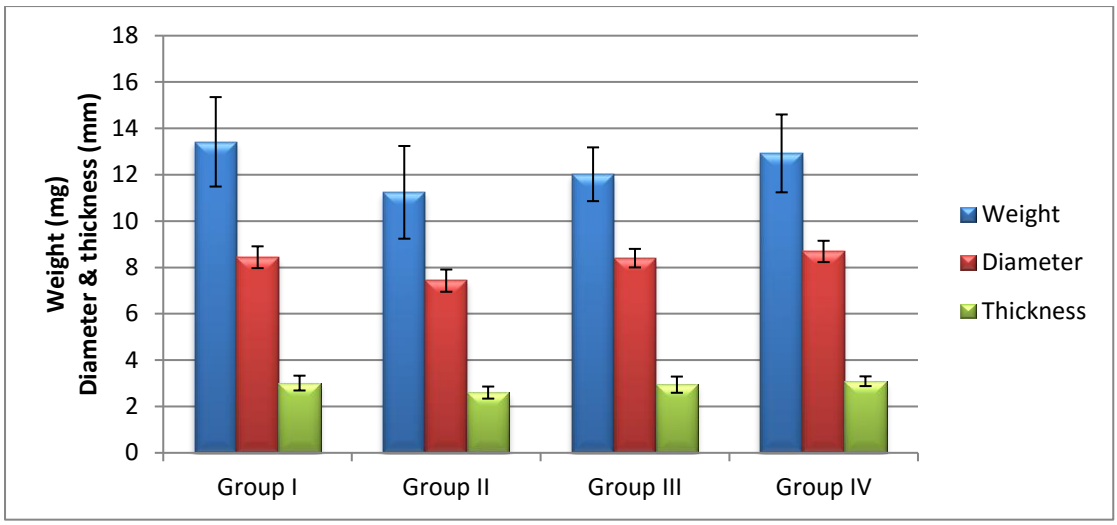


Fig. 12 : Placental weight, thickness and diameter in different groups

Table 11: Groupwise comparison of placental weight, diameter and thickness

Groups compared	p-value		
	Weight	Diameter	Thickness
I Vs II	<0.01	<0.01	<0.01
I Vs III	<0.01	>0.05	>0.05
I Vs IV	>0.05	>0.05	>0.05
II Vs III	<0.05	<0.01	<0.01
II Vs IV	<0.01	<0.01	<0.01
III Vs IV	<0.01	>0.05	>0.05

On histological study of haematoxylin and eosin stained section of placenta, the labyrinth zone of group I showed maternal sinusoids and the trophoblastic septa, which was composed of trilaminar trophoblastic epithelium and fetal capillary. Numerous cytotrophoblastic cells with large spherical nucleus were seen around the maternal sinusoids which were full of maternal blood. Two layers of synctiotrophoblasts with small rounded nuclei (syncytiotrophoblast I and syncytiotrophoblast II from the maternal sinusoid side) were present under cytotrophoblastic layer which collectively formed

trophoblastic septa. Numerous fenestrated fetal capillaries lined by endothelium were also seen which allowed the maternal and fetal blood to come close together for the exchange of substances through placental barrier (Fig. 60.A). The Mallory stained sections also revealed maternal sinusoids, fetal capillaries and the placental barrier more distinctly (Fig. 61.A). Channels present in the basal zone drained the maternal blood from the placenta. Numerous spongiotrophoblast cells were seen which formed the main structural component of the basal zone. The large nucleated trophoblastic giant cells were present below the spongiotrophoblast cells in contact with the labyrinth zone. Islands of glycogen cells were also seen scattered throughout the zone (Fig. 62.A).

In the haematoxylin and eosin stained placenta of group II, the cytoarchitecture of labyrinth zone was altered. The trophoblastic septa was thickened and disrupted at many places. The fibrinoid accumulations and dilation of maternal sinusoids were frequent. Frequent derangement of cytotrophoblast and synctiotrophoblast layers and congested maternal sinusoids were observed resulting into abnormal exchange of fetomaternal blood in comparison to group I. In some cases homogenous mucoid collection was also observed around the blood vessels. The overall width of this zone and arborisation of fetal vasculature were reduced (Fig. 60.B1-B3). The thickening of trophoblastic septa and congestion of maternal sinusoids was more prominently observed in Mallory stained sections (Fig. 61). The basal zone also showed marked degenerative changes as compared to control. The spongiotrophoblasts showed frequent pyknotic nuclei. Debris of the degenerated cells was a frequent picture. Vacuolar degeneration as well as karyorrhexis was seen in the nuclei of those cells. At many places cellular walls were also disrupted and cytoplasmic contents were extruded. The glycogen

cells were also degenerated and replaced by homogenous mass in most places. The zone also showed extensive vacuolization (Fig. 62.B).

In group III, the degenerative changes observed in group II were much reduced. In labyrinth zone the homogenous debris was reduced and also the placental barrier was thinner than in group II. But it still showed increased frequency of giant cells and thicker placental barrier than in group I. The sinusoids were dilated much less than in group II but slightly more in comparison to group I (Fig. 60.C & Fig. 61.C). In the basal zone few macrophages were observed but the trophoblastic cells were comparable to group I (Fig. 62.C). The overall picture showed partial protection from the disruptive changes of alcohol.

In group IV, the frequency of fetal capillaries was observed to be increased in labyrinth zone in both haematoxylin and eosin as well as Mallory stained slides. The placental barriers as well as maternal sinusoids were comparable with that of group I (Fig. 60.D & Fig. 61.D). In the basal zone the frequency of trophoblastic cells tend to be increased (Fig. 62.D). Overall features were comparable with group I.

Fetal kidney

Significant differential effect of the treatments was observed on weight of fetal kidney among the groups ($p<0.01$). The maximum weight was observed in group I and minimum in group II (Table 12, Fig. 13 & Fig. 56). Groupwise comparison revealed that the weight of fetal kidney was significantly decreased in prenatally alcohol-exposed fetuses of group II as compared to groups I ($p<0.01$), III ($p<0.05$) and IV ($p<0.01$). Folic acid administration along with alcohol significantly increased the weight in group III as compared to group II but it was still significantly lower than that in group

I ($p<0.05$). No significant difference in the weight was observed between groups I and IV ($p>0.05$) as well as between groups III and IV ($p>0.05$) (Tables 12, 13 & Fig. 13).

Table 12 : Weight of fetal kidney and histomorphometric parameters in different groups

Groups	Weight (mg) (Mean±SD)	Cortical thickness (µm) (Mean±SD)	Medullary Thickness (µm) (Mean±SD)	Nephrogenic zone thickness (µm) (Mean±SD)	Glomerular number (No./mm^2) (Mean±SD)	Renal corpuscle diameter (µm) (Mean±SD)
I	6.39±1.37	371.20±63.31	739.30±126.15	56.60±19.21	32.15±8.54	66.95±14.59
II	3.87±1.20	275.00±94.02	366.00±96.71	88.90±13.79	21.45±7.50	81.55±10.58
III	5.05±1.13	331.50±31.14	822.50±131.60	52.50±20.92	26.20±6.23	69.00±11.02
IV	5.48±0.90	362.30±63.88	849.40±121.93	40.20±12.20	33.35±9.07	78.15±14.04
F-value	12.132	4.222	34.880	15.059	9.754	6.172
p-value	<0.01	<0.05	<0.01	<0.01	<0.01	<0.01

Histological study of the cortex and medulla of haematoxylin and eosin stained sections of fetal kidney was done. In group I, the renal cortex revealed two zones i.e., the subcapsular zone (just beneath the capsule) which contained immature developing renal structures and the juxtamedullary zone which contained relatively more developed renal corpuscles surrounded by convoluted tubules. The subcapsular zone showed active nephrogenic zone which comprised of aggregates of basophilic undifferentiated mesenchymal cells that lacked proper organization. Beneath the nephrogenic zone there were numerous immature forms of renal structures such as cell condensates, renal vesicles, comma shaped and S shaped bodies. Some ureteric buds were also observed in the subcapsular zone as straight tubules ending with swollen ampullae. Aggregations of mesenchymal cells forming caps were seen in close association with the upper sides of the ureteric buds. Few medullary rays were also seen extending towards the cortex. The juxtamedullary renal corpuscles

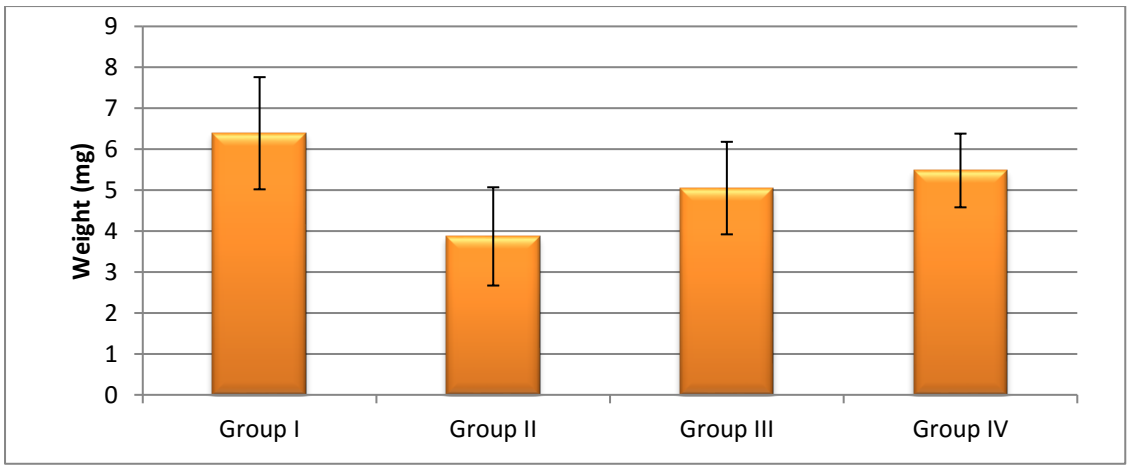


Fig. 13 : Weight of fetal kidney in different groups

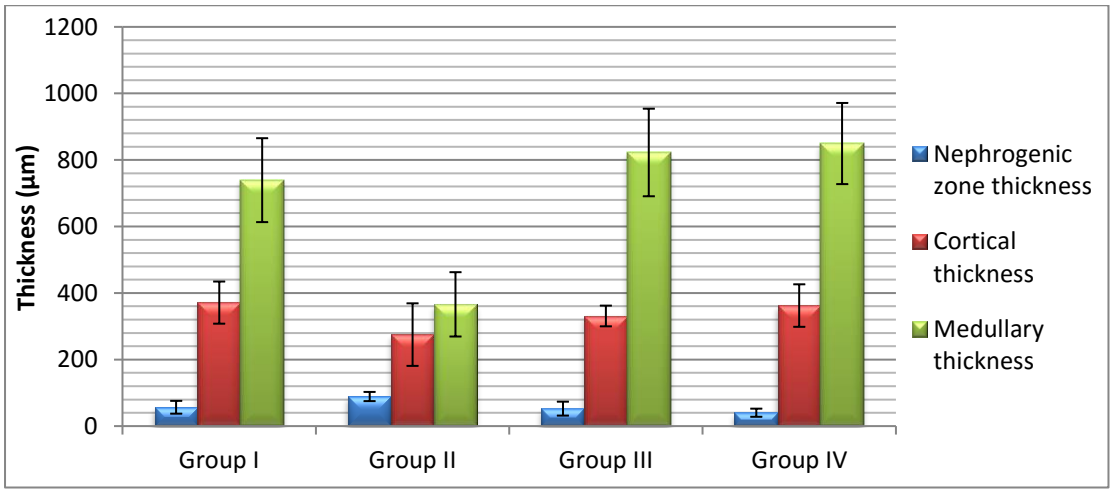


Fig. 14 : Nephrogenic zone thickness, cortical thickness and medullary thickness in different groups

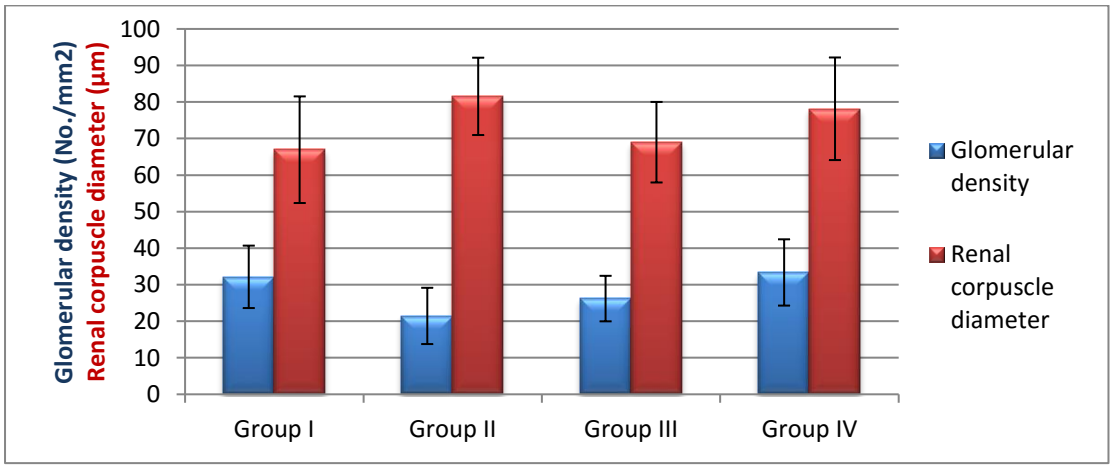


Fig. 15 : Glomerular density and diameter of renal corpuscles in different groups

were relatively larger and more mature form of renal corpuscles (Fig. 63.A). Glomerulus and urinary space lined by outer squamous parietal layer and inner columnar visceral layer was clearly visible at higher magnification (Fig. 64.A). The medulla contained the closely packed cross sections of collecting ducts, thin descending limb and thick ascending limbs of loop of Henle (Fig. 65.A)

Table 13 : Groupwise comparison of weight of fetal kidney and histomorphometric parameters

Groups compared	p-value					
	Weight	Cortical thickness	Medullary thickness	Nephrogenic zone thickness	Glomerular number	Renal corpuscles Diameter
I Vs II	<0.01	<0.05	<0.01	<0.01	<0.01	<0.01
I Vs III	<0.05	>0.05	>0.05	>0.05	>0.05	>0.05
I Vs IV	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05
II Vs III	<0.05	>0.05	<0.01	<0.01	>0.05	<0.05
II Vs IV	<0.01	<0.05	<0.01	<0.01	<0.01	>0.05
III Vs IV	>0.05	>0.05	>0.05	>0.05	<0.05	>0.05

In kidney of fetuses of group II, marked degenerative changes were observed in both the cortex as well as the medulla. In the cortex, necrosis of the developing glomeruli within the renal corpuscles was frequent resulting into its irregular shape and small size. The cells in the glomerulus were hyalinisd and they were shrunken as well as divided in some places (Fig. 63.B). In developing glomeruli some of the cells were vacuolated with pyknotic nuclei on higher magnification. There was karyorhexis as well as clumps of dark cells. The parietal layer of the Bowman's capsule was observed to be thickened may be due to the hypertrophy of lining cells. In toto the number of developing and developed glomeruli was markedly reduced. The urinary space of Bowman's capsules as well as lumen of convoluted tubules was dilated at many places. Hyalinised acidophilic mass were also observed in some tubules. There was hypertrophy of epithelial cells of the renal tubules and in some places

degeneration of tubular epithelia with simultaneous infiltration of mononuclear cells was observed under higher magnification (Fig. 64.B1 & 64.B2). In the medulla, the amount of connective tissue was markedly increased and the frequency of renal tubules and collecting ducts was decreased. Dilation of the collecting ducts and renal tubules was common feature. Cells lining the ducts and tubules showed degenerative changes. The cells were either vacuolated or hyalinised resulting into irregular margins (Fig. 65.B).

In group III, the degenerative changes induced by alcohol was much reduced by the folic acid co-treatment. Necrotic glomeruli were observed in some places but it was much reduced. The cells lining the Bowman's capsule as well as that of the tubules were comparable to that of group I. Dilation of the tubules and urinary space of Bowman's capsule had much reduced (Fig 63.C, Fig. 64.C1 & C2). In the medulla as well the number of tubules and ducts had dramatically increased with simultaneous decrease in interstitial matrix. The degenerative changes in the cells lining the tubules as well as those of ducts had much reduced (Fig. 65.C). In group IV, the overall picture was comparable with that of group I (Fig. 63.D, 64.D & 65.D) .

Similarly, the morphometric analysis revealed that the cortical thickness, medullary thickness, nephrogenic zone thickness, glomerular density and renal corpuscle diameter were significantly affected by the treatments among different groups (Table 12, Fig. 14 & Fig. 15). The cortical thickness and the medullary thickness were significantly reduced in fetal kidney of group II as compared to that of groups I and IV. The folic acid supplementation along with alcohol significantly increased the medullary thickness ($p<0.01$) in group III but not the cortical thickness ($p>0.05$) as compared to those in prenatally alcohol-exposed kidneys of group II. No statistical difference was observed in these parameters among groups I, III and IV ($p>0.05$). Nephrogenic zone was significantly thicker in alcohol exposed fetal kidney of group II as compared to remaining groups ($p<0.01$). Although this zone was thinnest in group IV, no

significant difference was observed among the groups I, III and IV ($p>0.05$). The glomerular density (No./mm^2) was significantly low in alcohol-exposed fetuses of group II as compared to groups I and IV ($p<0.01$). No significant difference in glomerular density was observed between groups II and III ($p>0.05$), although it was higher in group III. Similarly no significant difference ($p>0.05$) in the density was observed between groups I and IV as well as between groups I and III but it was significantly low ($p<0.05$) in group III as compared to that in group IV. The renal corpuscle diameter was significantly increased in alcohol-exposed fetuses of group II as compared to that in groups I ($p<0.01$) and III ($p<0.05$). It was also significantly higher in group IV as compared to that in group I. No significant difference in the diameter was observed between groups I and III as well as between groups II and IV (Tables 12, 13, Fig. 14 & 15).

Fetal liver

Table 14 : Weight of fetal liver in different groups

Groups	Weight (mg) (Mean±SD)
I	79.07±13.25
II	57.33±11.91
III	73.00±7.19
IV	80.40±23.62
F-value	7.245
p-value	0.01

The weight of fetal liver was also significantly affected in different experimental groups ($p<0.01$). The weight was maximum in group IV, followed by groups I, III and least in group II (Table 14, Fig. 16 & Fig. 57). Groupwise comparison using SNK test showed that prenatal alcohol intoxication from GD6 to GD15 significantly decreased the weight in group II as compared to control, i.e., group I ($p<0.01$). Folic acid supplementation along

with alcohol in group III significantly increased the weight ($p<0.05$) while no significant difference was observed among the groups I, III and IV ($p>0.05$) (Tables 14, 15 & Fig. 16).

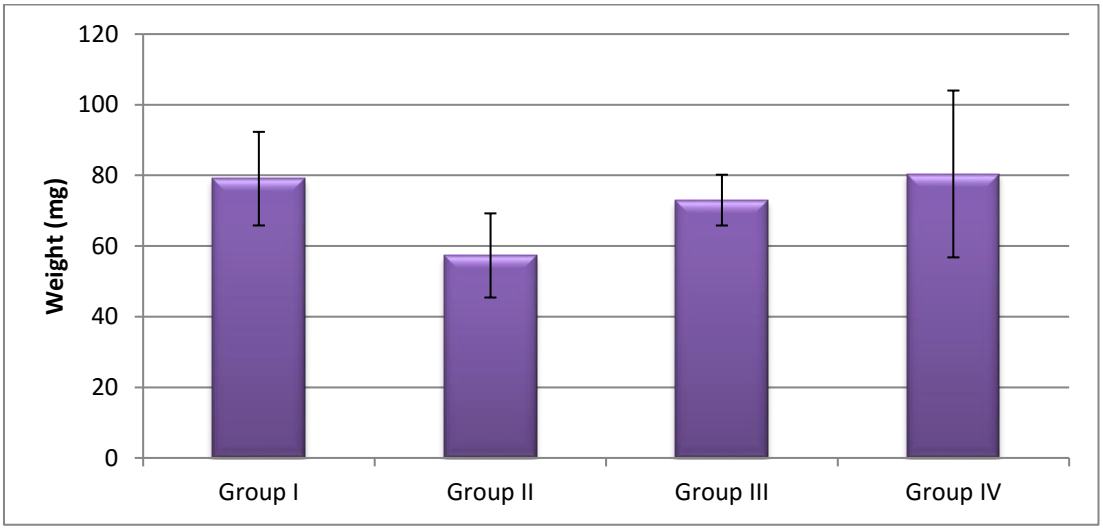


Fig. 16 : Weight of fetal liver in different groups

Table 15 : Groupwise comparison of weight of fetal liver

Groups compared	p-value
I Vs II	<0.01
I Vs III	>0.05
I Vs IV	>0.05
II Vs III	<0.05
II Vs IV	<0.01
III Vs IV	>0.05

The haematoxylin and eosin stained sections of group I showed developing hepatoblasts differentiating into hepatocytes which had started arranging themselves into cords around developing central veins. Sinusoids had started appearing in form of spaces in-between developing cords of hepatoblasts. Hematopoietic stem cells and megakaryocytes were frequently seen intermingled with developing hepatocytes (Fig. 66.A).

In group II, extensive degeneration of developing hepatoblasts and haemopoetic cells was seen. Homogenous acidophilic mass was seen along with the degenerating cells and marked fibrosis was observed along the lineage of degenerating cells. There was increase in cellular debris in the intercellular matrix. The hepatic laminae were disorganized and disrupted at many places due to which large empty lacunar spaces with loss of developing sinusoids and central veins was observed. The lacunar spaces were in plenty without any signs of the formation of central veins and sinusoids. The density of progenitor cells appeared to be considerably reduced and lymphocytic infiltration were common (Fig. 66.B).

In group III, the lacunar spaces were organizing themselves in the form of central veins. Large numbers of hepatoblasts as well as haematopoietic cells were present but the frequency of haematopoietic cells were comparatively more than in controls. The process of differentiation of hepatoblasts into hepatocytes was observed. Empty lacunar spaces were still seen but only at a few places and size of these spaces was considerably reduced (Fig. 66.C).

In group IV, the formation of central veins and sinusoids was more organized than in group III. Haematopoietic cells and hepatoblasts were observed. The differentiating hepatoblasts into hepatocytes were seen surrounding the developing central veins and sinusoids. The cellular population was slightly more than in group I, may be because of increased amount of haematopoietic cells (66.D).

PAS staining showed that decreased PAS positive material and increased megakaryocytes throughout the sections were observed in liver of group II. In group III, the megakaryocytes were decreased but still more than that in group I and PAS positive material was comparable to that of group I. In group I and IV, the PAS positive material was comparable and found more around the developing central veins (Fig. 67).

Fetal brain

Fetal brain weight and relative brain weight

The fetal brain weight and relative brain weight differed significantly among the different groups ($p<0.01$). The maximum weight was observed in fetuses of group I and minimum in those of group II (Table 16, Fig. 17, Fig. 18 & Fig. 58).

Group wise comparison revealed that prenatal alcohol exposure during the period of organogenesis significantly reduced the fetal brain weight and relative brain weight ($p<0.01$) in fetuses of group II as compared to remaining groups. However exposure to folic acid along with alcohol significantly increased ($p<0.01$) these parameters in group III as compared to alcohol-exposed fetuses of group II. Although fetal brain weight was significantly low ($p<0.05$) in group III as compared to that in group I, the relative brain weight didn't differ significantly ($p>0.05$). No significant differences ($p>0.05$) in these parameters were observed between groups I and IV as well as between III and IV (Tables 16, 17, Fig 17 & Fig. 18).

Table 16 : Fetal brain weight and relative brain weight in different groups

Groups	Weight of brain (mg) (Mean±SD)	Relative brain weight (%) (Mean±SD)
I	7.5±0.6	7.18±0.77
II	5.0±0.5	5.41±0.55
III	6.7±0.9	6.48±0.96
IV	7.0±0.6	6.57±0.40
F-value	27.319	13.094
p-value	<0.01	<0.01

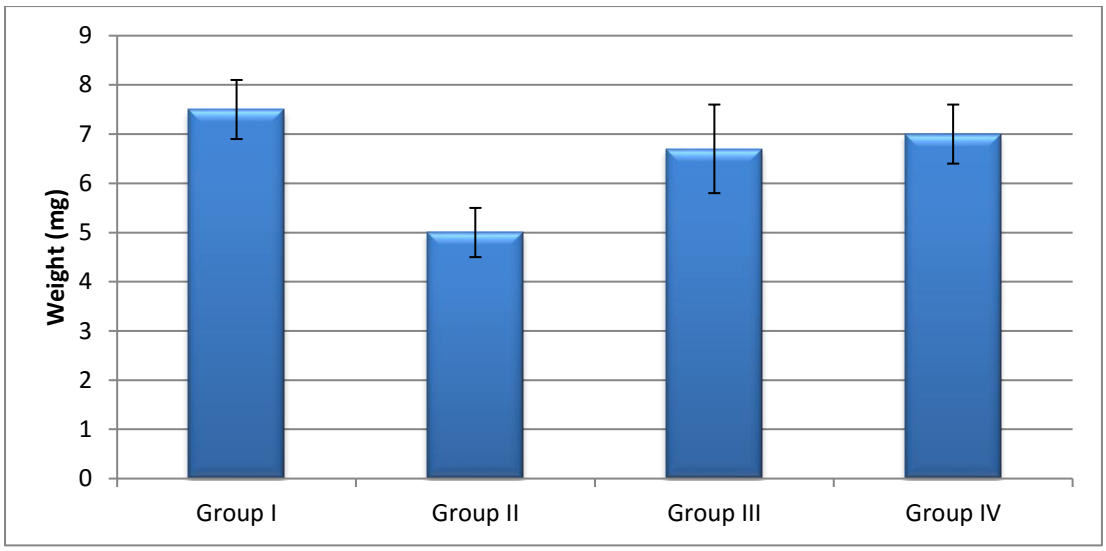


Fig. 17 : Weight of fetal brain in different groups

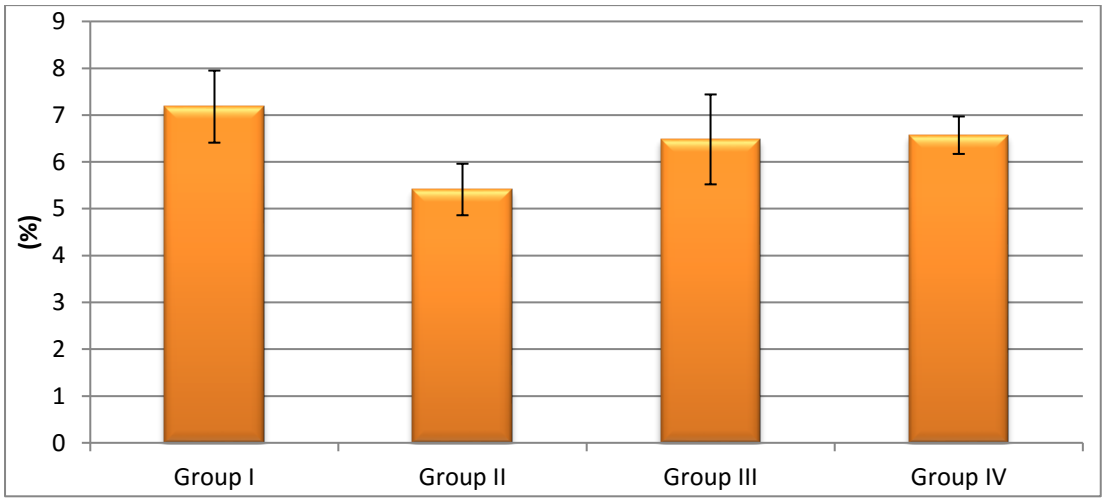


Fig. 18 : Relative brain weight of fetus in different groups

Table 17 : Groupwise comparison of fetal brain weight and relative brain weight

Groups compared	p-value	
	Weight of brain	Relative brain weight
I Vs II	<0.01	<0.01
I Vs III	<0.05	>0.05
I Vs IV	>0.05	>0.05
II Vs III	<0.01	<0.01
II Vs IV	<0.01	<0.01
III Vs IV	>0.05	>0.05

Fetal brain Malondialdehyde (MDA) and reduced glutathione (GSH) levels

The fetal brain MDA and GSH levels were measured from the brain homogenate. Significant effect of the treatments was observed in fetal brain MDA and GSH levels in different groups ($p<0.01$) (Table 18, Fig. 19 & Fig. 20). Group wise comparison showed that the prenatal alcohol exposure significantly increased the MDA level and decreased GSH level in brain tissue of fetuses of group II as compared to remaining groups. Exposure to folic acid along with alcohol significantly reversed the scenario in group III as compared to those in group II. The GSH level was still significantly low ($p<0.01$) in the brain of group III as compared to that in the brain of group I and IV fetuses while no such difference was observed in MDA level. No significant difference ($p>0.05$) was observed between groups I and IV for both these parameters (Tables 18, 19, Fig. 19 & Fig. 20).

Table 18 : Fetal brain MDA and GSH levels in different groups

Parameters	Group I	Group II	Group III	Group IV	F-value	p-value
MDA(nM/gm)	5.11 ± 1.03	14.29 ± 4.31	8.04 ± 1.98	5.07 ± 1.01	24.462	<0.01
GSH(mM/gm)	0.50 ± 0.05	0.25 ± 0.03	0.34 ± 0.05	0.51 ± 0.08	35.636	<0.01

Table 19 : Groupwise comparison of fetal brain MDA and GSH levels

Groups compared	p-value	
	MDA	GSH
I Vs II	<0.01	<0.01
I Vs III	>0.05	<0.01
I Vs IV	>0.05	>0.05
II Vs III	<0.01	<0.05
II Vs IV	<0.01	<0.01
III Vs IV	>0.05	<0.01

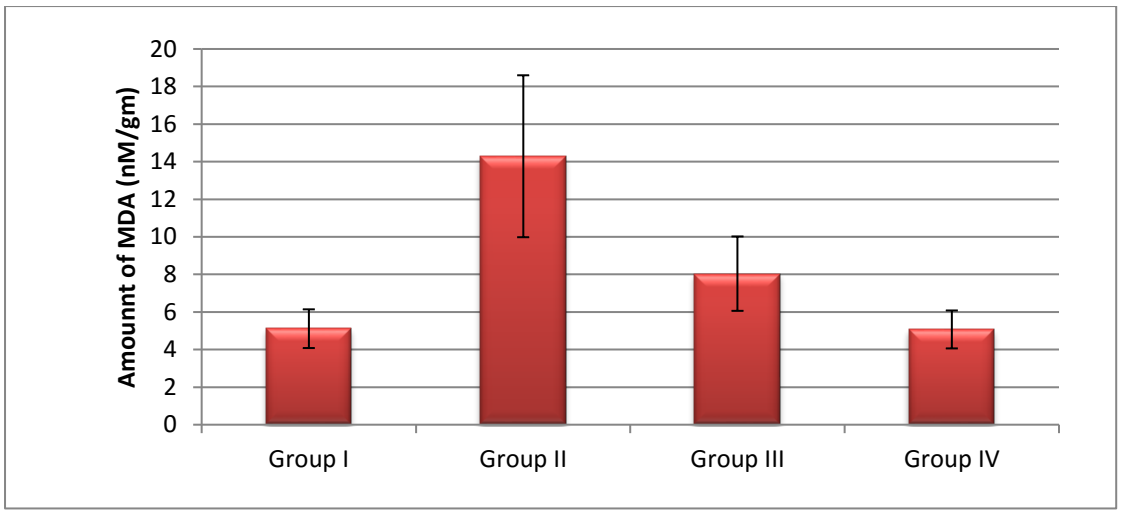


Fig. 19 : Fetal brain MDA level indifferent groups

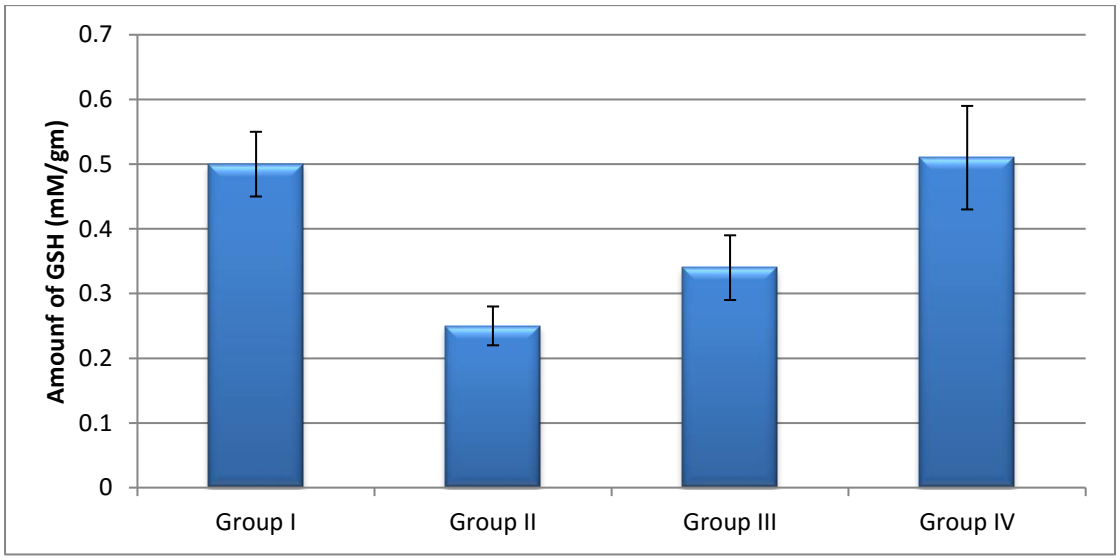


Fig. 20 : Fetal brain GSH level in different groups

Histological study

Histological study was done in developing cerebral hemisphere, hippocampus and the cerebellum. Only the left side of brain was included for the study so that comparison could be made among the different groups.

Cerebrum

Haematoxylin and eosin stained sections of developing cerebral hemisphere revealed five different zones in all the groups at low magnification (100x). From outside inward they were marginal, cortical, intermediate,

subventricular and ventricular zones (Fig. 68.A). The ventricular zone and the subventricular zone contained mitotically active precursor cells that were migrating towards the surface. Ventricular zone was the histologically defined region surrounding the ventricles while the subventricular zone was located outside the ventricular zone and formed by the cells that developed and migrated from ventricular zone. The marginal zone and the cortical plate were the fields that consisted largely of postmitotic or differentiating cells that were migrated from ventricular and subventricular zones. The intermediate zone is an architectonic subdivision that was located between cortical zone and subventricular zone which contained growing axons, migrating postmitotic neurons and cells in glial lineages. Except for some disintegration and disorganization of intermediate zone in group II, no other difference could be made at this magnification among the different groups (Fig. 68.A-D).

At higher magnification (400x), the differentiating cells of marginal and the cortical plate were clearly visible in group I. A number of large sized developing neurons and small sized deeply staining developing and differentiating glial cells were also observed. Below the cortical zone developing white matter was also seen (Fig. 69.A). In group II, the number of glial cells tended to be increased as compared to group I. The developing neurons showed frequent pyknosis. The cells were sparsely arranged with increased intervening intercellular matrix and edematous spaces giving spongiform appearance. Degenerating cells and gliosis were also frequently seen (Fig. 69.B). In group III, recoveries of cells were seen. They were arranged in layers as in group I. They showed recovery from the deleterious effect of alcohol on brain. The total picture was comparable to group I (Fig. 69.C). In group IV the layering of the cellular arrangement was not present as in groups I and III but the cellular density was observed to be markedly increased (Fig.69.D). It may be due to the effect of folic acid on cellular proliferation.

Table 20 : Histomorphometric parameters of cerebrum of fetus on GD 18 in different groups

Parameters	Group I (Mean±SD)	Group II (Mean±SD)	Group III (Mean±SD)	Group IV (Mean±SD)	F-value	P-value
Total thickness (µm)	784.22±66.02	665.72±20.21	746.06±43.03	768.98±65.93	8.115	<0.01
Thickness of marginal zone (µm)	31.80±2.45	27.18±2.85	29.66±5.36	31.03±3.22	2.464	>0.05
Thickness of cortical plate (µm)	252.82±13.12	187.56±20.89	242.86±25.52	254.55±8.35	24.150	<0.01
Thickness of ventricular zone (µm)	102.48±5.40	125.16±7.83	96.62±8.09	102.36±14.48	22.663	<0.01
Cells in cortical plate (Cells/mm^2)	18450±333.81	10600±709.12	17500±632.45	19050±2016.36	96.745	<0.01

Morphometric analysis showed that the total thickness of cerebral hemisphere, cortical plate, ventricular zone and the number of cells in cortical plate significantly differed between the groups while the thickness of marginal zone didn't differ significantly when analyzed by one way ANOVA (Table 20 & Fig. 21-23). The total thickness of cerebral hemisphere and the thickness of marginal zone were maximum in group IV and minimum in group II. Similarly, the cortical zone was thickest in group IV and thinnest in group II. Inversely, the ventricular zone was thickest in group II and thinnest in group IV. The number of cells in the cortical plate was maximum in group IV and minimum in group II (Table 20 & Fig. 21-23).

Groupwise comparison showed that the total thickness of cerebral hemisphere from marginal to ventricular zone was significantly less in alcohol-exposed fetuses of group II as compared to remaining groups. Although maximum thickness was observed in fetuses of group I, no significant difference was observed among groups I, III and IV ($p>0.05$). Similarly, the thickness of cortical plate was also significantly reduced in fetuses of group II as compared to groups I, III and IV ($p<0.01$). No such difference was observed among groups I, III and IV ($p>0.05$) though maximum thickness was observed in group IV fetuses. In contrast, the thickness of ventricular zone was significantly more in cerebrum of group II fetuses as compared to remaining

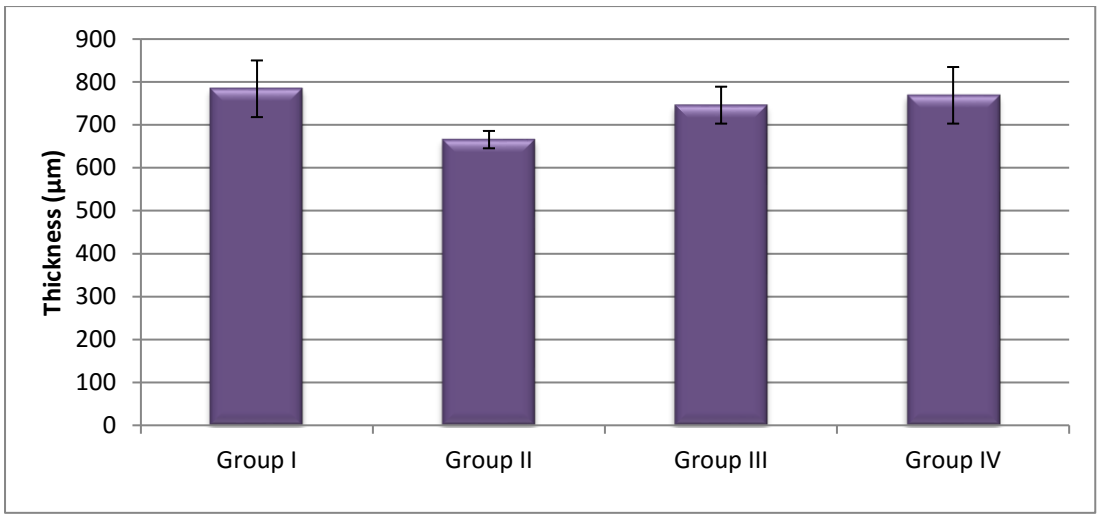


Fig. 21 : Total thickness of cerebrum in different groups

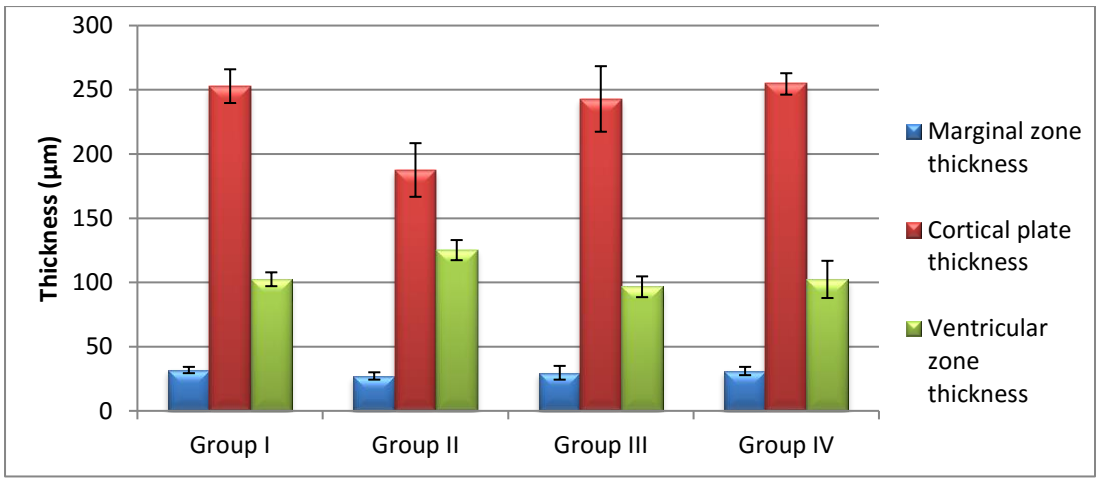


Fig. 22 : Thickness of marginal zone, cortical plate and ventricular zone in different groups

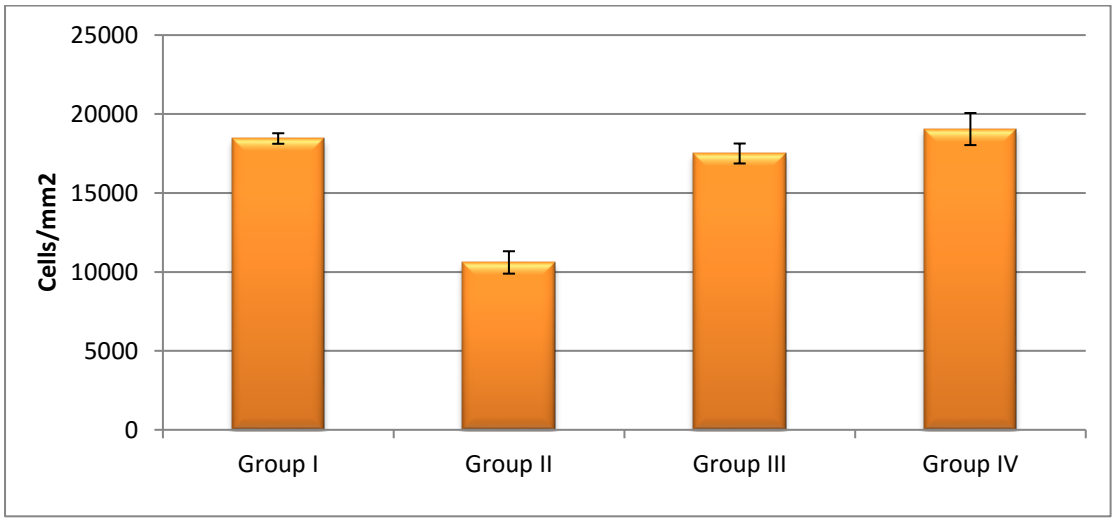


Fig. 23 : Cellular density in cortical plate of different groups

groups (p>0.01) while no such difference was observed among groups I, III and IV (p>0.05). The density of cells (cells/mm^2) was significantly reduced in the cortical plate of group II fetuses as compared to remaining groups (p<0.01). No significant difference was observed among groups I, III and IV (p>0.05), though the density of cells was maximum in group IV fetuses (Tables 20, 21 & Fig. 21-23).

Table 21 : Groupwise comparison of histomorphometric parameters of fetal cerebrum

Groups compared	p-value			
	Total thickness	Thickness of cortical plate	Thickness of Ventricular zone	Number of cells in cortical plate
I Vs II	<0.01	<0.01	<0.01	<0.01
I Vs III	>0.05	>0.05	>0.05	>0.05
I Vs IV	>0.05	>0.05	>0.05	>0.05
II Vs III	<0.05	<0.01	<0.01	<0.01
II Vs IV	<0.01	<0.01	<0.01	<0.01
III Vs IV	>0.05	>0.05	>0.05	<0.05

Hippocampus

The haematoxylin and eosin stained sections of the developing hippocampus showed the c- shaped Ammon's horn and the dentate gyrus. No difference was observed among different groups at low magnification (100x). Under higher magnification (400x), the study was focused on CA3 region. In group I, numerous neuroblasts and developing rounded glial cells were seen in haematoxylin and eosin stained sections in the CA3 region of hippocampus (Fig. 70.A). In group II, the number of neuroblasts was greatly decreased. Large number of edematous spaces appeared among the cells giving spongiform appearance. Many of the cells were enlarged in size, vacuolated showing rupture of their cytoplasmic membrane. Many pyknotic nuclei were also observed at many places. There was marked reduction in the number of

cells present in CA3 region. The enlarged cells were bigger in size than those of group I. Degeneration of the cells were generalized feature (Fig. 70.B). In group III also the frequency of cells in the pyramidal cell layer was reduced in comparison to that of group I but degeneration was much less than in group II (Fig. 70.C). In group IV, the cells were comparable to that of group I (Fig. 70.D).

Cerebellum

Haematoxylin and eosin stained sections of developing cerebellar cortex of group I showed three different zones. Outermost external granular layer that consisted of mitotically active precursor cells that have migrated from internal germinal layer followed by a thin developing molecular layer and the inner granular layer. Th external granular layer was in process of adding granule cells in the internal granular layer. In the internal granular layer spherical cells was observed. Some of the cells looked triangular migrating towards the surface of the internal granular layer; still many of the cells were spherical in shape in order to differentiate into Purkinje cells. Few small sized developing neuroglial cells were frequently seen in different layers (Fig. 71.A). In group II, the population of cells was much reduced in the inner granular layer. The cells were enlarged in size showing ballooning degeneration. At some of the places cellular debris was seen. The cells migrating from the external granular layer had lost their rounded shape and were clumped together and become much smaller. At some places, vacuolated cells were also seen showing the signs of degeneration. Karyolysis and karyorrhexis were generalized features (Fig. 71.B). In group III, the thickness of inner granular layer was increased in comparison to group II and also the number of cells looked to be increased. Only few cells were observed undergoing degenerative process. This showed protection of the cells from alcohol insult (Fig. 71.C). In group IV, the frequency of the cells present in the internal granular layer was comparable to that of group I. Differentiating Purkinje cells were seen to be migrating towards

the surface of internal granular layer. Overall features were comparable with group I (Fig. 71.D).

Postnatal Growth

The offspring from eight pregnant dams in each group were weighed every week from their birth till PND 70. The differences in their weights among the groups were statistically significant throughout whole period except on the PND 70 when the significant difference disappeared, although the mean weight of pups of group II remained lower than the other groups, while the weights of the offspring of groups I, III and IV were comparable. These results showed that prenatal alcohol intoxication not only affects the prenatal growth as shown earlier but also affects the postnatal growth, while folic acid administration induced catch up growth (Tables 22, 23 & Fig. 24).

Table 22 : Weight of fetus in different postnatal days

Postnatal days	Weight of Pups (g) (Mean± SD)				F-value	p-value
	Group I	Group II	Group III	Group IV		
PND 0	1.52±0.10	1.36±0.22	1.50±0.17	1.91±0.15	16.567	<0.01
PND 7	2.61±0.16	1.87±0.41	2.59±0.14	2.61±0.16	17.691	<0.01
PND 14	4.60±0.22	3.59±0.36	4.34±0.34	4.09±0.16	18.441	<0.01
PND 21	6.15±0.30	5.06±0.48	5.63±0.64	5.07±0.24	11.082	<0.01
PND 28	9.33±0.77	7.16±0.13	8.31±1.62	7.09±0.41	10.667	<0.01
PND 35	13.06±1.05	9.05±0.31	11.10±2.13	9.50±1.27	14.349	<0.01
PND 42	14.81±0.82	12.85±0.82	14.91±1.03	15.12±1.86	6.075	<0.01
PND 49	17.90±1.27	13.90±0.84	17.79±1.27	17.14±1.64	17.160	<0.01
PND 56	19.36±0.84	14.95±0.47	18.44±1.40	18.44±1.52	23.301	<0.01
PND 63	20.65±1.53	16.20±0.35	20.72±1.46	19.73±1.49	21.336	<0.01
PND 70	21.37±1.41	19.92±0.60	21.50±1.44	21.60±1.31	3.250	>0.05

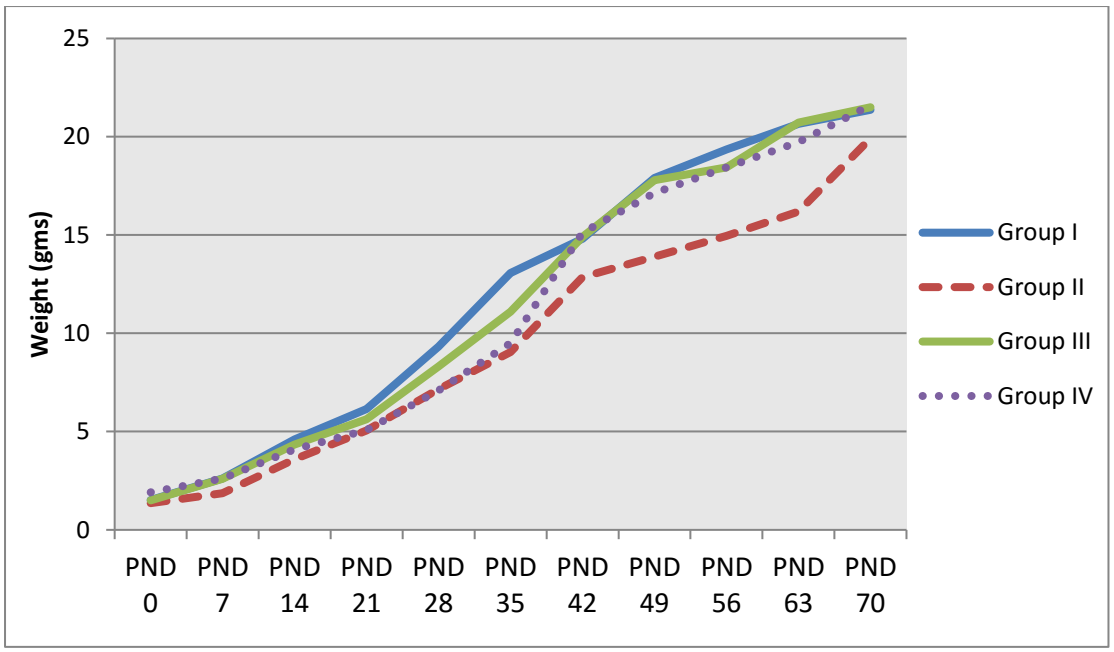


Fig. 24 : Postnatal growth of pups of different groups

Table 23 : Groupwise comparison of weight of fetus in different postnatal days.

Groups compared	p-value									
	PND 0	**PND 7**	**PND 14**	**PND 21**	**PND 28**	**PND 35**	**PND 42**	**PND 49**	**PND 56**	**PND 63**
I Vs II	>0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
I Vs III	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05
I Vs IV	<0.01	>0.05	<0.01	<0.01	<0.01	<0.01	>0.05	>0.05	>0.05	>0.05
II Vs III	>0.05	<0.01	<0.01	>0.05	>0.05	<0.05	<0.01	<0.01	<0.01	<0.01
II Vs IV	<0.01	<0.01	<0.01	>0.05	>0.05	>0.05	<0.01	<0.01	<0.01	<0.01
III Vs IV	<0.01	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Behavioral study

Open field test

Open field test was used to measure the locomotor activity and exploratory behavior. The values for ambulation, rearing, freezing time, grooming and number of faecal boli were significantly different in different

Table 24 : Open field scores in different groups

Groups	Ambulation (Mean±SD)	Rearing (Mean±SD)	Freezing time (Secs) (Mean±SD)	Grooming (Mean±SD)	Faecal boli (Mean±SD)
I	134.50±20.61	25.88±9.52	8.62±2.39	3.63±1.02	2.75±0.19
II	98.00±13.37	12.00±3.12	19.25±2.05	3.13±0.99	5.00±1.60
III	119.88±20.00	25.12±6.38	6.62±2.33	1.38±0.52	1.75±0.49
IV	169.00±31.77	35.38±11.26	4.25±0.83	1.88±0.54	1.38±0.42
F-value	12.876	11.018	26.065	5.960	8.145
p-value	<0.01	<0.01	<0.01	<0.01	<0.01

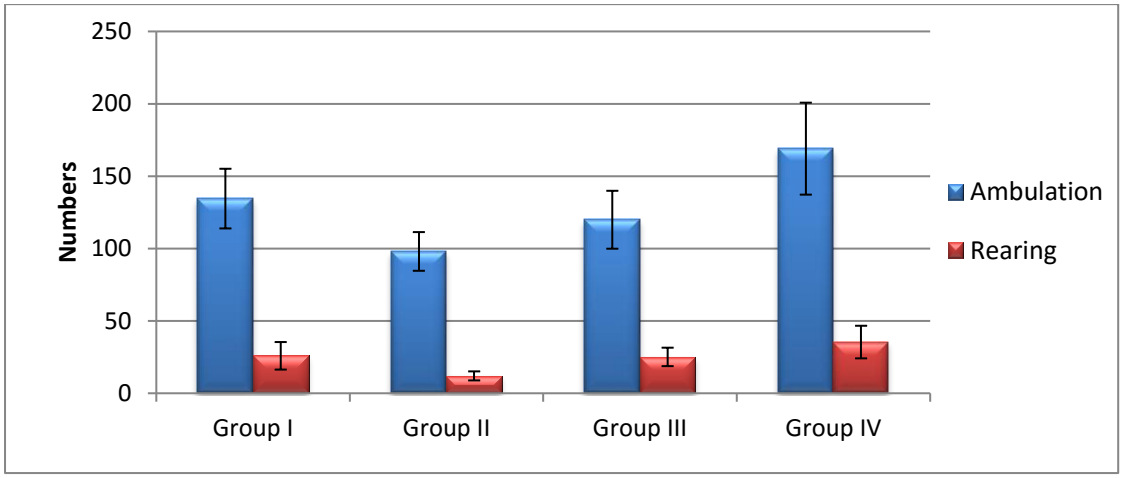


Fig. 25 : Ambulation and rearing scores of different groups of mice in open field test

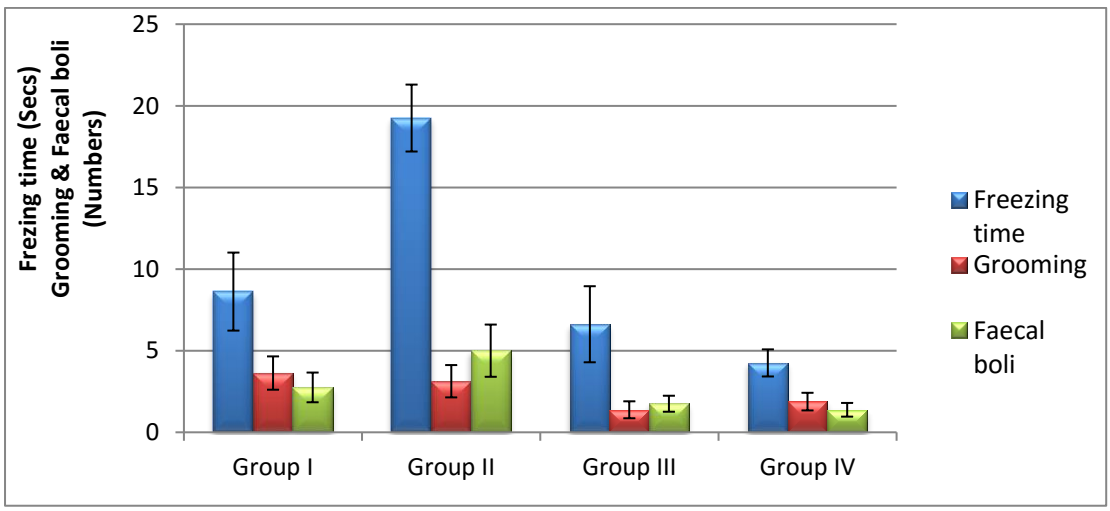


Fig. 26 : Freezing time, grooming and faecal boli scores of different groups of mice in open field test

groups of mice when analyzed by one way ANOVA and Kruskal-Wallis test. Highest ambulation and rearing scores were observed in group IV and lowest in group I. Similarly freezing time and defecation was maximum in group II and minimum in group IV while the grooming score was highest in group I and lowest in group III (Table 24, Fig. 25 & Fig. 26).

Table 25 : Groupwise comparison of different parameters open field test

Groups compared	p-value				
	Ambulation	**Rearing**	**Freezing time**	**Grooming**	**Faecal boli**
I Vs II	<0.05	<0.05	<0.01	>0.05	<0.05
I Vs III	>0.05	>0.05	>0.05	<0.01	>0.05
I Vs IV	<0.05	>0.05	>0.05	<0.05	>0.05
II Vs III	>0.05	<0.05	<0.01	<0.01	<0.01
II Vs IV	<0.01	<0.01	<0.01	<0.01	<0.01
III Vs IV	<0.01	>0.05	<0.05	>0.05	>0.05

Post hoc analysis indicated significant decrease in ambulation and rearing and increase in freezing time in group II when compared with group I ($p<0.01$) and group IV ($p<0.01$). But when groups II and III were compared, group III showed significant increase in rearing scores ($p<0.01$) and significant decrease in freezing time ($p<0.01$) but the increase in ambulation scores were not significant ($p>0.05$). The data show that alcohol intoxication during pregnancy significantly decreased locomotor activity in offspring and folic acid administration reduced this deleterious effect of alcohol. The defecation score or number of faecal boli was significantly higher in group II when compared with group I ($p<0.05$), group III ($p<0.01$) and group IV ($p<0.01$) but no significant difference was observed among groups I, III and IV. Grooming didn't significantly differ in group II as compared to that in group I but it was significantly lower in group III and IV as compared to groups I and II (Table 24, Table 25, Fig. 25 & Fig. 26).

Elevated plus maze test

In the elevated plus maze test there was significant difference between the groups ($p<0.01$) for number of entries into open arm, closed arm and central square as well as time spent in those areas when analyzed by one way ANOVA and Kruskal-Wallis test. Highest open arm entries and open arm duration was observed in group I, highest central square entries and central square duration in group IV, highest closed arm duration in group II and highest closed arm entries in group IV (Table 26, Fig. 27 & Fig. 28).

Table 26 : Number of entries and time spent in different areas of elevated plus maze in different groups

Groups	Number of entries (Mean±SD)			Time spent (Mean±SD) (Secs)		
	Open arm	Central square	Closed arm	Open arm	Central square	Closed arm
Group I	6.38±2.13	17.88±3.98	13.88±4.19	46.00±12.95	58.62±15.52	195.38±31.95
Group II	4.00±1.31	15.25±5.06	12.50±2.56	12.25±3.45	44.50±6.14	243.25±18.33
Group III	3.50±1.31	14.38±2.82	11.62±1.85	27.75±10.52	52.50±14.43	219.75±19.45
Group IV	6.00±2.27	24.50±4.11	21.00±2.73	40.38±12.26	68.25±6.63	191.38±16.48
F-value	4.987	10.084	16.674	6.096	6.740	10.655
p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Post hoc analysis showed that open arm entries were not significantly different in prenatal alcohol treated mice in group II as compared to remaining groups($p>0.05$) while it was significantly lower in group III as compared to that in group I ($p<0.05$) and group IV ($p<0.05$) pups. Central square entries were also not significantly different in group II mice in comparison to those in groups I and III mice, but it was significantly higher in group IV when compared with groups I ($p<0.01$), II ($p<0.01$) and III ($p<0.01$). The closed arm entries also didn't significantly differ in group II as compared to that in groups I and III, but it was significantly higher ($p<0.01$) in group IV as compared to remaining groups. In contrast to the number of entries, the mice in group II

spent significantly less time in open arms as compared to that in groups I ($p<0.01$), III ($p<0.01$) and IV ($p<0.01$) mice. Although mean open arm duration was highest in group I, than in groups III and IV, but the values were not statistically significant when compared among the groups ($p>0.05$). Central square duration was lower in group II mice as compared to remaining groups but didn't significantly differ from that in groups I and III. However, it was significantly lower as compared to that in group IV. No significant difference in central square duration was observed between groups I and III. Similarly, no difference in the duration was observed between groups I and IV but it was significantly lower in group III as compared to that in group IV ($P<0.05$).

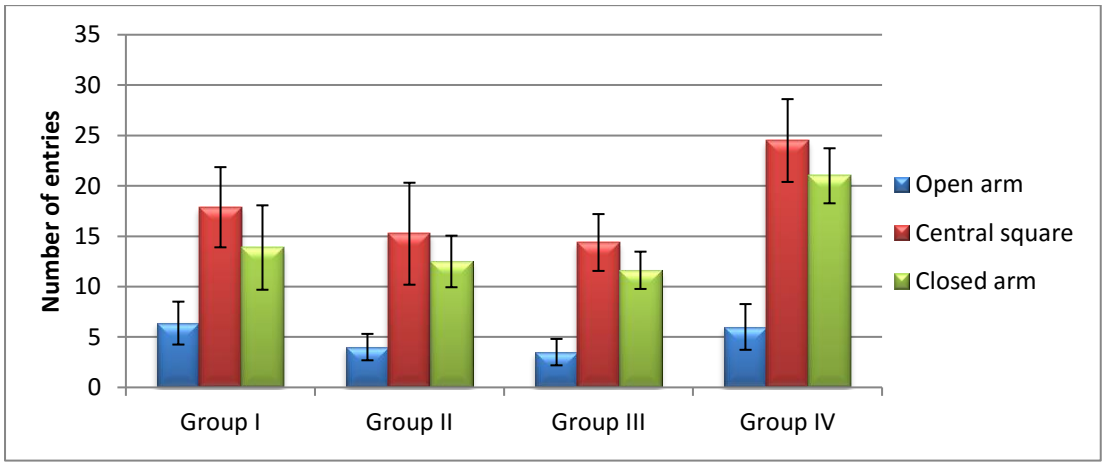


Fig. 27 : Number of entries in different areas of elevated plus maze

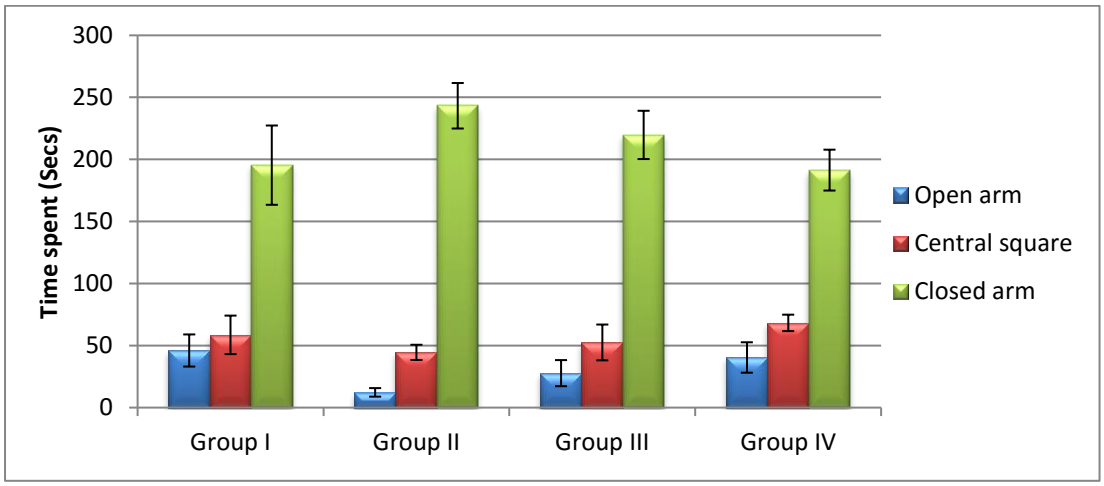


Fig. 28 : Time spent in different areas of elevated plus maze

Closed arm duration was significantly higher in group II as compared to that in groups I and IV ($p<0.01$) but didn't significantly differ from that in group III, although it was higher than that in group III. No significant difference in time spent in closed arm was observed among groups I, III and IV ($p>0.05$). The data show that ethanol administration during pregnancy significantly increased the anxiety and decreased the exploration in offspring in terms of increased closed arm duration and decreased open arm duration and folic acid exposure along with ethanol reversed this effect (Table 26, Table 27, Fig. 27 & Fig. 28).

Table 27 : Groupwise comparison of number of entries and time spent in different areas of elevated plus maze

Groups compared	p-value					
	Number of entries			Time spent		
	Open arm	Central square	Closed arm	Open arm	Central square	Closed arm
I Vs II	>0.05	>0.05	>0.05	<0.01	>0.05	<0.01
I Vs III	<0.05	>0.05	>0.05	>0.05	>0.05	>0.05
I Vs IV	>0.05	<0.05	<0.01	>0.05	>0.05	>0.05
II Vs III	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05
II Vs IV	>0.05	<0.01	<0.01	<0.01	<0.01	<0.01
III Vs IV	<0.05	<0.01	<0.01	>0.05	<0.05	>0.05

Morris water maze test

Morris water maze test was used to access the learning and memory of the mice. The test was performed in four different sessions, namely, place acquisition, probe trial, reversed acquisition and visual platform.

Place acquisition session was done for 5 consecutive days. The mean escape latency of each day was pooled to get the overall mean escape latency and compared among the groups. The overall mean escape latency was found to be significantly different between the groups ($P<0.01$) (Table 28 & Fig. 29). Pups in group II took more time to locate the platform followed by the pups of

groups I, III and least by the pups of group IV. On post hoc analysis, the time taken by pups to locate the platform i.e., escape latency, in group II was significantly higher as compared to those in groups I($p<0.01$), III($p<0.01$) and IV($p<0.01$), while no significant difference was observed among groups I, III and IV ($p>0.05$) (Tables 28, 29 & Fig. 29).

Table 28 : Overall escape latency during place acquisition of Morris water maze test

Groups	Overall escape latency (Secs) (Mean±SD)
I	33.82±11.72
II	48.90±18.08
III	33.72±14.97
IV	22.60±9.89
F-value	11.138
p-value	<0.01

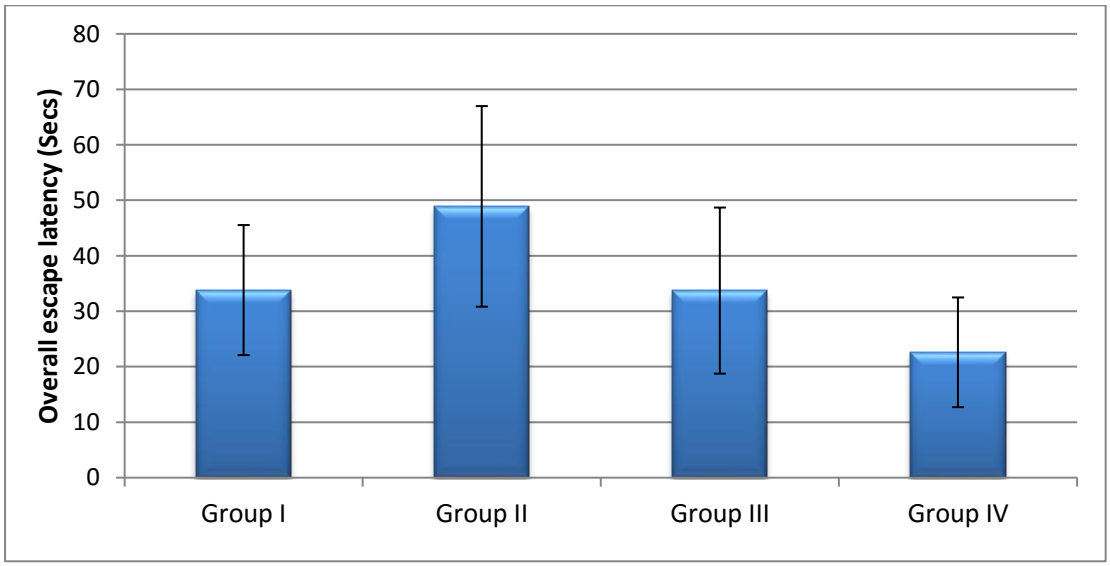


Fig. 29 : Overall escape latency during place acquisition of Morris water maze test

Table 29 : Groupwise comparison of overall escape latency during place acquisition

Groups compared	p-value
I Vs II	<0.01
I Vs III	>0.05
I Vs IV	>0.05
II Vs III	<0.01
II Vs IV	<0.01
III Vs IV	>0.05

Table 30 : Day wise comparison of escape latency during place acquisition of Morris water maze test

Groups	Escape latency (Mean±SD) (Secs)				
	Day 1	Day 2	Day 3	Day 4	Day 5
I	55.25±19.18	45.00±19.79	35.50±18.06	21.25±8.78	12.12±7.04
II	71.88±5.59	60.75±5.09	45.75±7.70	42.12±11.75	24.00±5.50
III	62.75±14.68	45.38±15.60	28.50±8.16	18.25±6.27	13.75±2.38
IV	59.00±15.58	22.88±7.51	14.75±7.48	9.88±2.99	6.50±0.76
F-value/χ^2-value	F=1.898	F=10.838	χ^2=19.534	F=22.812	χ^2=19.807
p-value	>0.05	<0.01	<0.01	<0.01	<0.01

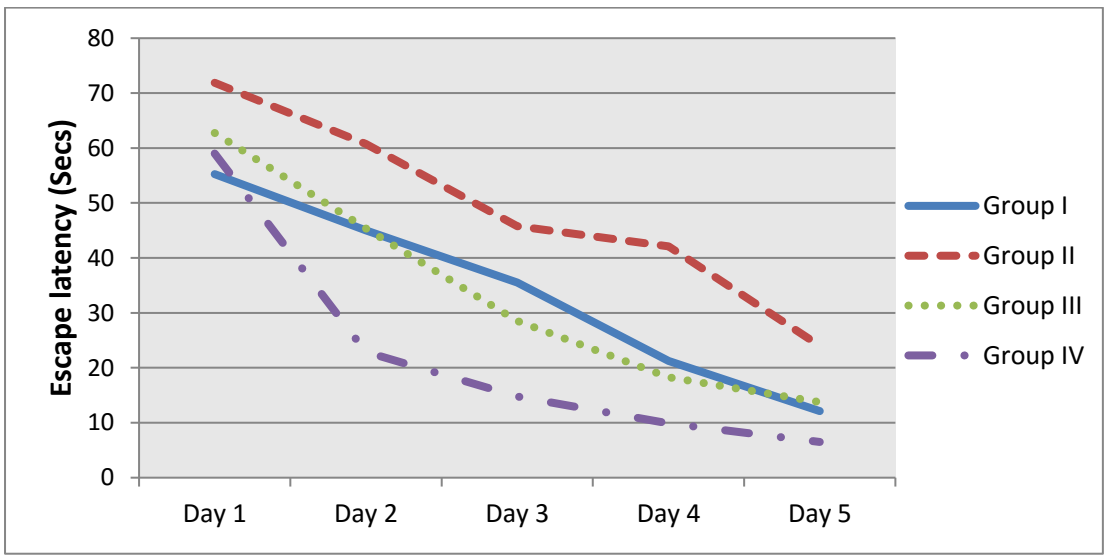


Fig. 30 : Learning curve of mice different groups during place acquisition of Morris water maze test

On day wise comparison of place acquisition of Morris water maze test, there was no significant difference between the groups in escape latency on the first day of the training ($p>0.05$) but on 2nd , 3rd, 4th and 5th day, the difference was significant ($p<0.01$) (Table 30 & Fig. 30).

Table 31 : Groupwise comparison of escape latency during place acquisition of Morris water maze test in different days

Groups compared	p-value				
	Day 1	Day 2	Day 3	Day 4	Day 5
I Vs II	>0.05	>0.05	>0.05	<0.01	<0.01
I Vs III	>0.05	>0.05	>0.05	>0.05	>0.05
I Vs IV	>0.05	<0.01	<0.01	<0.05	>0.05
II Vs III	>0.05	>0.05	<0.05	<0.01	<0.01
II Vs IV	>0.05	<0.01	<0.01	<0.01	<0.01
III Vs IV	>0.05	<0.05	>0.05	>0.05	<0.01

On post hoc analysis on the 2nd day, no significant difference in escape latency was observed for pups in group II as compared to that in groups I and III, although the escape latency was higher in group II as compared to foresaid groups. However, the escape latency was significantly lower in group IV pups as compared to remaining groups. On 3rd day of place acquisition, the escape latency in group II was significantly higher than that in groups III and IV, but didn't differ significantly from that of group I. Again, the escape latency for group IV was significantly lower as compared to groups I and II, but didn't differ significantly from that of group III. On the 4th day of the test, the escape latency for group II was significantly higher ($p<0.01$) as compared to remaining groups. No significant difference in escape latency was observed between groups I and III as well as between groups III and IV, however, in group IV it was significantly lower as compared to that in group I ($p<0.05$). On the 5th day, again the escape latency in group II was significantly higher as

compared to that in remaining groups. No significant difference was observed between groups I and III as well as groups I and IV ($p>0.01$) but it was significantly higher ($p<0.01$) in group III as compared to that in group IV (Tables 30, 31 & Fig. 30). The data showed that prenatal alcohol administration significantly decreased the learning ability of mice while folic acid administration increased the same.

Table 32 : Time spent in the quadrant (which initially contained platform) during probe trial of Morris water maze test

Groups	Time spent (Secs) (Mean±SD)
I	47.50±6.33
II	27.69±4.39
III	37.62±3.42
IV	49.12±4.61
F-value	30.819
p-value	<0.01

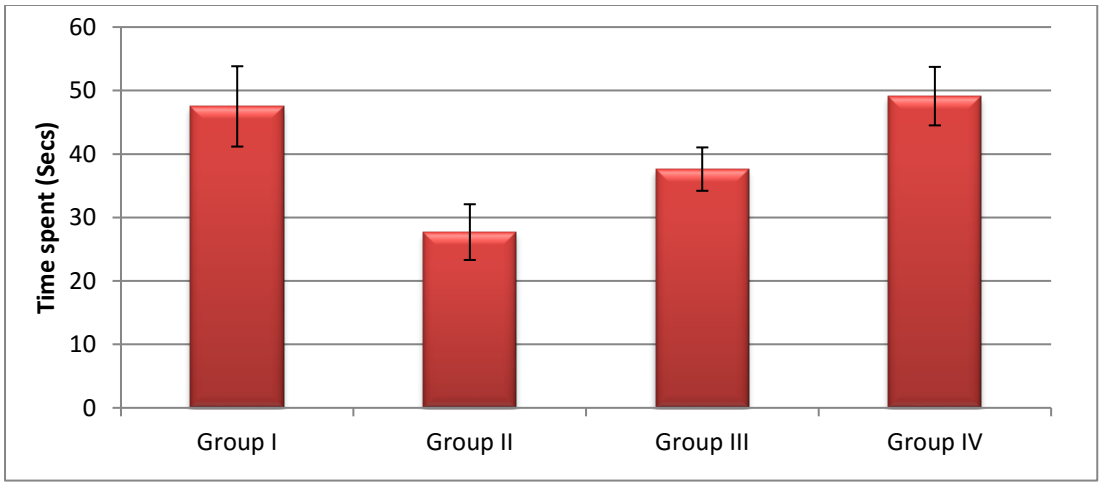


Fig. 31 : Time spent in the quadrant during probe trial of Morris water maze test

The memory of mice was tested during probe trial by observing the time of permanence in the quadrant that initially contained the platform. There was significant difference in time of permanence in the quadrant among the

different groups ($p<0.01$). The mice in group IV spent comparatively longer duration and that of group II stayed for least duration in the quadrant (Table 32 & Fig. 31). On post hoc analysis the mice of group II spent significantly less time in the quadrant as compared to groups I ($p<0.01$), III ($p<0.01$)and group IV ($p<0.01$). Similarly, the groups I ($p<0.01$) and IV ($p<0.01$) mice spent significantly longer time as compared to that of group III while no significant difference between groups I and IV was observed (Table 32, Table 33 & Fig. 31).

Table 33 : Groupwise comparison of time spent during the probe trial

Groups compared	p-value
I Vs II	<0.01
I Vs III	<0.01
I Vs IV	>0.05
II Vs III	<0.01
II Vs IV	<0.01
III Vs IV	<0.01

The probe trial session was followed by reversed acquisition in which the hidden platform was placed in the opposite quadrant than that was during place acquisition and again the escape latency was observed for 5 consecutive days. The overall mean escape latency significantly differed between the groups ($p<0.01$). Again, the overall mean escape latency was highest for group II and least for group IV (Table 34 & Fig. 32).

On post hoc analysis the escape latency of pups of group II was significantly higher as compared to groups I ($p<0.01$), III ($p<0.01$)and IV($p<0.01$) while no significant difference was observed among groups I, III and IV ($p>0.05$) (Tables34, Table 35 & Fig. 32).

Table 34 : Overall escape latency during reversed acquisition of Morris water maze test

Groups	Overall escape latency (Secs) (Mean±SD)
I	30.60±13.58
II	47.47±16.72
III	32.25±11.37
IV	20.70±8.68
F-value	11.021
p-value	<0.01

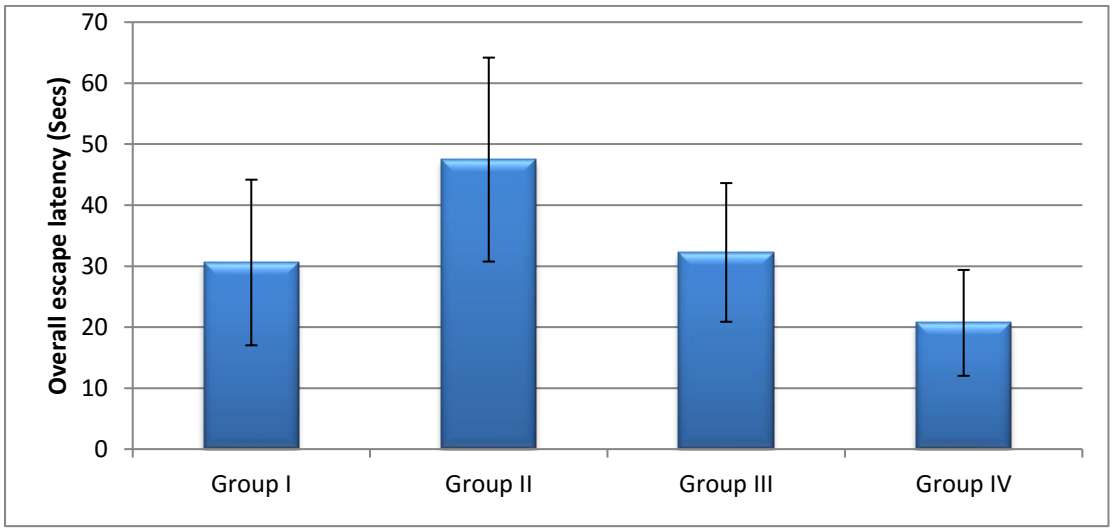


Fig. 32 : Overall escape latency during reversed acquisition of Morris water maze test

Table 35 : Groupwise comparison of overall escape latency during reversed acquisition

Groups compared	p-value
I Vs II	<0.01
I Vs III	>0.05
I Vs IV	>0.05
II Vs III	<0.01
II Vs IV	<0.01
III Vs IV	>0.05

Table 36 : Day wise comparison of escape latency during reversed acquisition of Morris water maze test

Groups	Escape latency (Secs) (Mean±SD)				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
I	63.87±22.43	43.75±13.81	20.87±4.96	14.75±4.33	9.75±2.71
II	69.75±20.07	53.75±16.01	49.50±16.77	35.87±23.22	28.50±13.72
III	61.75±15.45	42.25±12.43	32.50±10.78	14.25±2.71	10.50±2.82
IV	46.12±13.14	22.25±6.56	16.87±7.29	10.62±4.24	7.62±3.32
F-value/χ^2-value	F=2.240	F=8.642	F=14.405	χ^2=17.470	χ^2=19.653
p-value	>0.05	<0.01	<0.01	<0.01	<0.01

During day wise comparison of reversed acquisition session of Morris water maze test, on the 1st day of the test, the difference in escape latencies of different groups was insignificant ($p>0.05$) while during the following four days, the difference among the groups was significant ($p<0.01$) (Table 36 & Fig. 33).

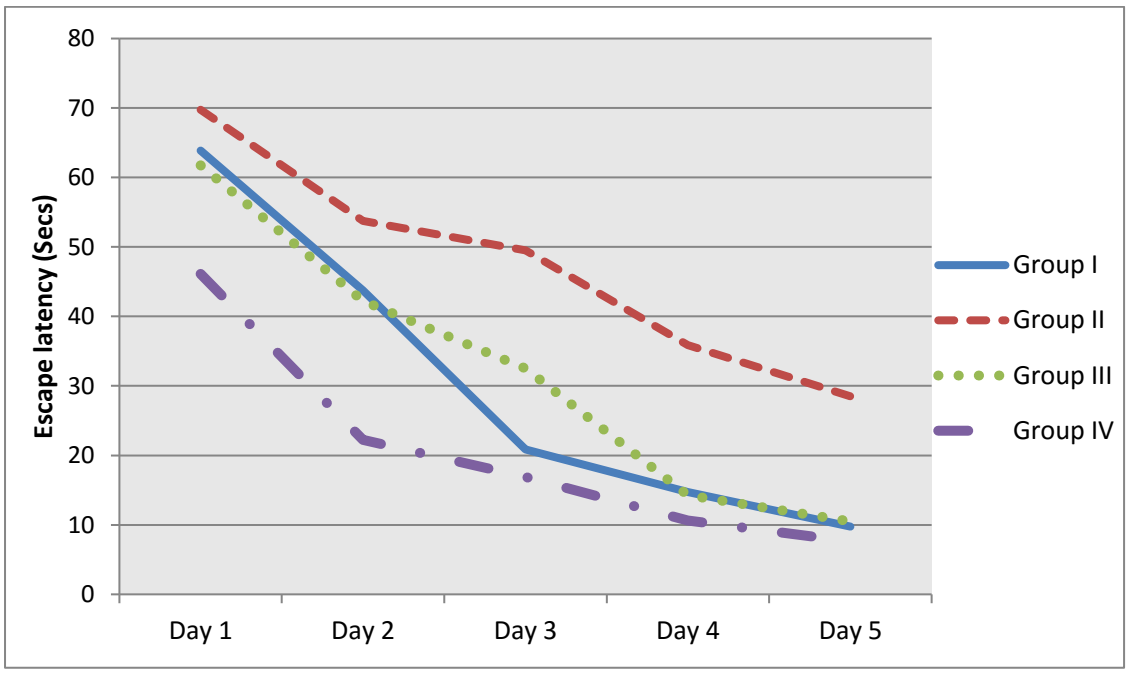


Fig. 33 : Learning curve of mice different groups during reversed acquisition of Morris water maze test

Table 37 : Groupwise comparison of escape latency during reversed acquisition of Morris water maze test in different days

Groups compared	p-value				
	Day 1	Day 2	Day 3	Day 4	Day 5
I Vs II	>0.05	>0.05	<0.01	<0.01	<0.01
I Vs III	>0.05	>0.05	>0.05	>0.05	>0.05
I Vs IV	>0.05	<0.05	>0.05	>0.05	>0.05
II Vs III	>0.05	>0.05	<0.05	<0.01	<0.01
II Vs IV	>0.05	<0.01	<0.01	<0.01	<0.01
III Vs IV	>0.05	<0.05	<0.05	>0.05	>0.05

On the 2nd day of the test, no significant difference was observed in escape latency among groups I, II and III ($p>0.05$). However, the escape latency in group IV was significantly low as compared to remaining groups. On the 3rd day, the escape latency of pups of group II was significantly higher as compared to that in groups I ($p<0.01$), III ($p<0.05$) and IV ($p<0.01$). Similarly, the escape latency of group IV pups was significantly lower ($p<0.05$) as compared to that in group III, while no such difference ($p>0.05$) was observed between groups I and III as well as groups I and IV. On the 4th day, again the escape latency of group II pups was significantly higher as compared to remaining groups ($p<0.01$), while no such difference was observed among groups I, III and IV ($p>0.05$). On the 5th day, the result was same as that in 4th day of the test (Table 36, Table 37 & Fig. 33). The result showed that although pups in all groups found difficulty in learning to locate hidden platform initially but the folic acid exposed pups learned faster than alcohol exposed pups.

During cued version, although the least escape latency was observed in group IV and highest in group II, no statistical difference was found among the groups (Table 38 & Fig. 34).

Table 38 : Escape latency during cued version of Morris water maze test

Groups	Escape latency (Secs) (Mean±SD)
I	10.62±2.67
II	13.87±2.75
III	12.12±4.38
IV	10.12±3.39
F-value	2.004
p-value	>0.05

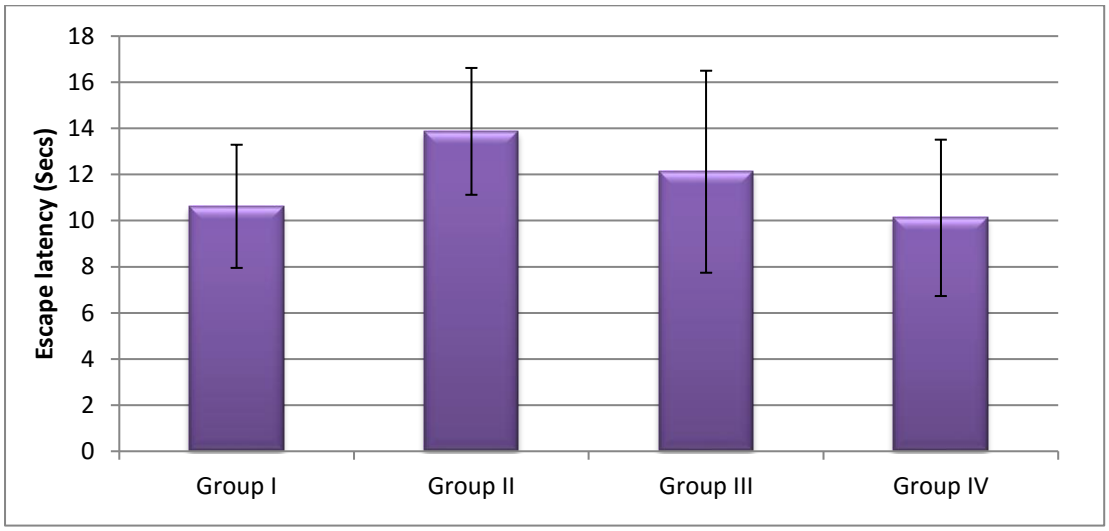


Fig. 34 : Escape latency during cued version of Morris water maze test

Behavior despair test

During behavior despair test, significant difference among the groups ($p<0.01$) was observed for the immobility time. The highest immobility time was observed in group II and lowest in group IV (Table 39 & Fig. 35). On post hoc analysis, the immobility time for prenatally alcohol exposed pups of group II was significantly higher as compared to remaining groups ($p<0.01$). The immobility time of Group III was also significantly higher as compared to that of group IV ($p<0.01$). No significant difference in the immobility time was observed between groups I and III as well as I and IV during the analysis ($p>0.05$) although lowest was observed in pups of group IV (Table 39, Table 40 & Fig. 35).

Table 39 : Immobility time during behavior despair test in different groups

Groups	Immobility time (Secs) (Mean±SD)
I	82.88±34.51
II	207.50±29.32
III	118.38±32.25
IV	63.00±17.39
F-value	38.574
p-value	<0.01

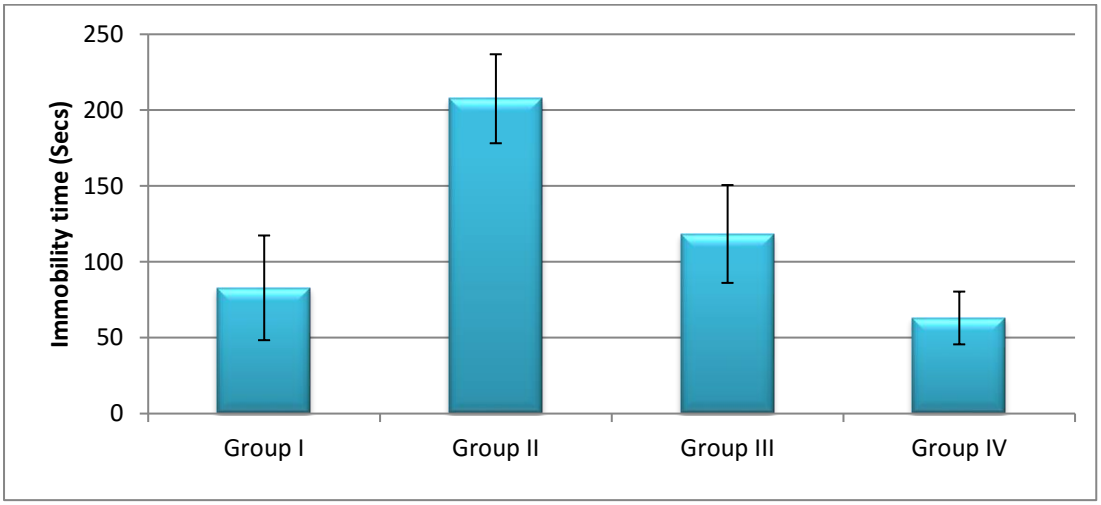


Fig. 35 : Immobility time during behavior despair test

Table 40 : Groupwise comparison of immobility time during behavior despair test

Groups compared	p-value
I Vs II	<0.01
I Vs III	>0.05
I Vs IV	>0.05
II Vs III	<0.01
II Vs IV	<0.01
III Vs IV	<0.01

Tail suspension test

Significant difference was observed between the groups in the parameter of immobility time during tail suspension test. The highest immobility time was observed in group II and the lowest in IV (Table 41 & Fig. 36). On post hoc analysis, the immobility time was significantly higher in prenatal alcohol-exposed pups of group II as compared to groups I ($p<0.01$), III ($p<0.05$) and IV ($p<0.01$). Similarly, the immobility time in group III was significantly higher than in group IV ($p<0.05$) but no significant difference was observed between groups I and IV as well as groups I and III ($p>0.05$) (Tables 41, 42 & Fig. 36).

Table 41 : Immobility time during tail suspension test in different groups

Groups	Immobility time (Secs) (Mean±SD)
I	84.88±12.36
II	159.50±45.61
III	118.50±34.18
IV	71.50±9.71
F-value	14.125
p-value	<0.01

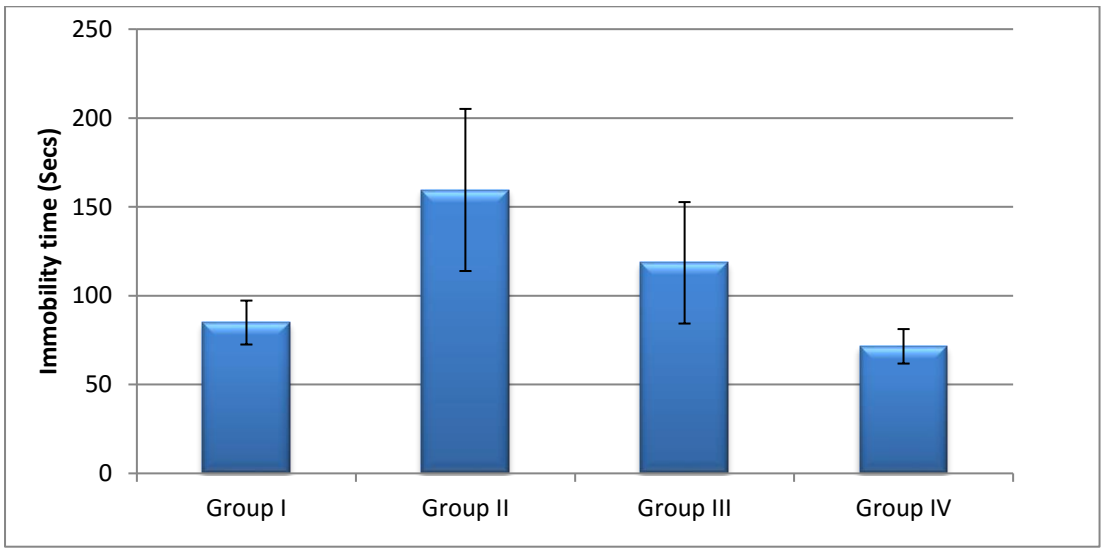


Fig. 36 : Immobility time during tail suspension test

Table 42 : Groupwise comparison of immobility time during tail suspension test

Groups compared	p-value
I Vs II	<0.01
I Vs III	>0.05
I Vs IV	>0.05
II Vs III	<0.05
II Vs IV	<0.01
III Vs IV	<0.05

Brain of pups

Brain weight and relative brain weight

Brain weight of the pups at PND 70 significantly differed between the groups ($p<0.01$). Minimum brain weight was seen in group II and maximum in group I (Table 43, Fig. 37 & Fig. 59). Although, the relative brain weight was lowest in group II and highest in group III but they didn't differed significantly ($p>0.05$) when compared among the different groups (Table 43).

Table 43 : Brain weights and relative brain weight of pups on PND 70

Groups	Weight of brain (g)	Relative brain weight (%)
I	0.53±0.02	2.34 ±0.15
II	0.45 ±0.02	2.22±0.19
III	0.50 ±0.02	2.43±0.13
IV	0.52± 0.04	2.32±0.25
F-value	12.817	2.003
p-value	<0.01	>0.05

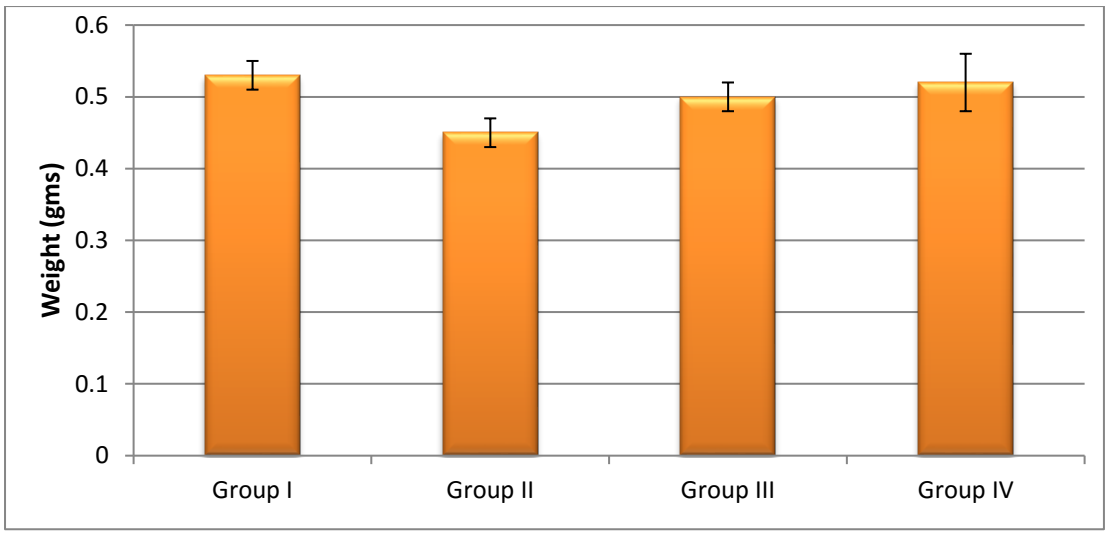


Fig. 37 : Brain weight of pups of different groups

Table 44 : Groupwise comparison of brain weight of pups

Groups compared	p-value
I Vs II	<0.01
I Vs III	>0.05
I Vs IV	>0.05
II Vs III	<0.01
II Vs IV	<0.01
III Vs IV	.>0.05

Post hoc analysis showed that the decrease in brain weight in group II due to prenatal alcohol intoxication was significant ($p<0.01$) as compared to group I. In utero folic acid exposure along with alcohol significantly ($p<0.01$) increased the brain weight in group III as compared to group II. Brain weights in groups III and IV as compared to group I was not significant (Tables 43, 44 & Fig. 37).

Histological study

Cerebrum

The haematoxylin and eosin stained sections of cerebral frontal lobe revealed six layers in the grey matter. From outside inward they were

molecular, outer granular, outer pyramidal, inner granular, inner pyramidal and polymorphic layers. All the layers were normally arrange in group I. Large number of granules cells were seen in layers II and IV and pyramidal cells in layers III and V. The neuroglial cells were also frequently seen in different layers (Fig. 72.A1 & Fig. 72.A2). In group II, the typical six layered arrangement of the frontal cortex was disorganized. Edematous spaces had appeared in layer I, vasodilation was also seen. The neuroglial cells were markedly enlarged in size. In layer II, the neurons showed marked degeneration. In layer III also cellular degeneration and decrease in their number was observed. In layers IV, V and VI also marked degeneration of the cells as well as decrease in their frequency was seen. Both the granule cells and pyramidal cells looked to be decreased in density (Fig. 72.B1 & Fig. 72.B2). In group III, a better laminar arrangement of cells was observed. Some of the cells were observed to undergoing degeneration. The number of glial cells was comparatively increased. The overall picture was better than group II but still not as good as in group I (Fig. 73.C1 & Fig. 73.C2). In group IV, the cellular frequency was increased in all the layers as compared to group I. The layered arrangement of the cells was observed. The overall picture was comparable to that of group I (Fig. 73.D1 & Fig. 73.D2).

In sections stained with Nissl's stain in group I, Nissl's granules of pyramidal cells in layers III and V were prominent and appeared as compact bodies in the form of flakes and granules. The granules were arranged around the nucleus and especially at the proximal part of dendrites. The intensity of the Nissl's granules was more in pyramidal cells of layer V than those in layer III (Fig. 74.A & Fig. 75.A).

In group II, the pyramidal cells in both the layers were faintly stained as compared to group I cells indicating decreased Nissl's granules content in their

cytoplasm. In both the layers III and V, the number of cells looked to be decreased. Their size was markedly reduced and the size of nucleus appeared to be increased in comparison to the cellular size. Many cells in layer V lost their pyramidal shape with dissolution of Nissl's substances and disruption of nuclear membrane was observed (Fig. 74.B & Fig. 75.B).

In group III, the pyramidal cells looked to be nearly equal to the size of controls in layer III. The intracellular Nissl's granules were increased as well as the number of cells. In layer V, the large pyramidal cells showed their normal shape though the size of the cells looked to be smaller than those of group I and IV. The intracellular Nissl's granules were normally distributed (Fig. 74.C & Fig. 75.C). In group IV, the cells looked to comparable to those of group I. Increase in the size and number of cells was observed in comparison to group III. In layer V also, the large pyramidal cells were comparable to that of group I. In both layers III and V of the alcohol group II the cells were decreased in number, shrunken size as well as intracellular Nissl's granules were reduced as compared to remaining groups (Fig. 74.D & Fig. 75.D).

In Golgi stained sections of group I, numbers of pyramidal cells of layer V with their axons as well as apical and basal dendrites were clearly seen. The arborizations of dendrites were also clearly visible. The branching of apical and basal dendrites was clearly observed (Fig. 76.A). There was similar appearance of the cells in group III as well as group IV (Fig. 76.C & Fig. 76.D). But in group II, markedly reduced arborization was observed in comparison to groups I, III and IV (Fig. 76.B).

Histomorphometric analysis was done on Nissl stained sections. The sections showed that the thickness of grey matter, number of cells in different layers as well as the diameter of pyramidal neurons in layers III and V were significantly affected by the treatments among the different groups ($p<0.01$).

The grey matter was thickest in group IV and thinnest in group II. The number of neurons in layer V of the cerebral cortex of group IV was maximum and it was minimum in group II. The diameter of the pyramidal cells of layer III and V was maximum in group IV and minimum in group II (Table 45 & Fig. 38-40).

Table 45 : Histomorphometric parameters of pup's cerebrum on PND 70 in different groups

Parameters	Group I (Mean±SD)	Group II (Mean±SD)	Group III (Mean±SD)	Group IV (Mean±SD)	F-value	P-value
Cortical thickness (µm)	862.89±57.88	740.80±17.26	853.88±26.86	923.25±62.98	22.240	<0.01
Number of neurons in layer II/III (cells/mm^2)	2130±333.50	1120±265.83	1840±259.06	1870±312.87	21.655	<0.01
Number of neurons in layer IV (cells/mm^2)	1630±176.70	720±147.57	1420±229.98	1550±158.11	52.829	<0.01
Number of neurons in layer V (cells/mm^2)	1350±212.13	820±244.04	1220±139.84	1360±241.29	14.060	<0.01
Diameter of Layer III pyramidal cells (µm)	13.90±0.91	10.41±0.99	13.04±1.52	14.14±1.43	28.538	<0.01
Diameter of layer V pyramidal cells (µm)	17.17±2.66	13.01±2.38	15.66±2.51	19.17±2.93	14.685	<0.01

Groupwise comparison revealed that the prenatal alcohol exposure in group II significantly decreased the thickness of cerebral cortex as well as the number of cells in layers II/III, IV and V of the cortex. The diameter of the pyramidal cells of layers III and V was reduced as compared to those in group I ($p<0.01$). There was significant decrease in the diameter of pyramidal cells of layer V and thickness of cerebral cortex in group III in comparison to those of group IV. No significant difference was observed in other parameters among the groups I, III and IV ($p>0.05$) (Tables 45, 46 & Fig. 38-40).

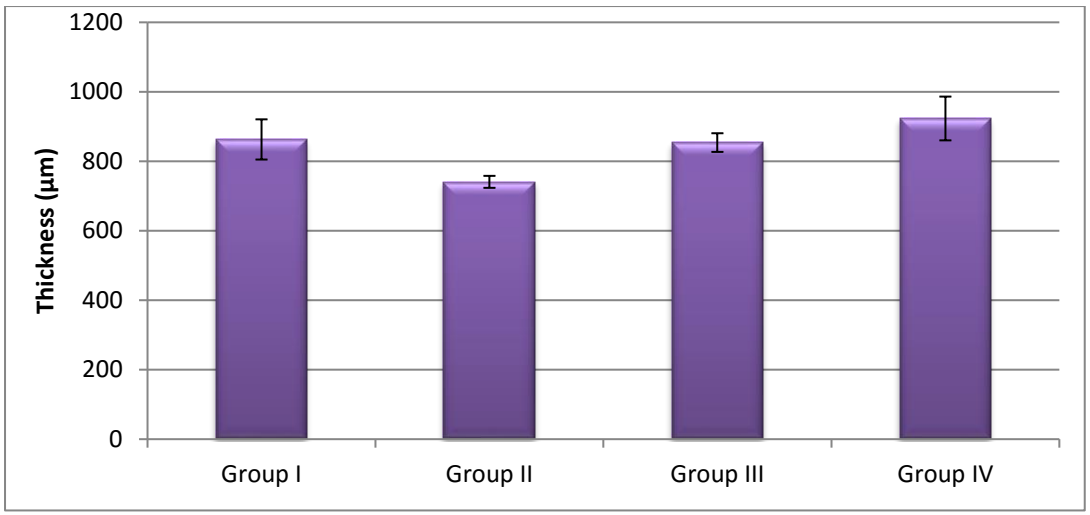


Fig. 38: Cortical thickness of different groups

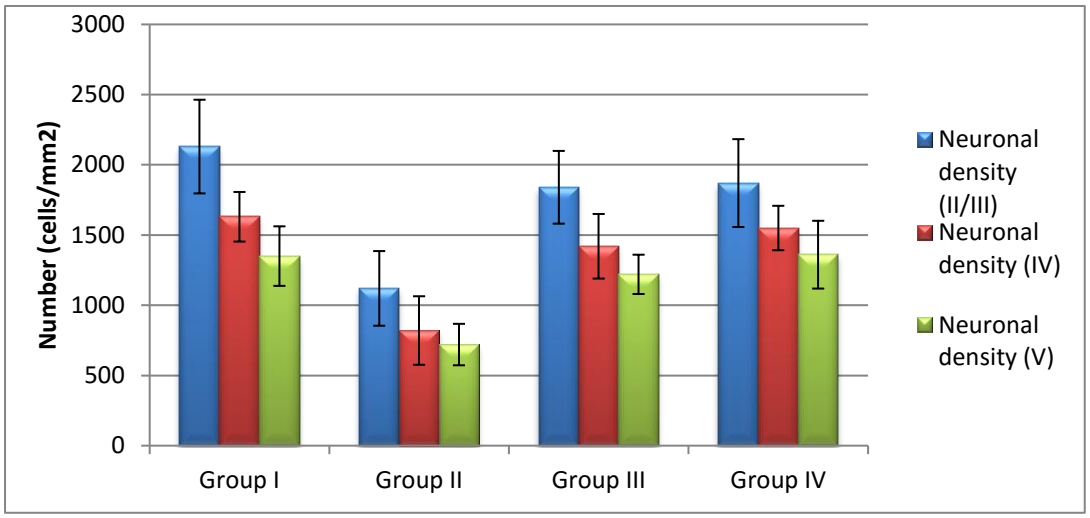


Fig. 39: Neuronal density in different layers of frontal cortex

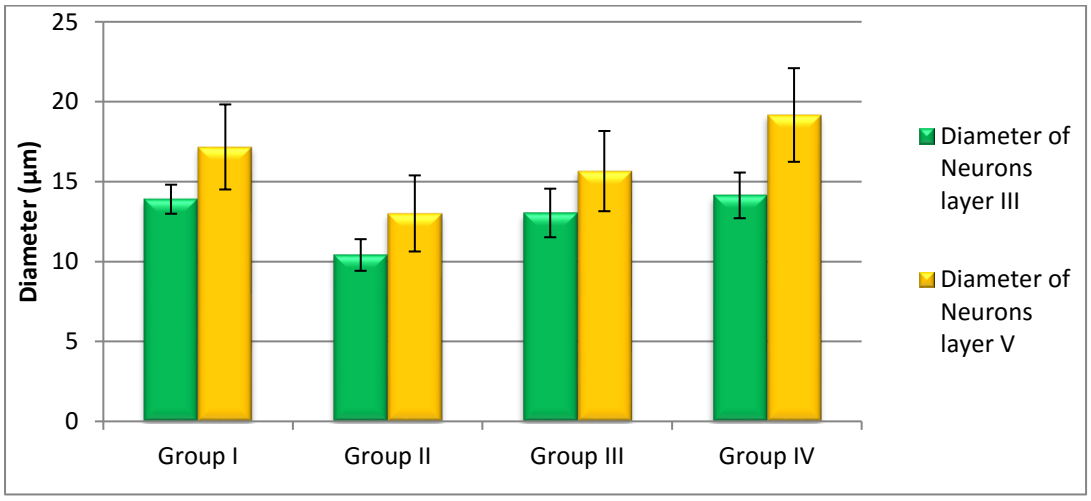


Fig. 40: Diameter of pyramidal cells of layer III and V of frontal cortex

Table 46 : Groupwise comparison of histomorphometric parameters of pup's cerebrum

Groups compared	p-value					
	Cortical thickness	No. of neurons Layer II/II	No. of neurons Layer IV	No. of neurons Layer V	Diameter pyramidal cell (III)	Diameter pyramidal cell (V)
I Vs II	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
I Vs III	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
I Vs IV	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
II Vs III	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05
II Vs IV	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
III Vs IV	<0.05	>0.05	>0.05	>0.05	>0.05	<0.01

Cerebellum

The haematoxylin and eosin stained sections of group I showed 3 layers in cerebellar cortex, the outer molecular layer, middle Purkinje cell layer and the inner granular layer. Small rounded granule cells and Golgi cells were observed in the granular layer. In the Purkinje cell layer, many large flask shaped cells with their apical dendrites were seen lying individually at intervals. Few neurons were also observed in outer molecular layer which were sparsely arranged. Glial cells were frequently observed in different layers (Fig. 77.A).

In group II, thickness as well as the density of granule cells was reduced in the granular layer. At many places there was clumping of granule cells. The Purkinje cells were also reduced in number and size. A few cells also showed degeneration. At places the cells were shrunken and had lost their intracellular contents. Debris of Purkinje cells was observed at some places. Some of the cells showed Pyknotic nuclei. Thus karyolysis and karyorrhexis and pyknotic nuclei were observed in Purkinje cell layer (Fig. 77.B). In group III, recovery of cells was observed in the Purkinje cell layer but the granular layer still

showed decrease in the frequency of granule cells (Fig. 77.C). In group IV, all the layers were comparable to those in group I (Fig. 77.D).

In Nissl stained sections, in group I, a few dispersed neurons and lightly stained neuroglial cells were distinctly observed. In the inner granular layer intensely stained, densely populated many granule cells were seen. Large flask shaped Purkinje cells with their dendrites and distinct nucleus and prominent central nucleolus was also visible. The Nissl's granules were distributed throughout the cytoplasm of the cells and denser near the base of dendrites (Fig. 78.A). In group II, the alteration of shapes of the Purkinje cells were clear. In some Purkinje cells the nuclei were found eccentrically and few neurons lacked nucleolus in their nucleus. The intensity of the Nissl' staining was also decreased in some cells due to fewer amounts of Nissl's granules in their cytoplasm. Some Purkinje cells were pyknotic and few had lost their shape, the amount of Nissl's granules was lost and the vesicular shape of nucleus was distorted. Rupture of the cytoplasmic membrane with extruded intracellular material was seen (Fig. 78.B). In group III, recovery of cells was seen. The Purkinje cells were resuming their flask shape and also the vesicular nucleus was observed. A few degenerated Purkinje cells were also observed. Overall there was comparative recovery in comparison to group II (Fig. 78.C). Granule cells and Golgi cells was observed in granular layer in all the groups thought the density was decreased in group II. In group IV, clear vesicular nuclei and intra cytoplasmic Nissl's granules was observed in the Purkinje cells. The total picture was comparable to that in group I (Fig. 78.D).

In Golgi staining the arborization of Purkinje cell dendrites was compared among the different groups. In group I, the apical dendrites arise as main process from the upper end of the cell body of the Purkinje cells which branch and extend through the molecular layer. This main dendrite repeatedly branches to from a large and fully formed dendritic arborization. The

arborizations were formed from lateral processes that emerged from the main dendrite. Numerous dendritic spines were also visible along the branches of the dendrites (Fig. 79.A). In group II, there was shrinkage of Purkinje cells resulting into its smaller size. Some of the soma of the cells also showed intracellular vacuolization. The dendritic arborization was much reduced as compared to group I. Some cells lacked arborization although they contained the main dendrite and its secondary branches. The number of dendritic spines was much reduced (Fig. 79.B). In group III also, the frequency of dendritic spines on the dendritic arborization looked to be increased in comparison to group II but slightly reduced as compared to those of groups I and IV (Fig. 79.C). In group IV, the dendritic arborization and dendritic spines was comparable to those of group I, rather the arborization was denser than group I (Fig. 79.D).

Table 47 : Histomorphometric parameters of pup's cerebellum on PND 70 in different groups

Parameters	**Group I (Mean±SD)**	**Group II (Mean±SD)**	**Group III (Mean±SD)**	**Group IV (Mean±SD)**	**F-value**	**P-value**
Thickness of cortex (µm)	283.70±37.47	232.16±40.41	280.43±55.47	279.04±55.14	7.888	**<0.01**
Thickness of folium (µm)	602.56±75.27	492.16±85.91	591.30±116.32	591.63±113.82	8.134	**<0.01**
Thickness of molecular layer (µm)	147.67±24.74	116.47±25.81	146.65±34.91	145.58±39.71	6.720	**<0.01**
Thickness of Granular layer (µm)	131.31±17.71	112.31±19.68	130.48±29.03	128.55±19.71	5.003	**<0.01**
No. of cells in molecular layer (cells/mm^2)	21.48±4.38	14.00±2.97	19.45±2.89	19.11±2.32	18.648	**<0.01**
No. of cells in Granular layer (cells/mm^2)	51.83±3.17	38.58±2.44	49.17±2.65	50.33±3.16	15.667	**<0.01**
Purkinje cell linear density (cells/mm)	28.78±4.90	20.28±7.06	26.60±4.81	27.21±5.33	5.356	**<0.01**
Purkinje cells diameter (µm)	16.27±1.96	13.75±1.62	15.64±2.69	15.33±2.26	6.035	**<0.01**

Histomorphometric analysis of the cerebellum was done in Nissl stained sections in all the four groups. It was observed that the thickness of folia, cerebellar cortex, molecular layer and granular layer was significantly affected due to treatments ($p<0.01$) among different groups. The number of cells in molecular and granular layers, the linear density and the diameter of the Purkinje cells was also significantly altered by different treatments during the intrauterine development ($p<0.01$) (Table 47 & Fig. 41-43).

Groupwise comparison revealed that alcohol intoxication during intrauterine life significantly decreased the thickness of molecular and granular layers, the cerebellar cortex as well as that of folium in group II as compared to those in group I ($p<0.01$). The folic acid supplementation along with alcohol in group III significantly increased the thickness of molecular and granular layers, cerebellar cortex and the folium as compared to group II ($p<0.01$). No significant difference in those parameters was observed among the groups I, III and IV ($p>0.05$). Similarly, the number of cells in molecular and granular layers and the Purkinje cell density was significantly reduced in prenatally alcohol-exposed pups of group II as compared to those in group I. Again the folic acid supplementation along with alcohol in group III significantly increased the number of cells in molecular and granular layers and Purkinje cell density as compared to those in group II. No significant difference in number of cells in molecular layer and Purkinje cell density was observed among the groups I, III and IV ($p>0.05$). The number of cells in granular layer also didn't significantly differ ($p>0.05$) between groups I and IV as well as between III and IV but the cells were significantly decreased in group III as compared to that in group I ($p<0.01$) (Tables 47, 48 & Fig. 41-43).

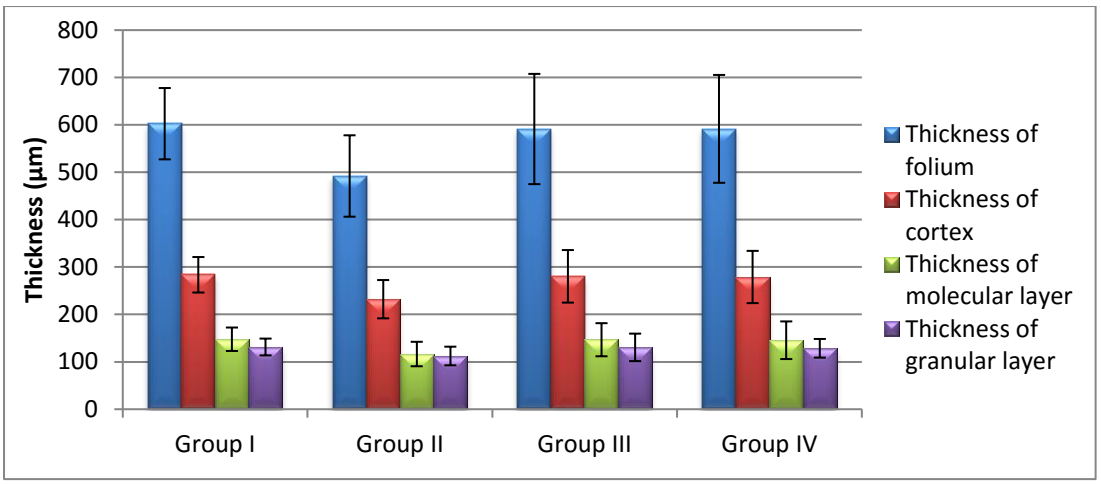


Fig. 41: Thickness of folium, cortex, molecular and granular layers in cerebellum

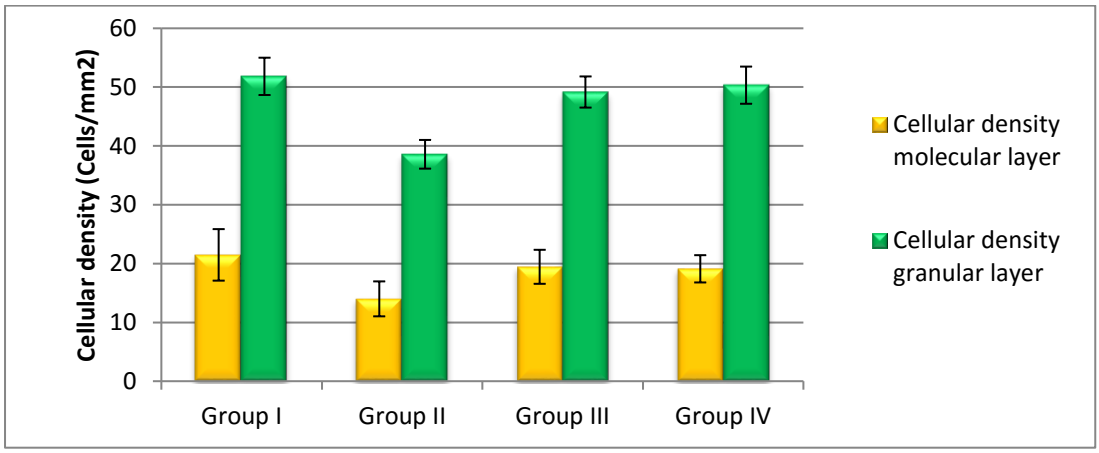


Fig. 42: Cellular density in molecular and granular layer in cerebellum

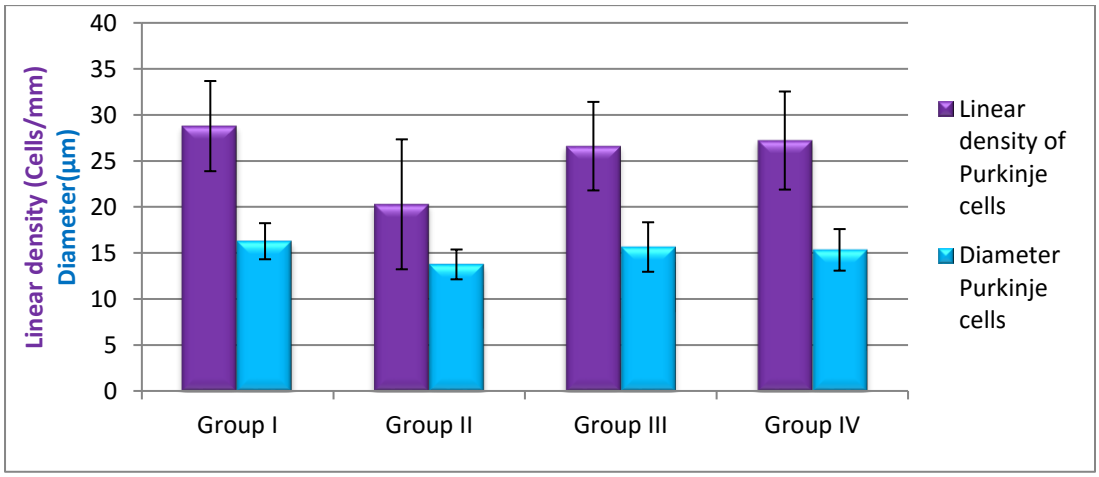


Fig. 43: Linear density of Purkinje cells and its diameter in cerebellum

Considerable shrinkage of Purkinje cells was observed in group II as shown by its decreased diameter as compared to group I ($p<0.01$). Again the

supplementation of folic acid along with alcohol significantly reduced ($p<0.05$) the shrinking effect of alcohol on Purkinje cells in group III as compared to that in alcohol-exposed pups of group II. Although maximum Purkinje cell diameter was in group I, no significant difference in the diameter was observed among the groups I, III and IV ($p>0.05$). The data showed that prenatal alcohol intoxication significantly altered the histomorphometric parameters in the cerebellum of the pups on PND 70 which were moderately neutralized by folic acid supplementation (Tables 47, 48 & Fig. 41-43).

Table 48 : Groupwise comparison of histomorphometric parameters of pups's cerebellum

Groups compared	p-value							
	Thickness of cortex	Thickness of folium	Thickness of molecular layer	Thickness of Granular layer	No. of cells in molecular layer	No. of cells in Granular layer	Purkinje cell linear density	Purkinje cells diameter
I Vs II	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
I Vs III	>0.05	>0.05	>0.05	>0.05	>0.05	<0.01	>0.05	>0.05
I Vs IV	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
II Vs III	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05	<0.05
II Vs IV	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01	<0.05	<0.05
III Vs IV	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Hippocampus

The haematoxylin and eosin stained sections showed different areas of hippocampal formation which are the hippocampus proper and the dentate gyrus. The hippocampus proper or Cornu Ammonis was divisible into different regions according to the distribution, shape, size and branching of axons and dendrites of neurons (CA1-CA4). Each of these regions consists of three layers; stratum molecularae (molecular layer), stratum pyramidale (pyramidal layer) and stratum multiforme (polymorphic layer).

In group I, the haematoxylin and eosin stained sections of the hippocampus contained large densely packed pyramidal cells with globular nuclei in CA3 region and medium sized densely packed pyramidal cells with spherical nucleus in CA1 region in the stratum pyramidale layer which were arranged in 4-6 layers. Few polymorphic neurons were also observed in stratum multiforme. Neuroglial cells were frequently seen in all the layers (Fig. 80.A & Fig. 81.A). In group II, the cytoarchitecture of the pyramidal cell layer was severely disturbed. The thickness of the layer was reduced. Numerous degenerating pyramidal cells were seen in both the CA1 as well as CA3 region. Frequent shrinkage of pyramidal cells with pyknotic nuclei in CA3 region was also observed. In some of the cells, pyknotic nuclei as well as vacuolation were seen. Pericellular lacunar spaces were present, may be due to edematous infiltration which was also responsible for compression of the cells. At some places granular cellular debris was observed. Neuroglial cells were also observed (Fig. 80.B & Fig. 81.B). In group III, there was moderate improvement in cellular picture in comparison to group II, still it was comparable to group I (Fig. 80.C & Fig. 81.C). In group IV, the cellular layer and the cells of the pyramidal layer were comparable to those of group I (Fig. 80.D & Fig. 81.D).

In Nissl stained sections of group I, the pyramidal cells with their apical dendrites were clearly distinguished in both CA1 zone and CA3 zone. The globular nucleus with prominent nucleoli was faintly stained while the perinuclear cytoplasm was darkly stained due to abundant amount of Nissl's granules (Fig. 82.A & Fig. 83.A). In group II, pyramidal cells in CA3 region were sparsely arranged and the intensity of Nissl's staining was reduced as compared to group I due to lesser amount of Nissl's granules. Disruption of cytoplasmic membrane and extrusion of the intracellular content was also seen. Cells were smaller in size and sparsely arranged. Some pyknosis as well as degenerated pyramidal cells were also observed. In CA1 region also the

pyramidal cells were lightly stained, the layers of cells were reduced and a few cells showed large vacuolation, probably may be undergoing degenerative process (Fig. 82.B & Fig. 83.B). In group III, the cellular condition was much improved in CA3 region and moderate Nissl's granules were observed in comparison to group II. The layers of the cells were also increased. In CA1 region also the cellular layer was increased (Fig. 82.C & Fig. 83.C). In group IV also, the thickness of CA1 and CA3 region and the cellular size was comparable to group I (Fig. 82.D & Fig. 83.D).

In Golgi stained sections, nerve cells, dendrites, axons and terminal arborizations were clearly visible in group I. The pyramidal cells were characterized by the apical dendrites and basal dendrites which projected into stratum moleculare and axons projecting into polymorphic layer towards the alveus. The apical dendrites arising from the apex of the pyramidal cell was a single, long, thick dendrite that branches several times forming vast dendritic arborization were clearly visible. The basal dendrites consisting of 3 to 5 primary dendrites were also seen (Fig. 84.A). In group II, the dendritic arborization was much reduced as compared to group I. Vacuolated cells and non branching dendrites were also common (Fig. 84.B). In group III, the branching was more than in group II but less than in groups I and IV which were comparable together (Fig. 84.C & Fig. 84.D)).

Histomorphometry was done in Nissl stained sections. The study showed that the thickness of pyramidal cell layer as well as density of cells in different regions of the pyramidal layer significantly differed due to different treatments in different groups ($p<0.01$). The minimum number of cells in different regions of pyramidal layer as well as its thickness was observed in group II while maximum number of cells in CA1 and the maximum thickness of CA1 and CA3 region were observed in group I. Maximum number of cells in CA3 region was observed in group IV (Table 49, Fig. 44 & Fig. 45).

Table 49 : Histomorphometric parameters of pup's hippocampus on PND 70 in different groups

Parameters	Group I (Mean±SD)	Group II (Mean±SD)	Group III (Mean±SD)	Group IV (Mean±SD)	F-value	P-value
Thickness of pyramidal layer of CA1 region (µm)	60.86±7.51	49.79±5.87	54.79±8.10	56.15±9.49	2.883	<0.05
Number of pyramidal cells in CA1 region (No./mm^2)	5401.20±502.91	3805.60±540.69	4742.10±766.28	5381.90±529.67	18.866	<0.01
Thickness of pyramidal layer of CA3 region (µm)	75.60±8.52	61.68±5.39	67.94±6.59	68.73±4.42	6.549	<0.01
Number of pyramidal cells in CA3 region (No./mm^2)	3412.70±634.74	1507.90±271.08	2916.70±957.75	3567.30±736.46	13.073	<0.01

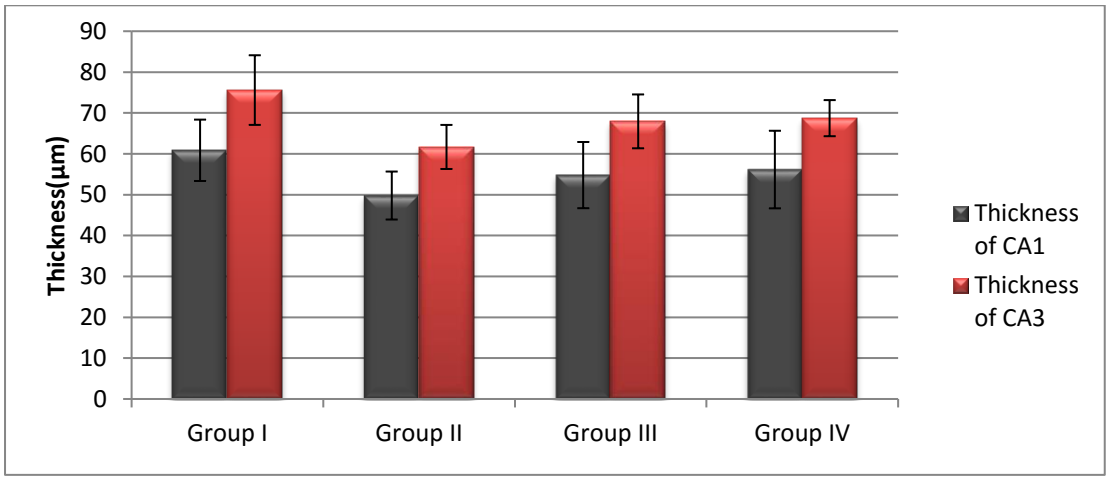


Fig. 44 : Thickness pyramidal layer of CA1 and CA3 region of hippocampus

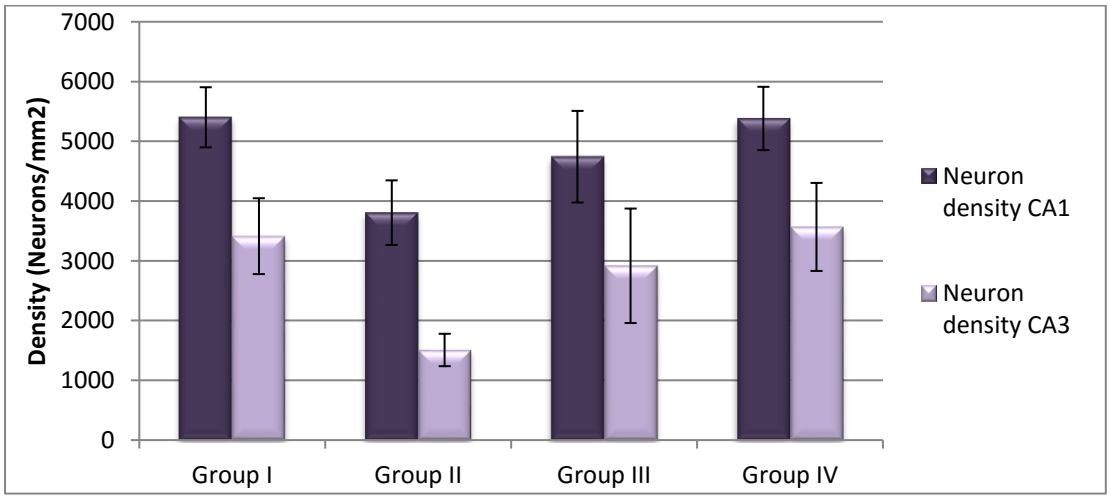


Fig. 45 : Neuronal density of pyramidal layer of CA1 and CA3 region of hippocampus

Groupwise comparison showed that the thickness of pyramidal layer of CA1 and CA3 regions as well as density of pyramidal cells in those regions significantly decreased in alcohol exposed pups of group II as compared to control ($p<0.01$). Folic acid co-treatment along with alcohol significantly increased the number of cells in group III as compared to those in alcohol-exposed pups of group II. Except for the decreased number of cells in CA1 region in group III as compared to that in group IV ($p<0.05$), no significant difference in other parameters was observed among groups I, III and IV ($p>0.05$) (Tables 49, 50, Fig. 44 & Fig. 45).

Table 50 : Groupwise comparison of histomorphometric parameters of pup's hippocampus

Groups compared	p-value			
	CA1 Thickness	**CA1 Neuronal density**	**CA3 Thickness**	**CA3 Neuronal density**
I Vs II	<0.05	<0.01	<0.01	<0.01
I Vs III	>0.05	>0.05	>0.05	>0.05
I Vs IV	>0.05	>0.05	>0.05	>0.05
II Vs III	>0.05	<0.01	>0.05	<0.01
II Vs IV	>0.05	<0.01	<0.05	<0.01
III Vs IV	>0.05	<0.05	>0.05	>0.05

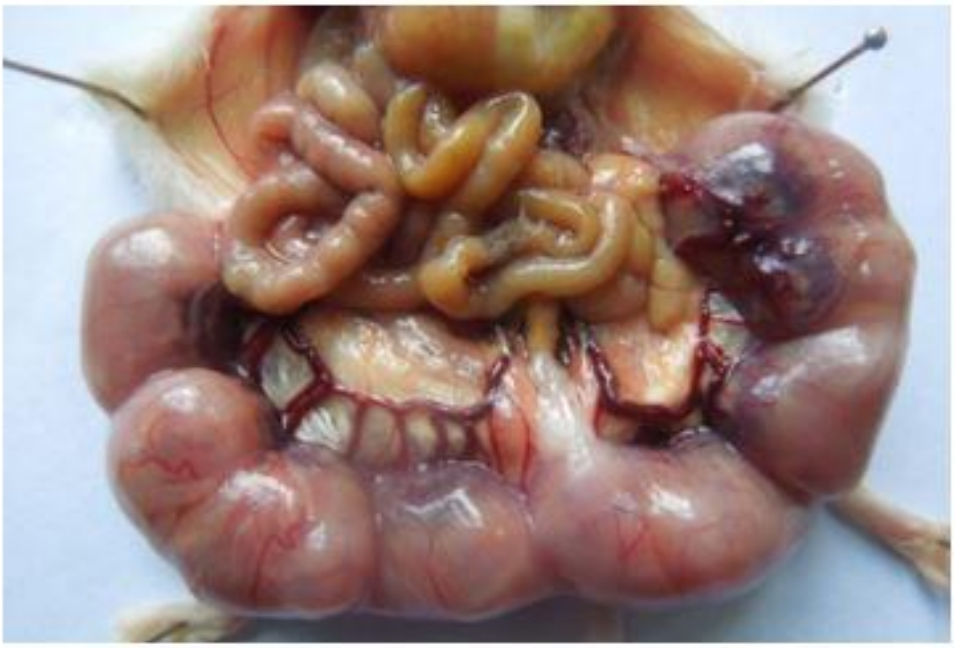


Fig. 46: Uterine horns of group I mice showing normal fetal implantations

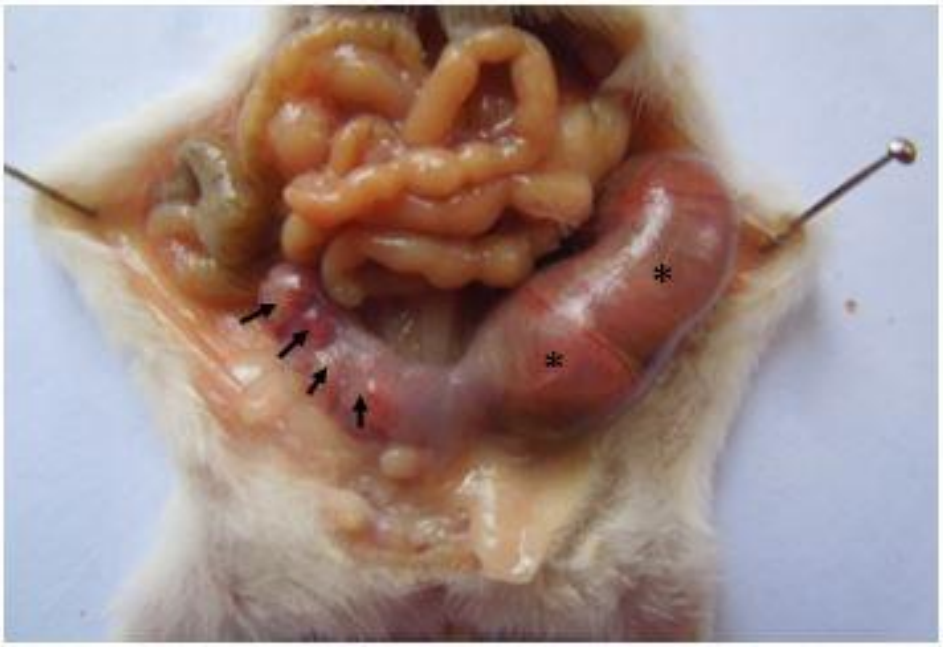


Fig. 47: Uterine horns of group II mice showing four resorptions (↖) on right horn and two fetuses in left horn (*)

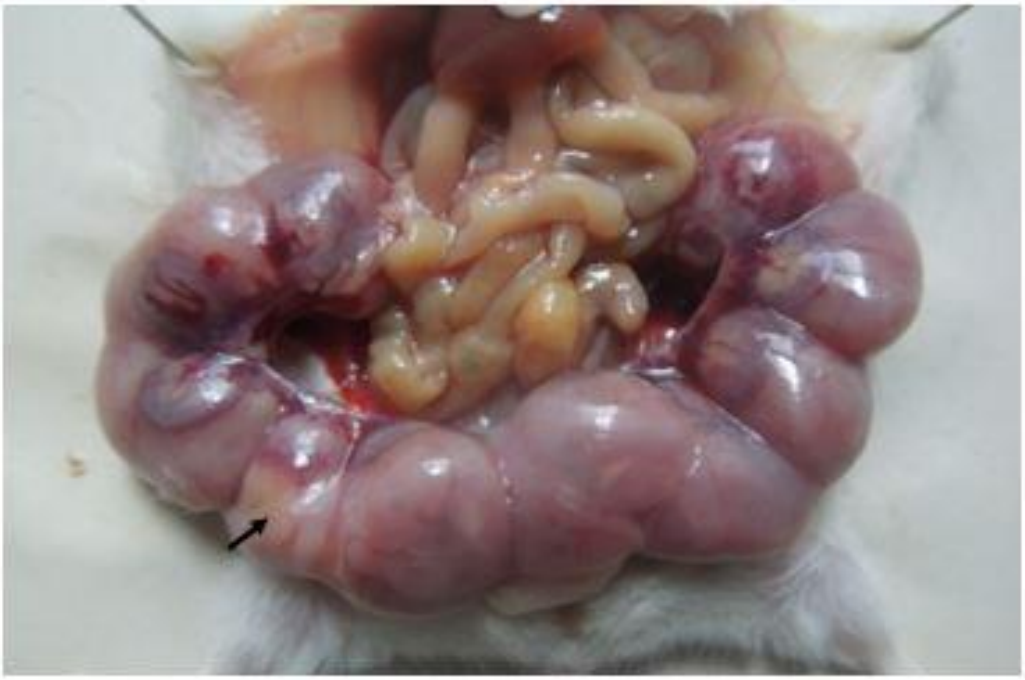


Fig. 48: Uterine horns of group III mice with one dead fetus (↖) in right horn

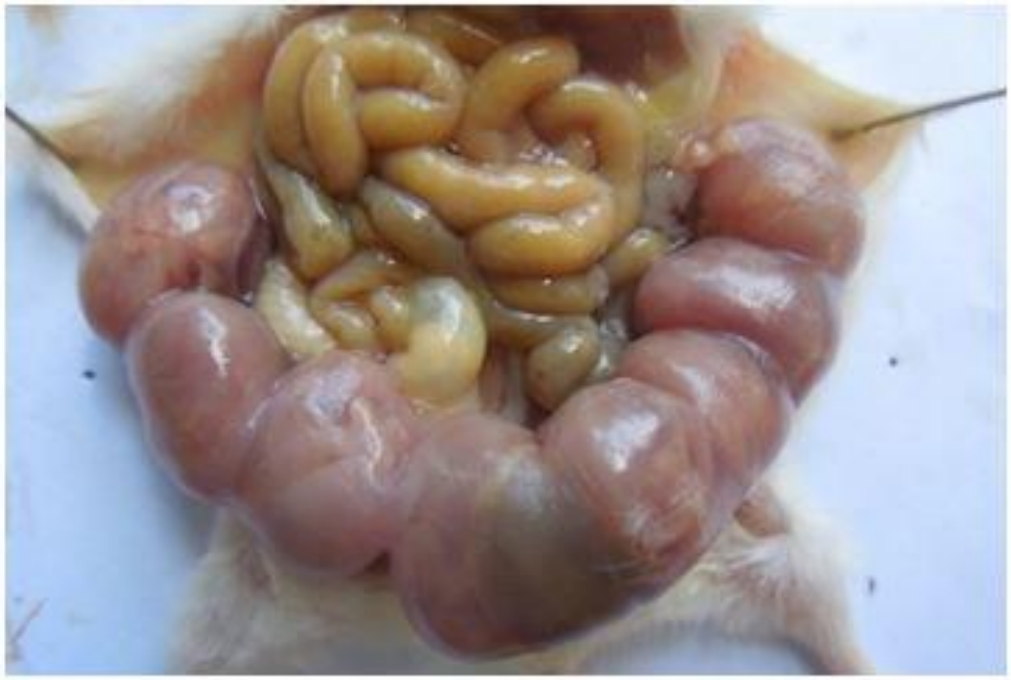


Fig. 49: Uterine horns of group I mice showing normal fetal implantations

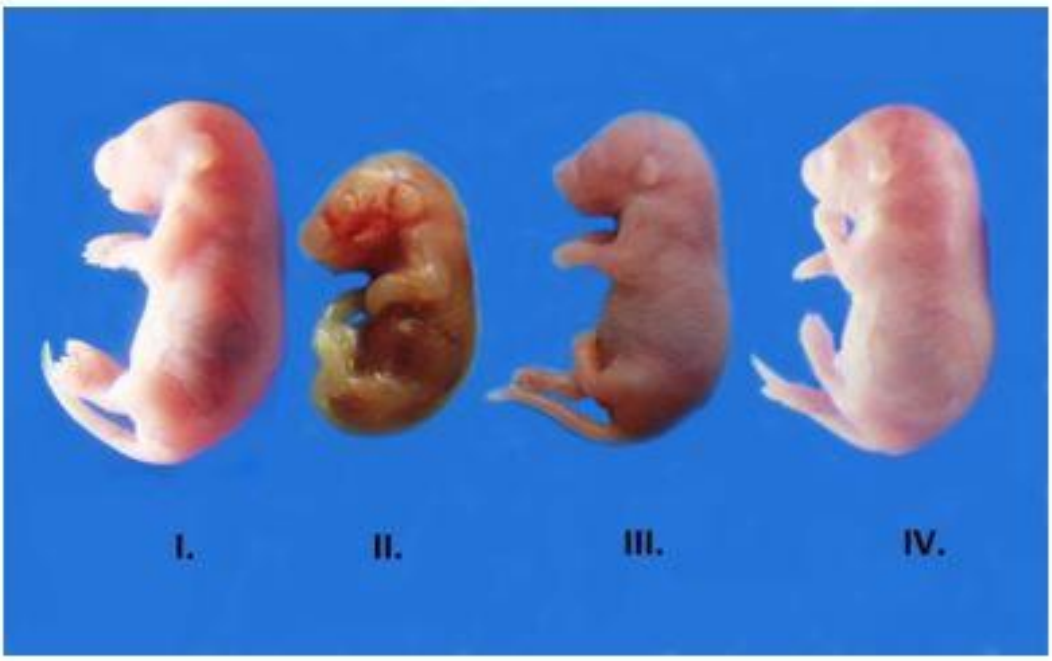


Fig. 50: Showing fetuses of different groups

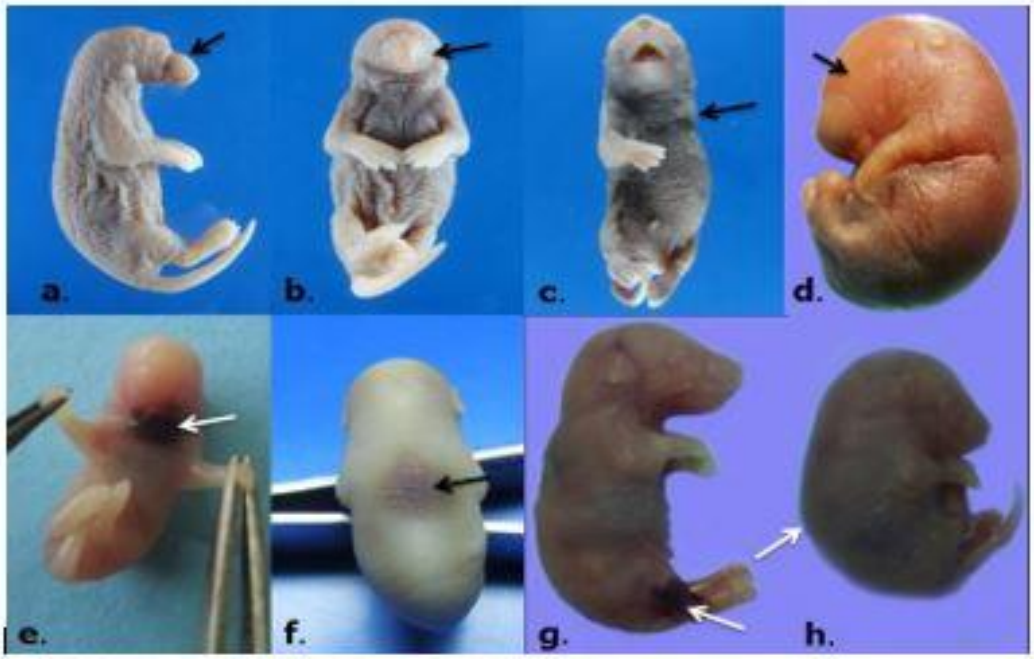


Fig. 51: Fetuses of group II with different congenital malformations: Fetuses with elongated snout with defect in development of face (a & b); a fetus with amelia of left fore limb (c); a fetus with anophthalmia (d); fetuses with hemorrhagic patches on front of neck(e), back (f) and hind limb (g); a fetus with lumbar kyphosis (h).

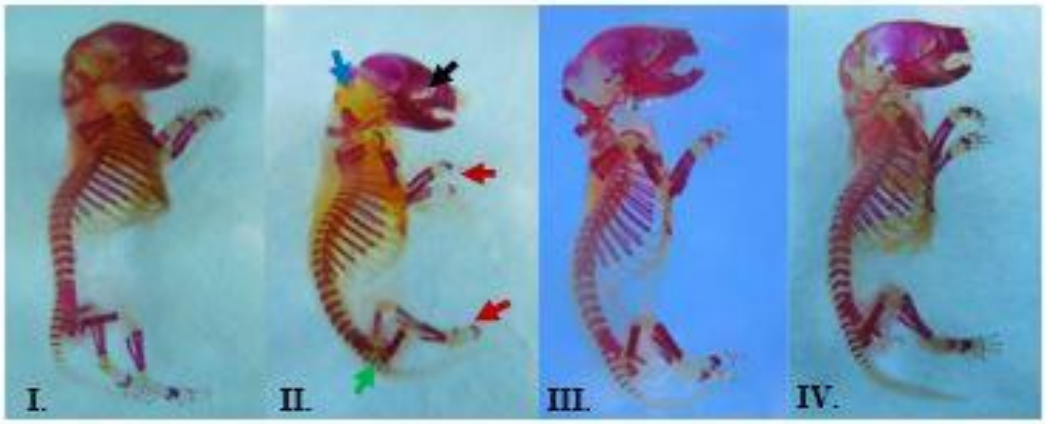


Fig. 52: A

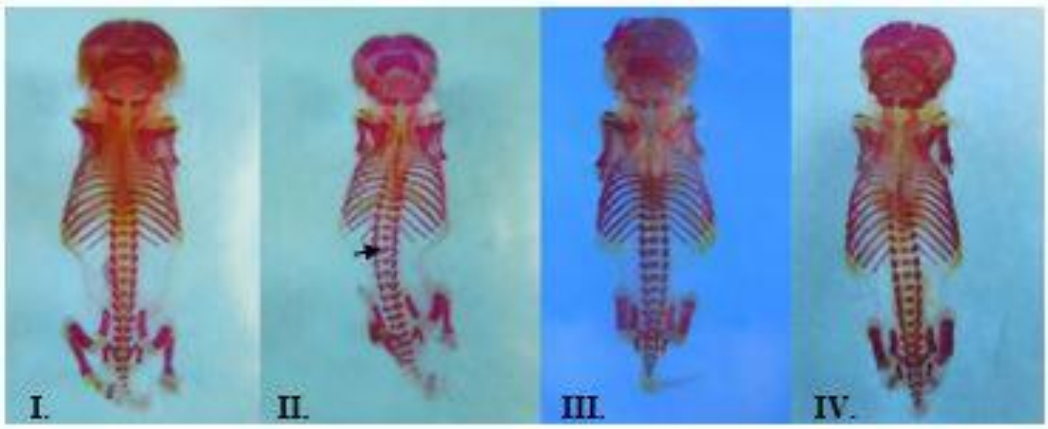


Fig. 52: B

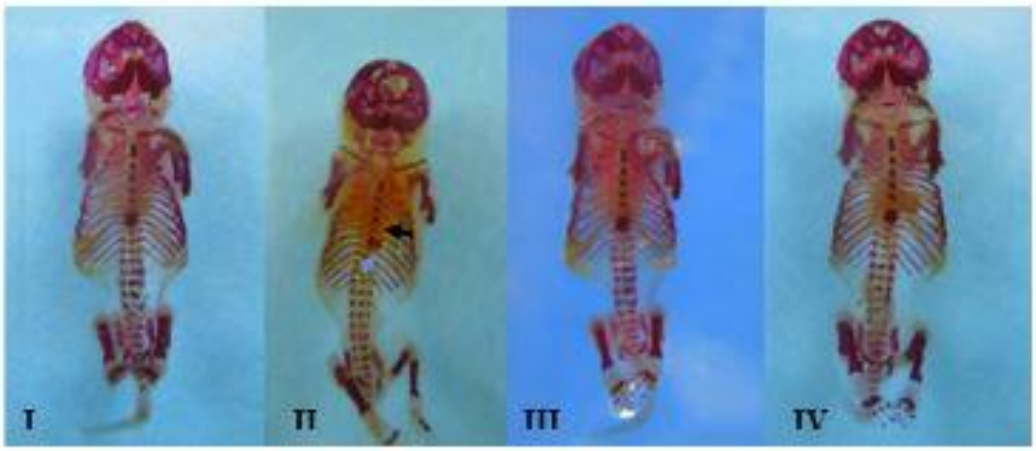


Fig. 52: C

Fig. 52: Photographs of alizarin red stained fetuses of different groups

A. Lateral view: Group II fetus showing reduced parietal & occipital bone ossification (blue arrow), enlarged orbit (black arrow), deficient phalangeal ossification of fore limb and hind limb (red arrow), and reduced ossification of coccygeal vertebrae (green arrow).
B. Dorsal view: Group II fetus showing scoliosis (lateral curvature) of vertebral column (black arrow).
C. Frontal view: Group II fetus showing missing fifth sternebrae (black arrow) and divided xiphoid process (blue arrow).

No such malformations were seen in remaining groups.

Fig. 53: Alizarin red stained fetus of group II showing faint staining of developing bones

Fig. 54: A fetus with its placenta of group I

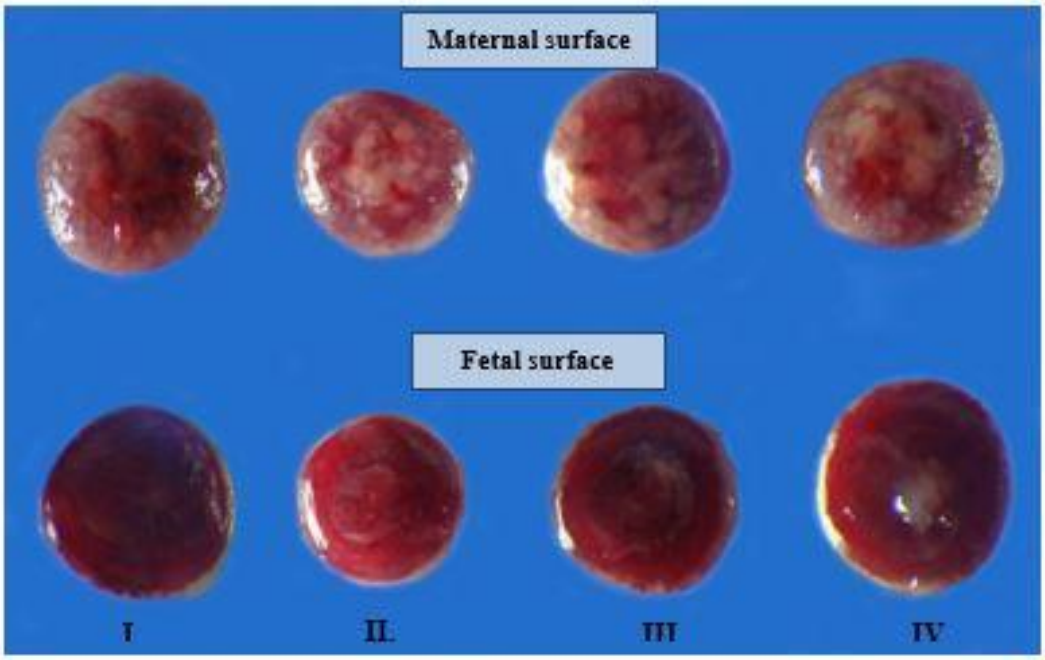


Fig. 55: Placenta of different groups

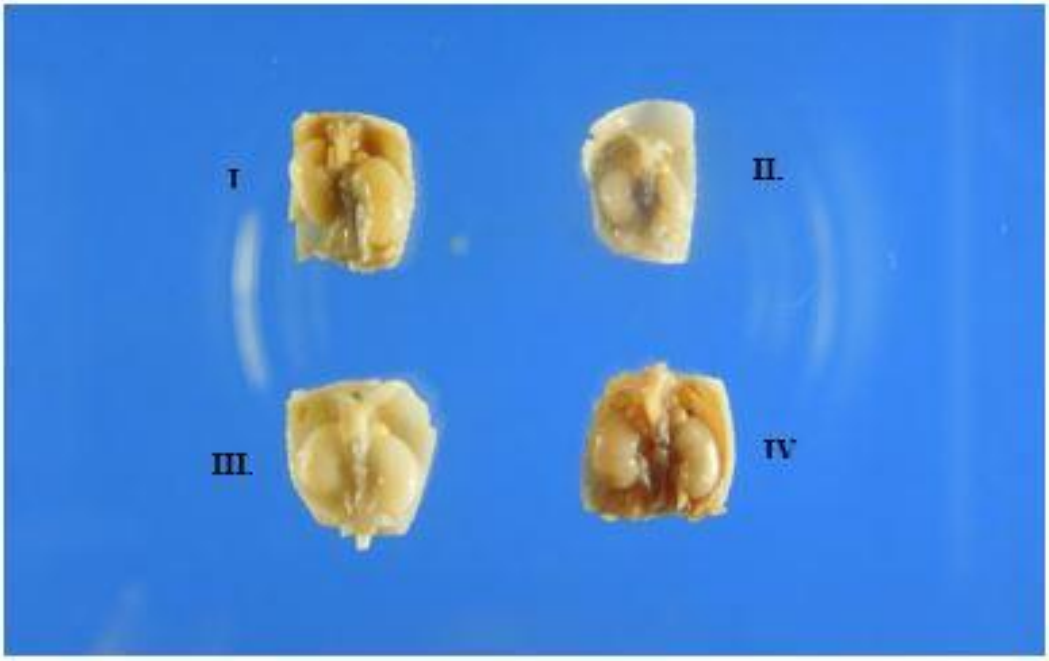


Fig. 56: Kidney of fetuses of different groups

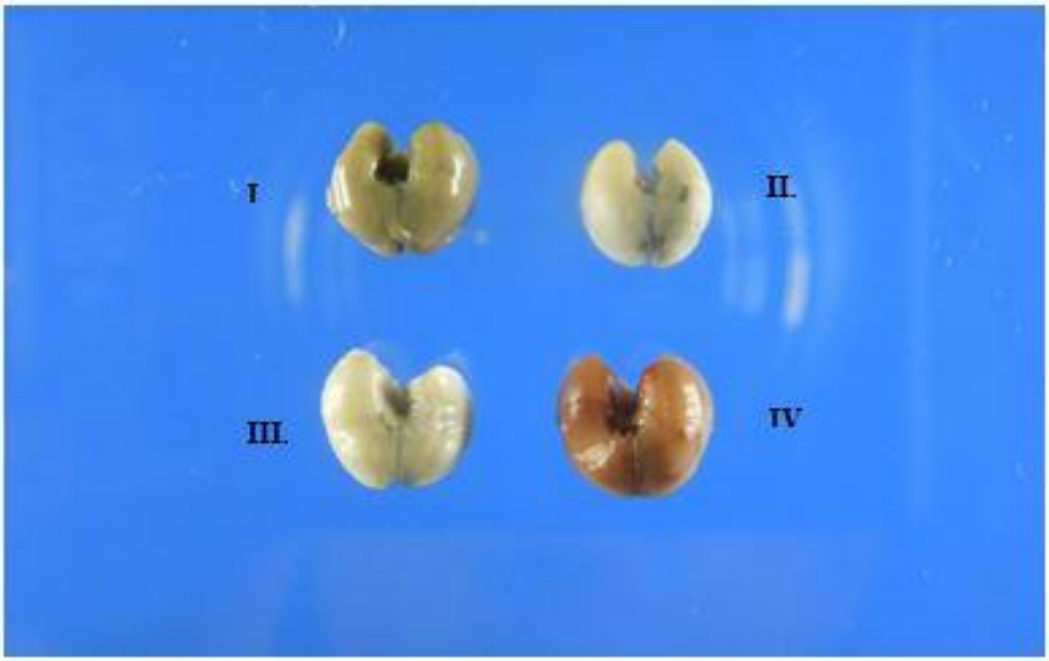


Fig. 57: Liver of fetuses of different groups

Fig. 58: Brain of fetuses (GD 18) of different groups

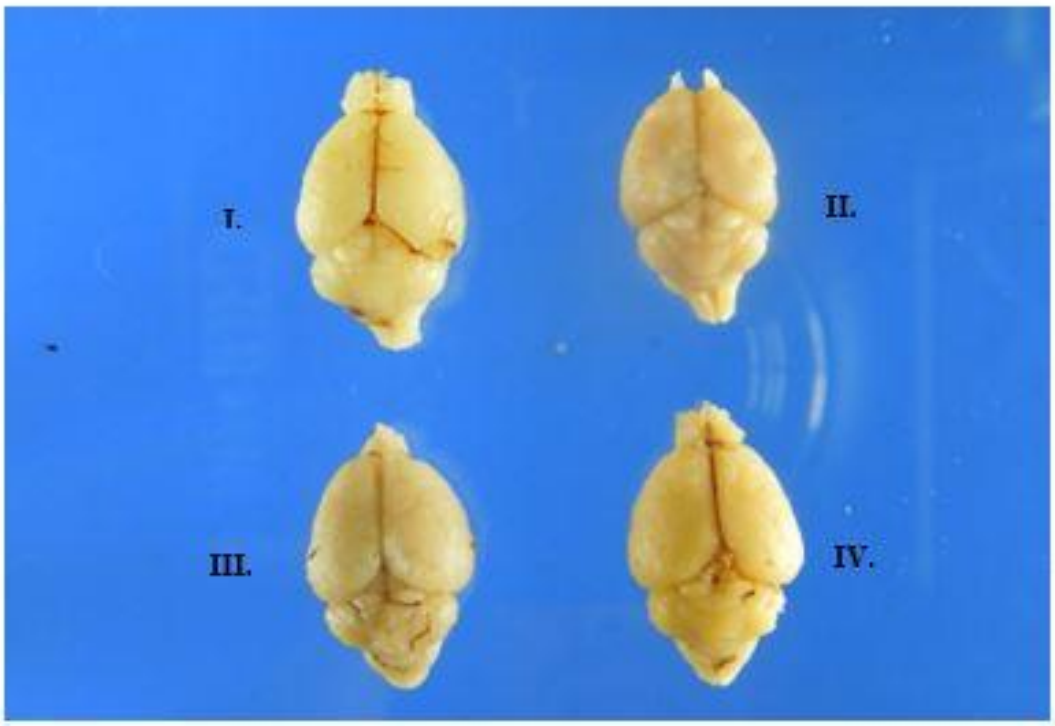
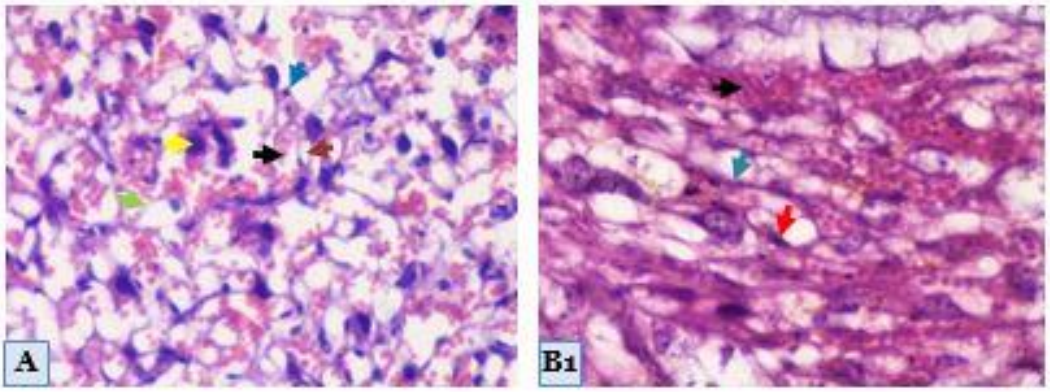
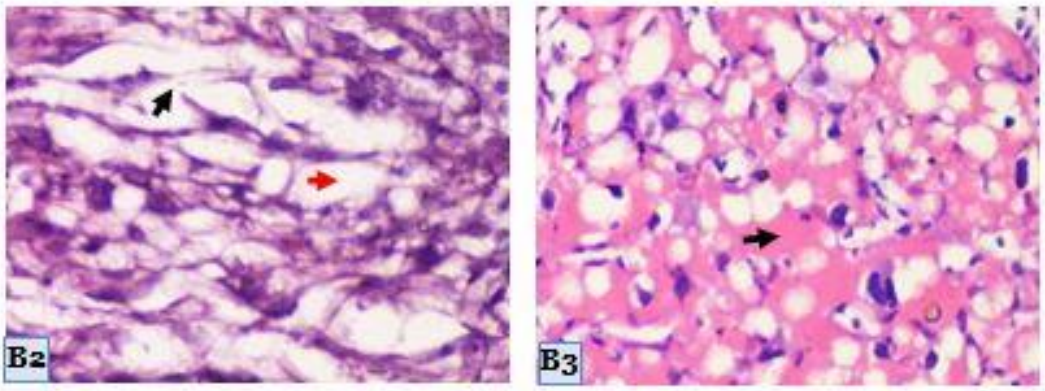


Fig. 59: Brain of pups (PND 70) of different groups


A
B1


B2
B3

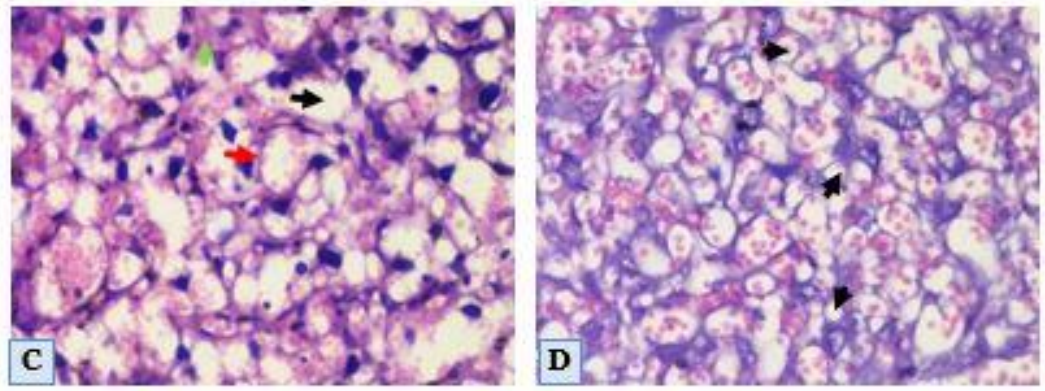

C
D

Fig. 60 : Photomicrograph of labyrinth zone of placenta (H&E stain X 400)

A. Group I placenta showing maternal sinusoids (black arrow), fetal capillaries (red arrow), cytotrophoblasts (yellow arrow), syncytiotrophoblasts (blue arrow) and placental barrier (green arrow).

B1. Group II placenta showing congested maternal sinusoids (black arrow), thickened placental barrier (blue arrow) and degenerating cells (red arrow).

B2. Group II placenta showing disruption of placental barrier (black arrow) and dilation of maternal sinusoids (red arrow).

B3. Group II placenta showing homogenous mucoid material (black arrow).

C. Group III placenta showing reduced dilation of maternal sinusoids (black arrow), reduced thickening of placental barrier (red arrow) and few homogenous debris (green arrow).

D. Group IV placenta showing increased number of fetal capillaries (black arrow).

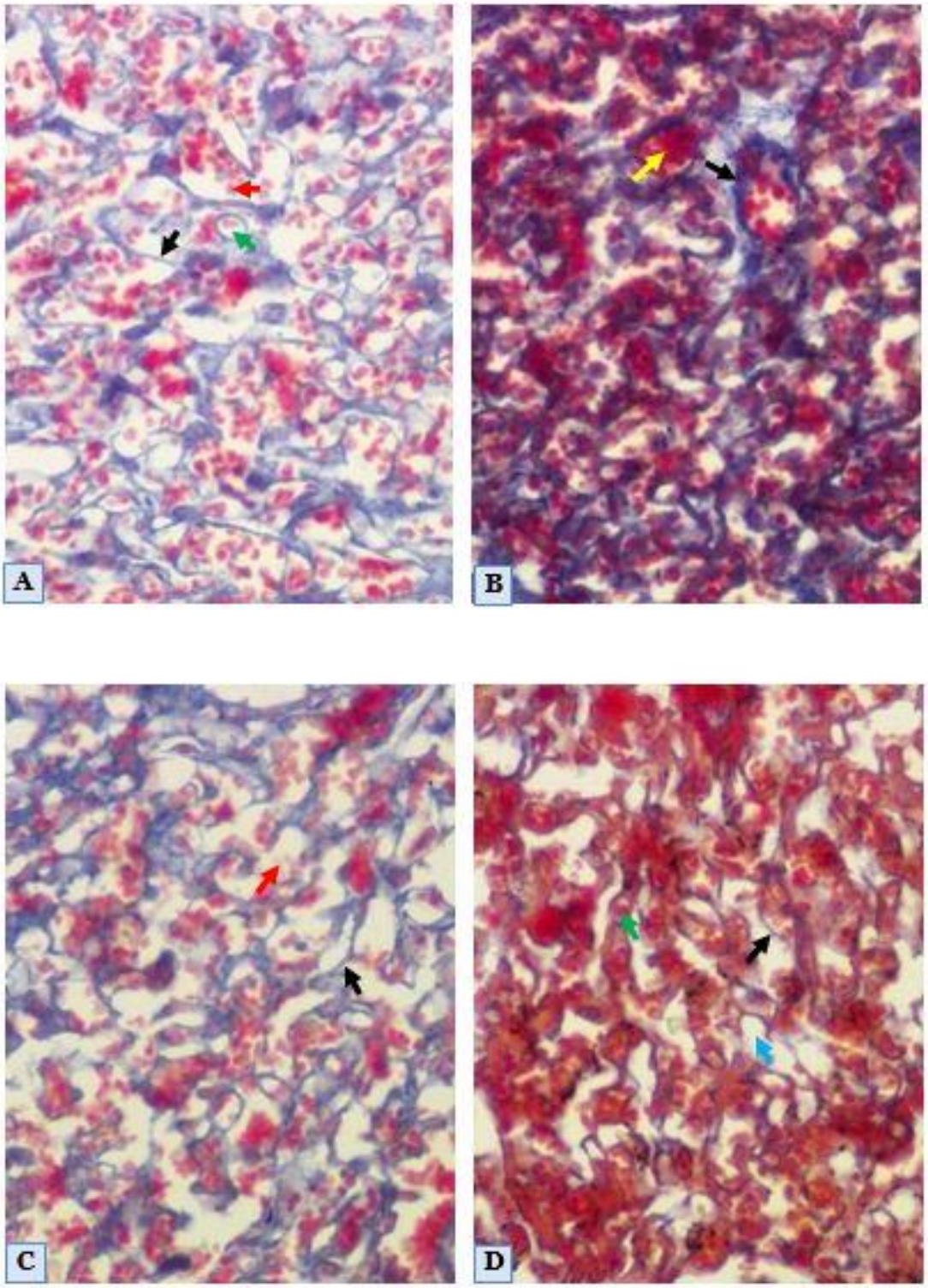

A
B
C
D

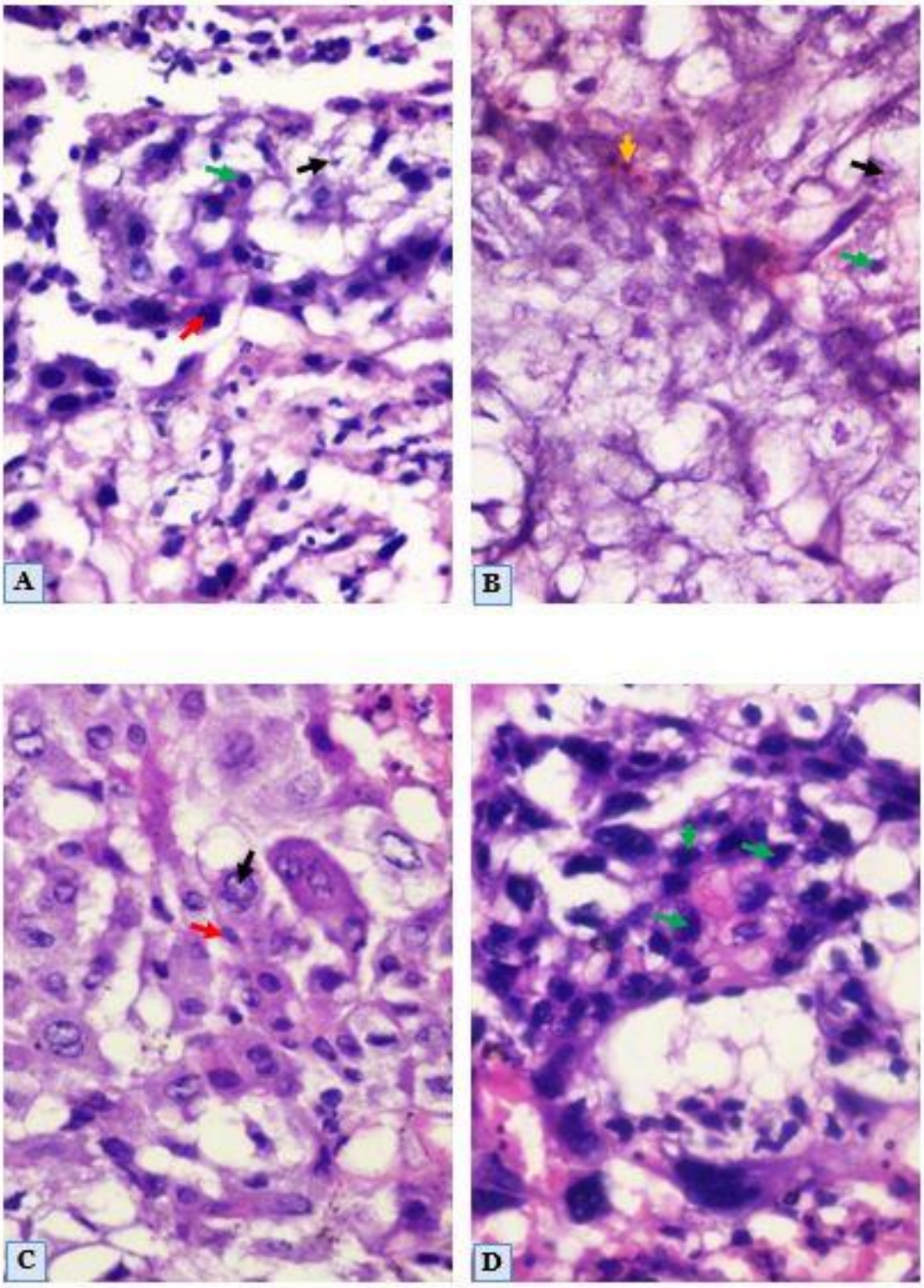
Fig. 61: Photomicrograph of labyrinth zone of placenta (Mallory stain X 400)

A. Placenta of group I showing placental barrier (black arrow), maternal sinusoids with RBC (red arrow) and fetal capillaries (green arrow).

B. Placenta of group II showing thickened placental barrier (black arrow) and congested maternal sinusoids (yellow arrow).

C. Placenta of group III showing reduced thickness of placental barrier (black arrow) and decreased dilation of maternal sinusoids (red arrow) as compared to group II

D. Placenta of group IV showing placental barrier (black arrow) and maternal sinusoids (blue arrow) and fetal capillaries (green arrow).


A
B
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D

Fig. 62 : Photomicrograph of basal zone of placenta (H&E stain X 400)

A. Group I basal zone showing spongiotrophoblasts (green arrow), giant trophoblasts (red arrow) and glycogen cells (black arrow).

B. Group II basal zone showing degenerating cells (green arrow), pyknotic nuclei (yellow arrow) and cellular debris (black arrow).

C. Basal zone of group III showing reduced degenerative changes of spongiotrophoblasts (red arrow) and giant trophoblasts (black arrow).

D. Basal zone of group IV showing increased trophoblastic cells (green arrow).

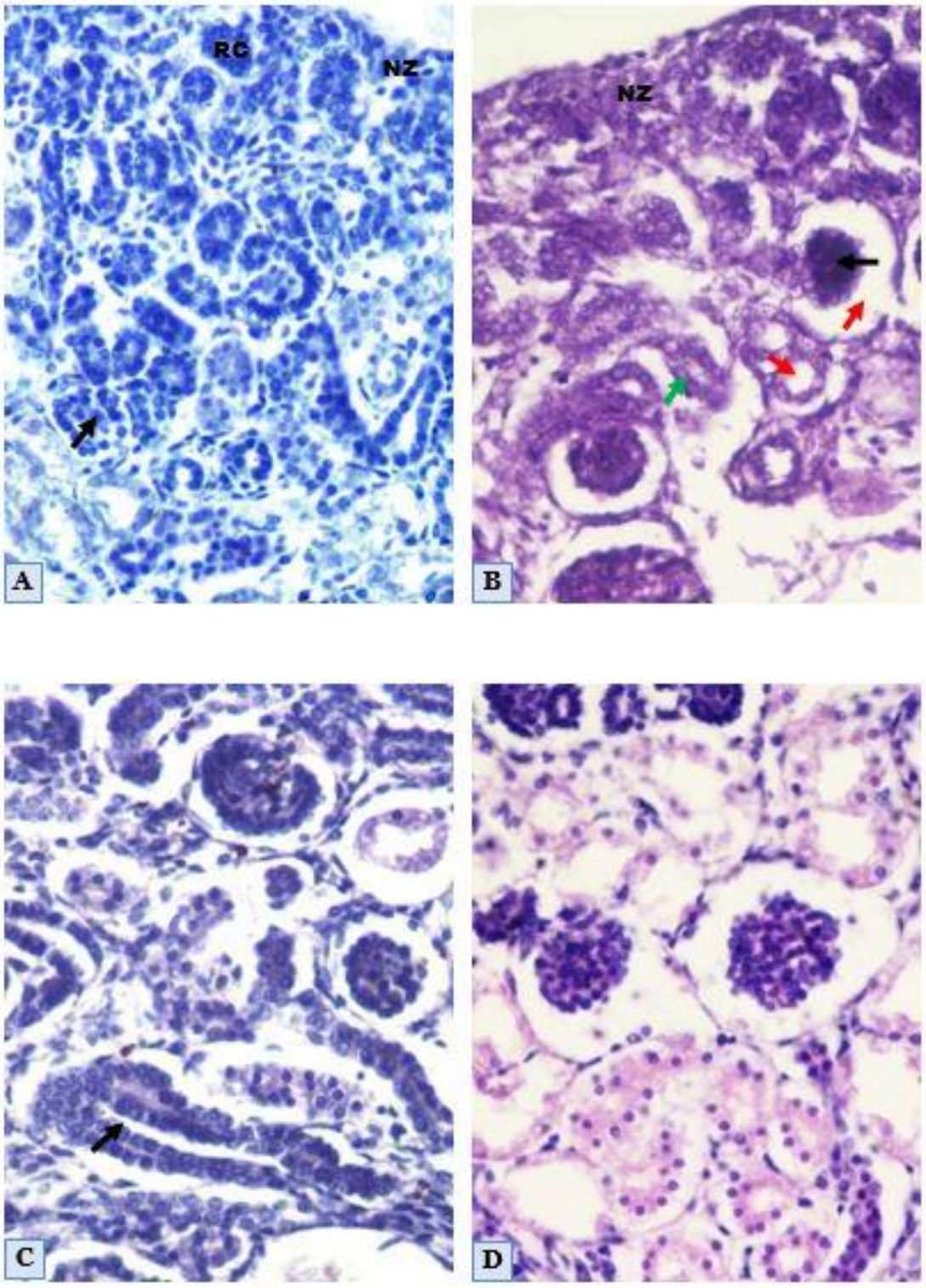

RC
NZ
A
NZ
B
C
D

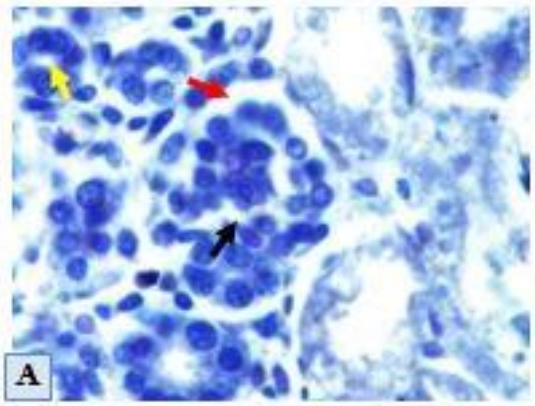
Fig. 63: Photomicrograph of cortex of fetal kidney (H&E stain X 400)

A. Renal cortex of group I showing subcapsular region containing nephrogenic zone (NZ) and developing renal corpuscles (RC) and juxtamedullary region containing relatively mature renal corpuscles (black arrow).

B. Renal cortex of group II showing thicker nephrogenic zone (NZ), necrotic glomeruli (black arrow), dilation of urinary space and tubules (red arrow) and hypertrophy and degenerating tubular epithelia (green arrow).

C. Renal cortex of group III showing reduced glomerular necrosis and reduced dilation of urinary space and tubules. A ureteric bud is also seen in the section (black arrow).

D. Renal cortex of group IV showing increased sized of renal corpuscles.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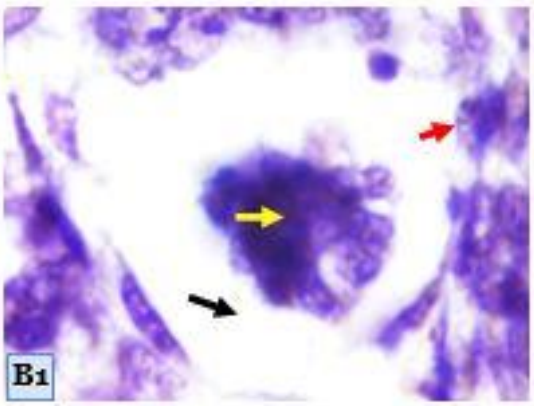
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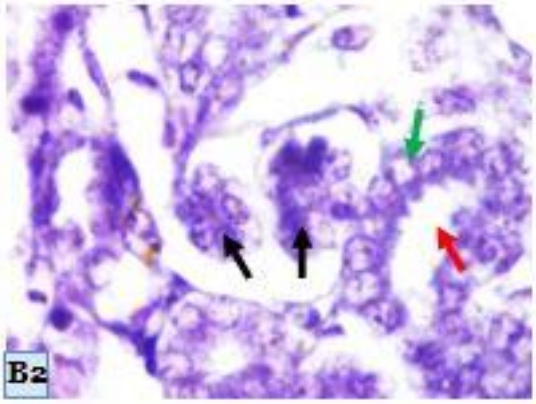
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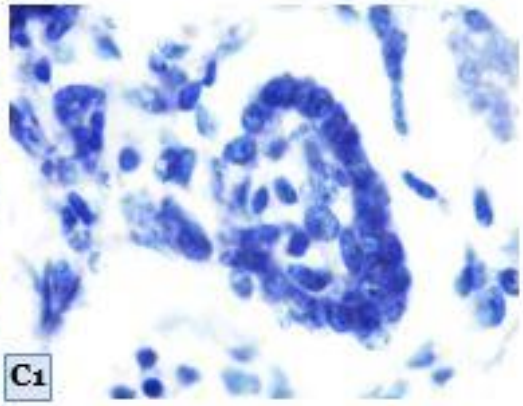
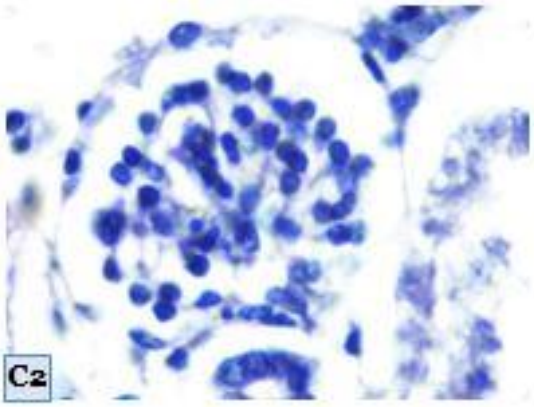

C1


C2

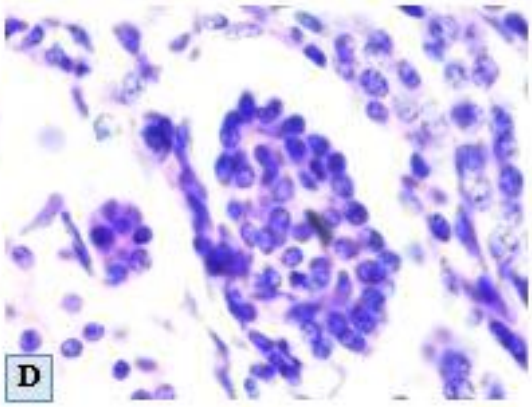

D

Fig. 64 : Photomicrograph of cortex of fetal kidney (H&E stain X 1000)

A. Renal corpuscles of group I showing glomerulus (black arrow) and Bowman's capsule (red arrow). Developing tubules are also seen (yellow arrow).

B1. Renal corpuscles of group II showing necrotic glomerulus (yellow arrow), dilated urinary space (black arrow) and degenerating parietal epithelia (red arrow) of Bowman's capsule.

B2. Renal cortex of group II showing necrotic and dividing glomeruli (black arrow), dilated tubules (red arrow) and hypertrophy and degenerating tubular epithelia (green arrow).

C1. Renal corpuscles of group III showing reduced glomerular necrosis and dilated urinary space.

C2. Renal corpuscles of group III showing glomerulus and urinary space

D. Renal corpuscles of group IV showing glomerulus and Bowman's capsule.

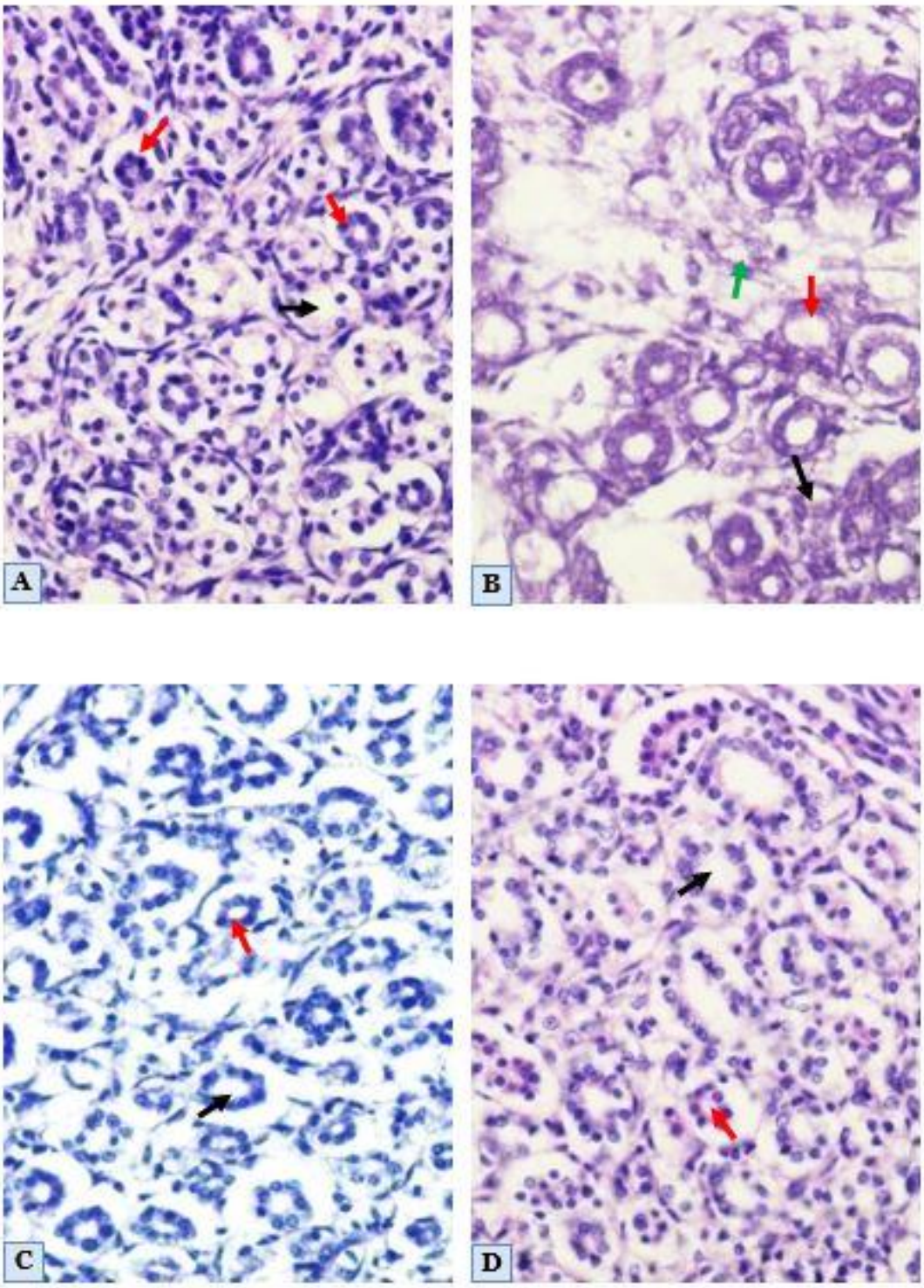

A
B
C
D

Fig. 65 : Photomicrograph of medulla of fetal kidney(H&E stain X 400)

A. Group I renal medulla showing collecting ducts (black arrow) and ascending and descending limbs of loop of Henle (red arrow).
B. Group II renal medulla showing decreased frequency of collecting ducts (red arrow) and tubules (black arrow), dilated structures, increased intertubular connective tissue (green arrow) and degenerating epithelial lining.
C. Group III renal medulla showing reduced degenerative changes and dilation of collecting ducts (black arrow) and renal tubules (red arrow).
D. Group IV renal medulla showing collecting ducts (black arrow) and renal tubules (red arrow).

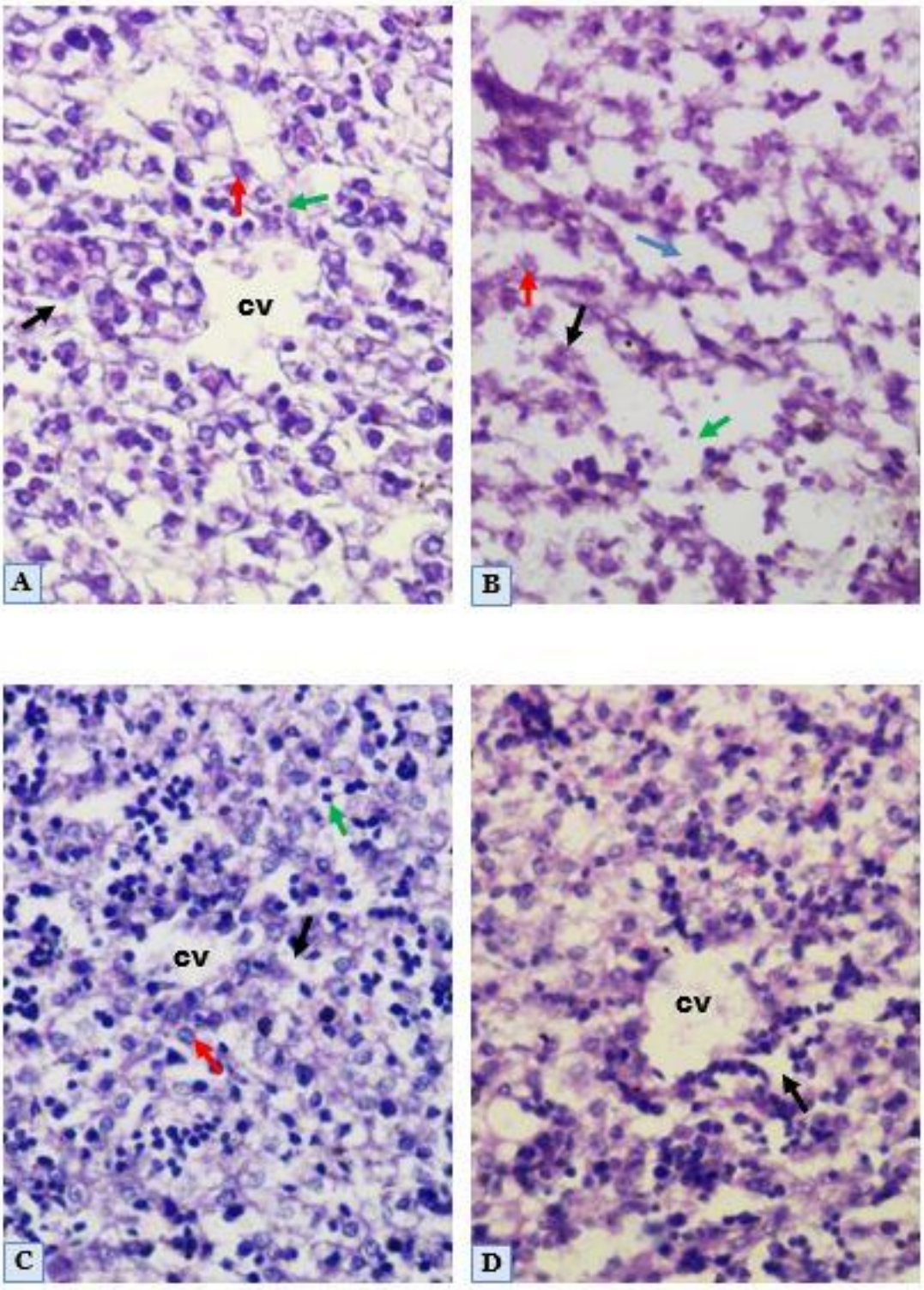

cv
A
B
cv
C
cv
D

Fig. 66: Photomicrograph of liver of fetus (H&E stain X 400)

A. Group I liver showing developing central vein (CV), sinusoids (black arrow), developing hepatoblasts arranging in lamina (red arrow) and hematopoietic cells (green arrow).
B. Group II liver showing extensive degeneration of hepatoblasts (black arrow), marked fibrosis (red arrow), disrupted hepatic lamina (green arrow), large empty lacunar spaces (blue arrow) and reduced cellular density.
C. Group III liver showing developing central vein (CV), sinusoids, large number of hepatoblasts (red arrow) and hematopoietic cells (green arrow) and reduced degenerative changes.
D. Group IV liver showing increased cellular population around developing central vein (CV) and sinusoids (black arrow).

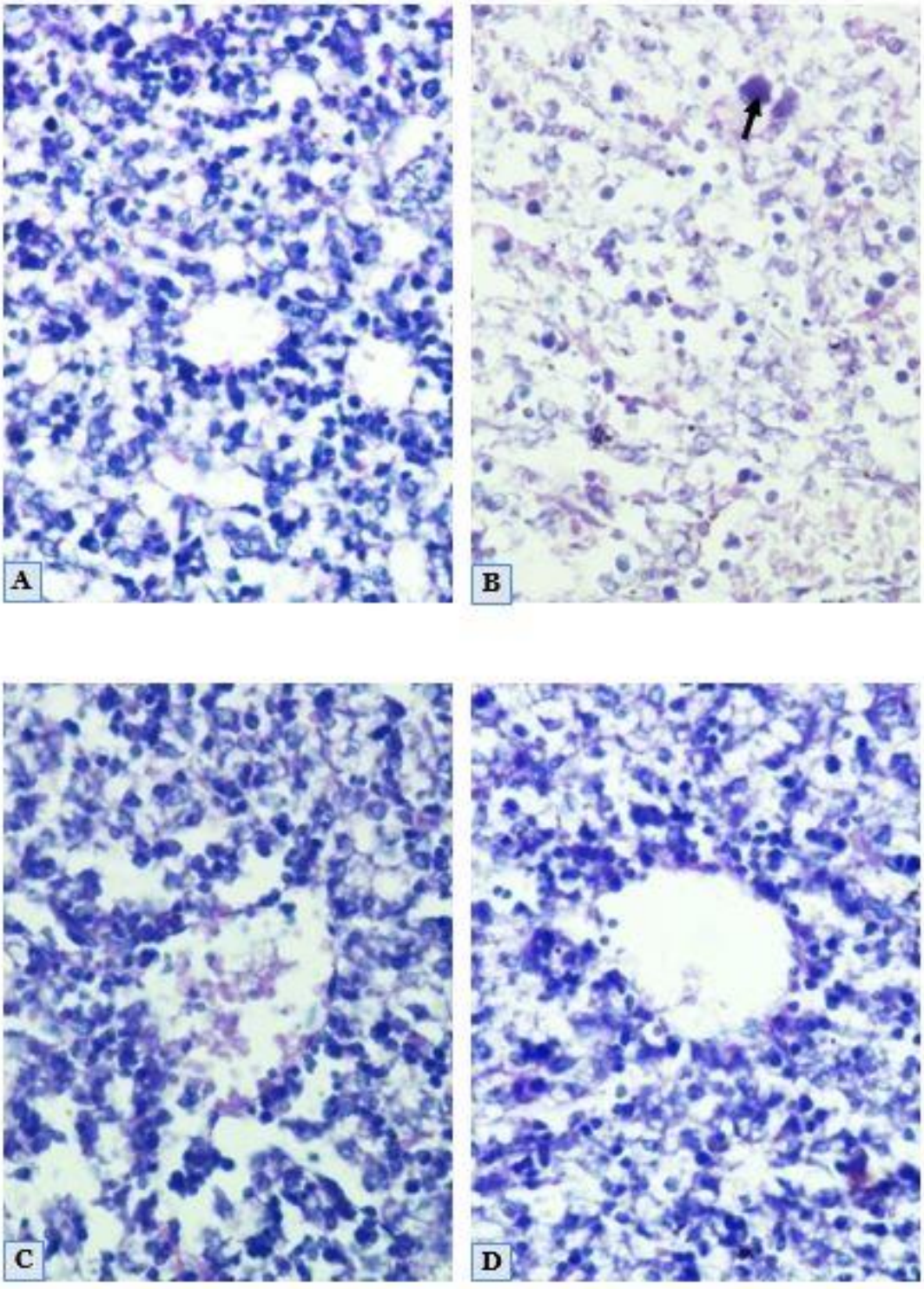

A
B
C
D

Fig. 67: Photomicrograph of liver of fetus (PAS stain X 400)

A. Group I liver showing negligible PAS positive material.
B. Group II liver showing reduced PAS positive material, cellular degeneration and fibrosis. A number of megakaryocytes are also visible (arrow).
C. Group III liver showing increased PAS positive material, increased cellular density and reduced degenerative changes as compared to group II.
D. Group IV liver showing negligible PAS positive material and increased cellular density.

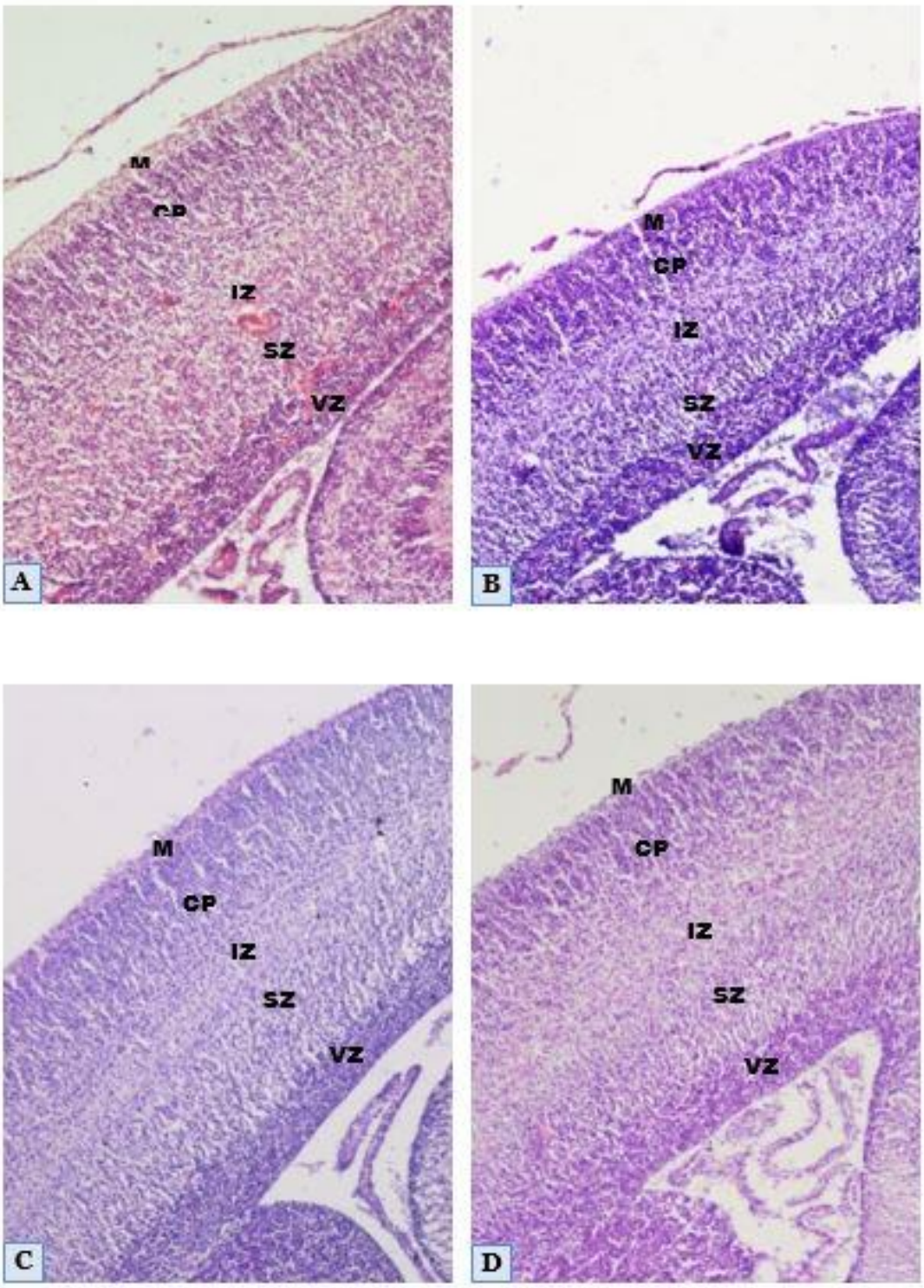

M
CP
IZ
SZ
VZ
A
M
CP
IZ
SZ
VZ
B
M
CP
IZ
SZ
VZ
C
M
CP
IZ
SZ
VZ
D

Fig. 68: Photomicrograph of fetal cerebrum (H&E stain X 100)

A. Group I cerebrum showing different zones: marginal zone (M), cortical plate (CP), intermediate zone (IZ), subventricular zone (SZ) and ventricular zone (VZ).
B. Group II cerebrum showing reduced thickness of the cerebrum and disintegration of intermediate zone.
C. Group III cerebrum showing different zones
D. Group IV cerebrum showing different zones

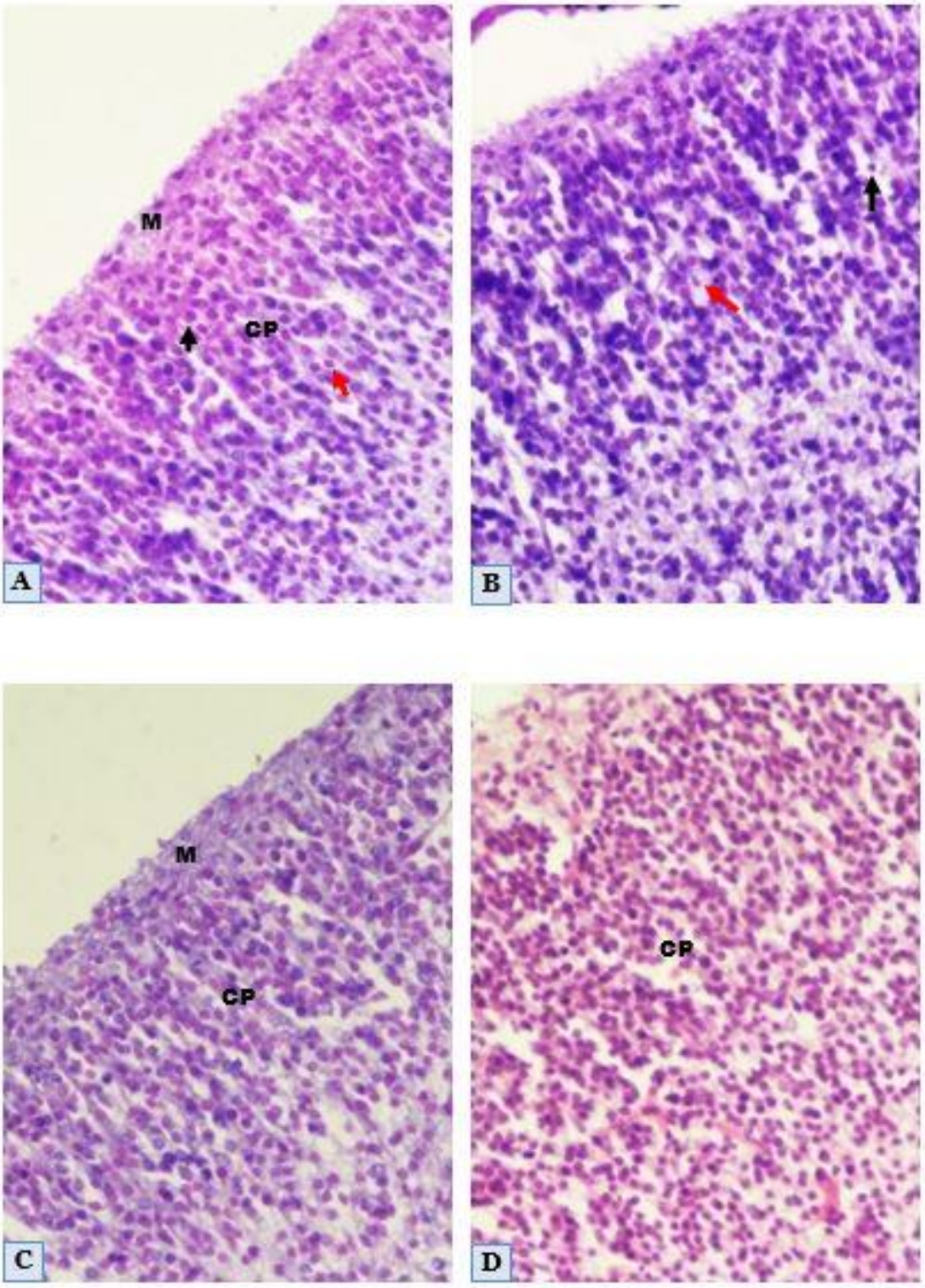

M
CP
A
B
M
CP
C
CP
D

Fig. 69: Photomicrograph of fetal cerebrum (H&E stain X 400)

A. Group I cerebrum showing large developing neuroblasts (black arrow) and small glial cells (red arrow) in the cortical plate (CP) and marginal zone (M) at different stages of differentiation.
B. Group II cerebrum showing degenerating cells (red arrow), pyknotic nuclei (black arrow), edematous spaces, sparsely arranged neuroblasts and increased glial cells in the cortical plate.
C. Group III cerebrum showing reduced degenerative changes in the marginal zone (M) and cortical plate (CP).
D. Group IV cerebrum showing increased cellular density in the cortical plate (CP).

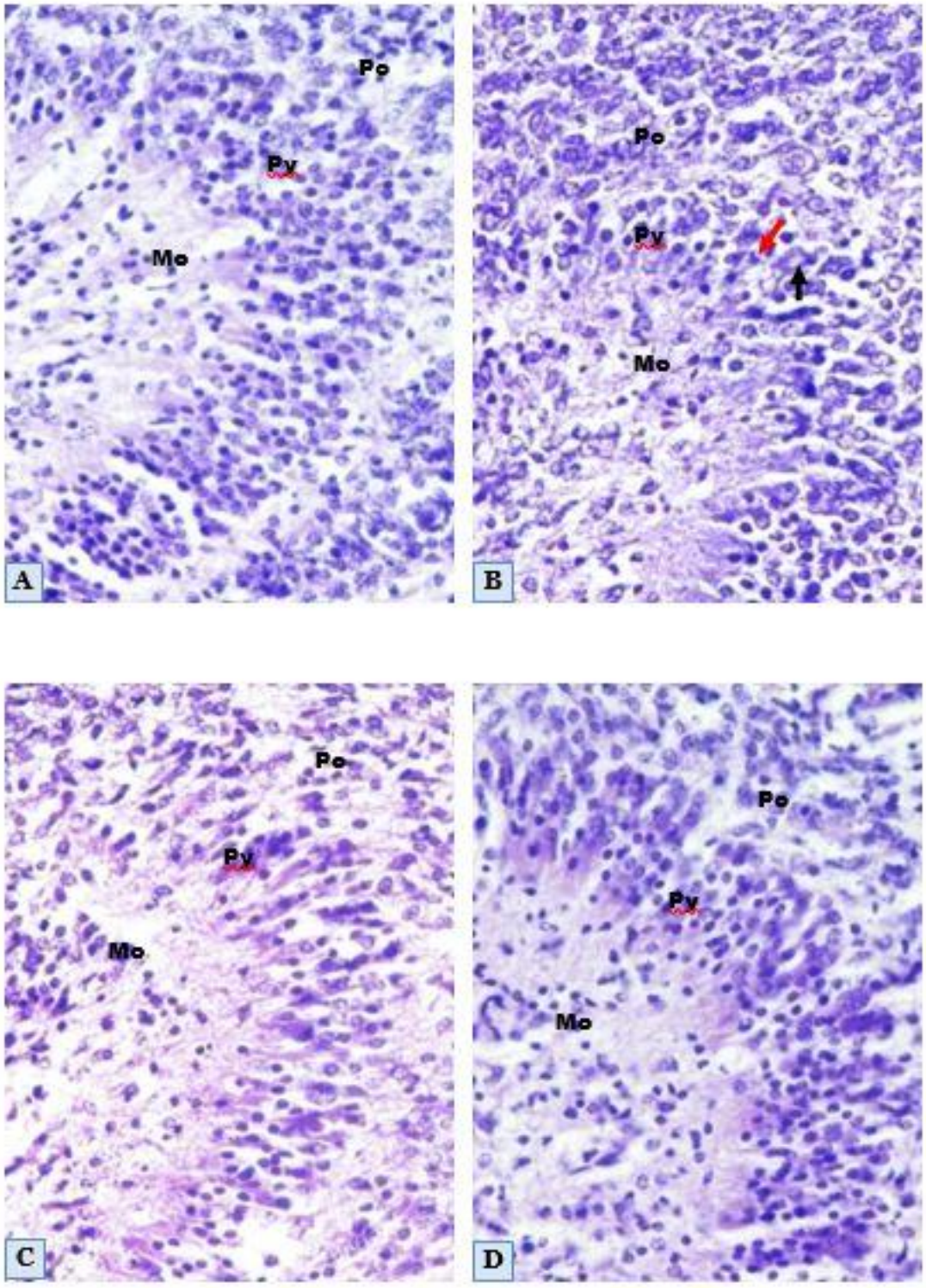

Po
Py
Mo
A
Po
Py
Mo
B
Po
Py
Mo
C
Po
Py
Mo
D

Fig. 70: Photomicrograph of CA3 region of hippocampus of fetus (H&E stain X 400)

A. Group I CA3 region showing polymorphic layer (Po), pyramidal cell layer (Py) and molecular layer (Mo).
B. Group II CA3 region showing degenerating cells (red arrow), vacuolated cells (black arrow), edematous spaces and decreased cellular density in pyramidal cell layer
C. Group III CA3 region showing reduced degenerative changes in pyramidal cell layer.
D. Group IV CA3 region showing increased cellular density in pyramidal cell layer

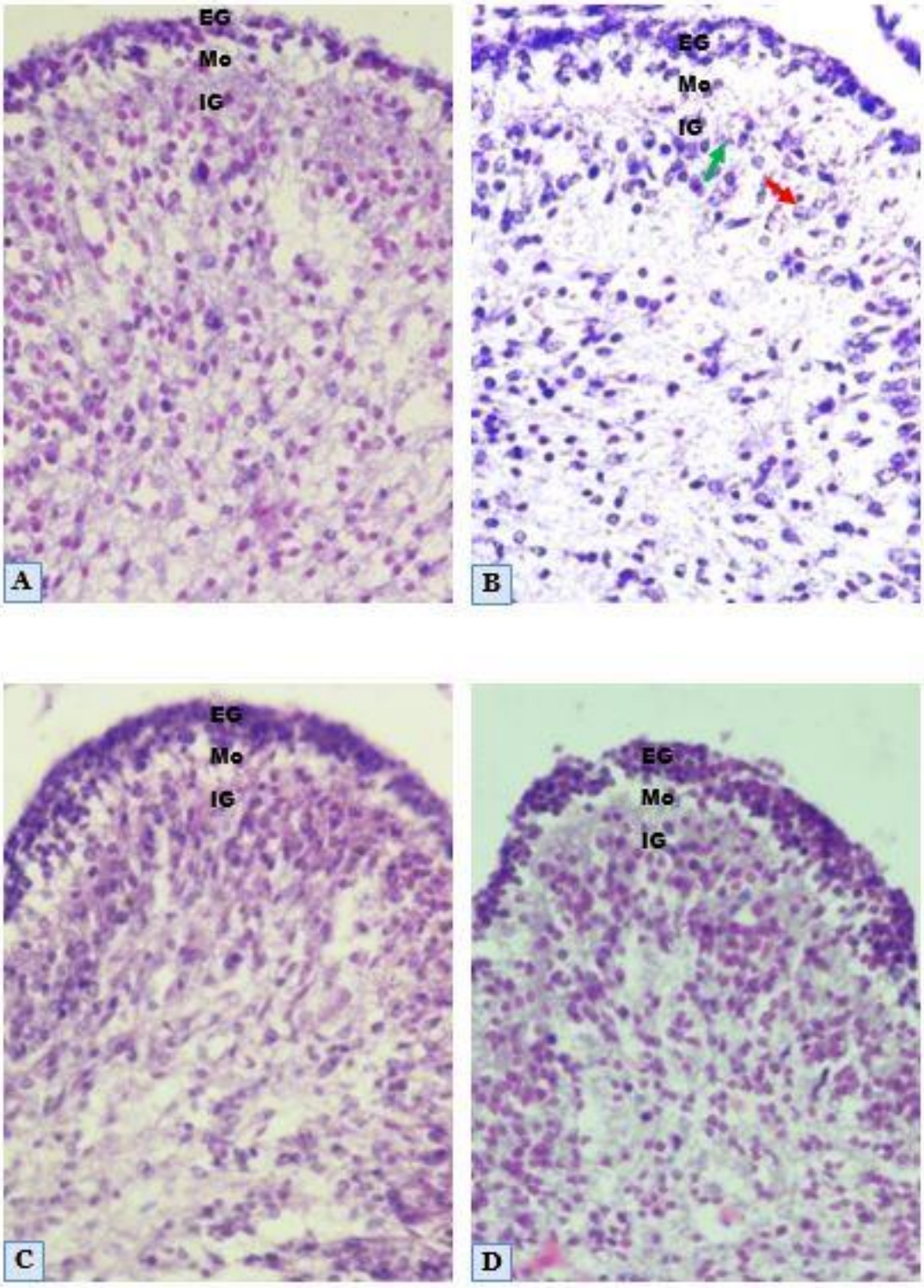

EG
Mo
IG
A
EG
Mo
IG
B
EG
Mo
IG
C
EG
Mo
IG
D

Fig. 71: Photomicrograph of cerebellum of fetus (H&E stain X 400)

A. Group I cerebellum showing external granular layer (EG), developing molecular layer (Mo) and inner granular layer (IG).
B. Group II cerebellum showing reduced cellular density, degenerating cells (red arrow) and cellular debris (green arrow) in inner granular layer.
C. Group III cerebrum showing increased cellular density and reduced degenerative changes in inner granular layer
D. Group IV cerebellum showing increased cellular density in inner granular layer

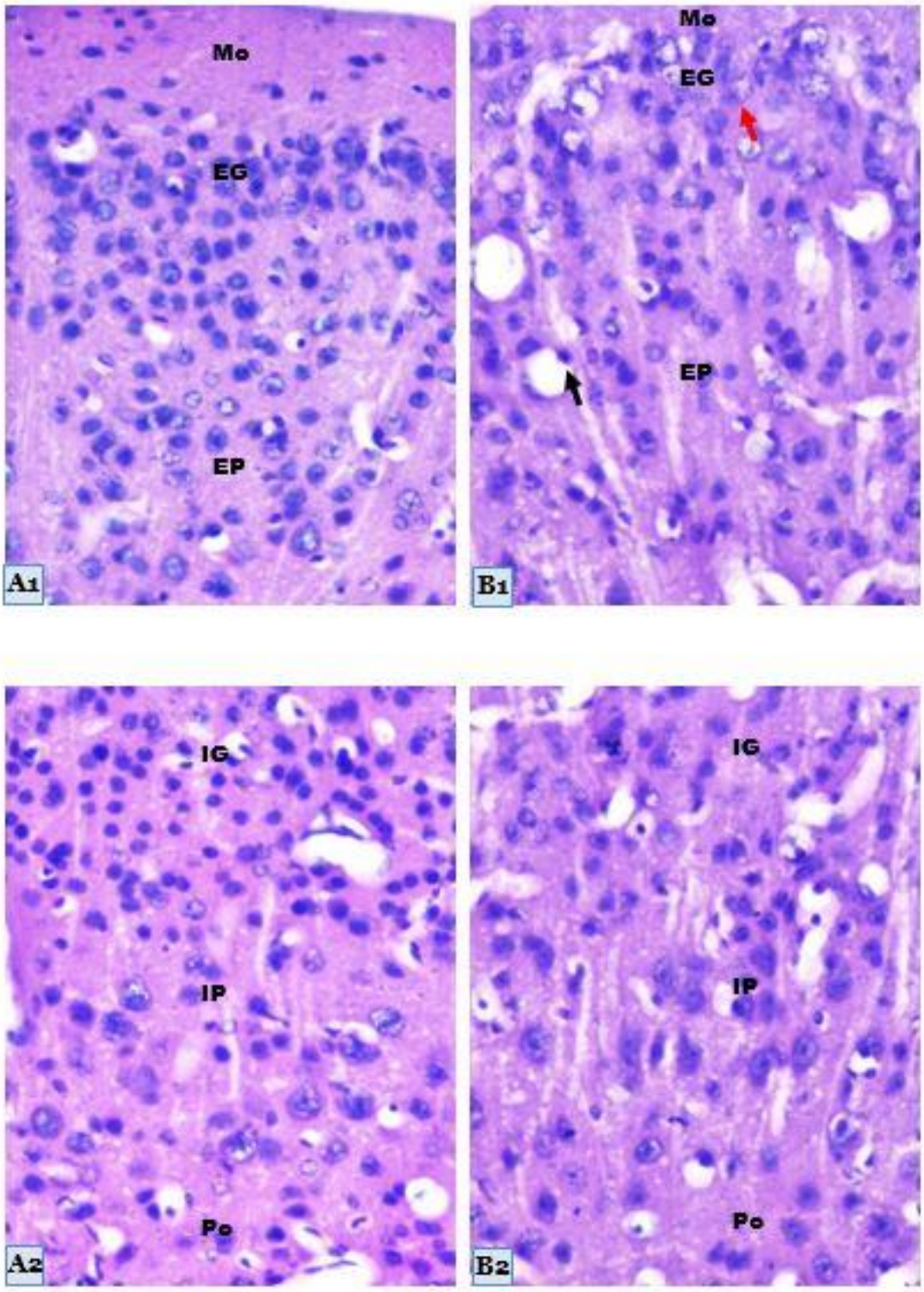

Mo
EG
EP
A1
Mo
EG
EP
B1
IG
IP
Po
A2
IG
IP
Po
B2

Fig. 72: Photomicrograph of frontal cortex of cerebrum of pups on PND 70 (H&E stain X 400)

A1& A2: Group I cerebrum showing different layers in the cerebral cortex: Molecular layer (Mo), External granular layer (EG), External Pyramidal layer (EP), Internal granular layer (IG), Internal pyramidal layer (IP) and polymorphic layer (Po).

B1 & B2: Group II cerebrum showing disorganization of different layers, edematous spaces, enlarged neuroglial cells (black arrow), marked degeneration (red arrow) and decreased cellular density.

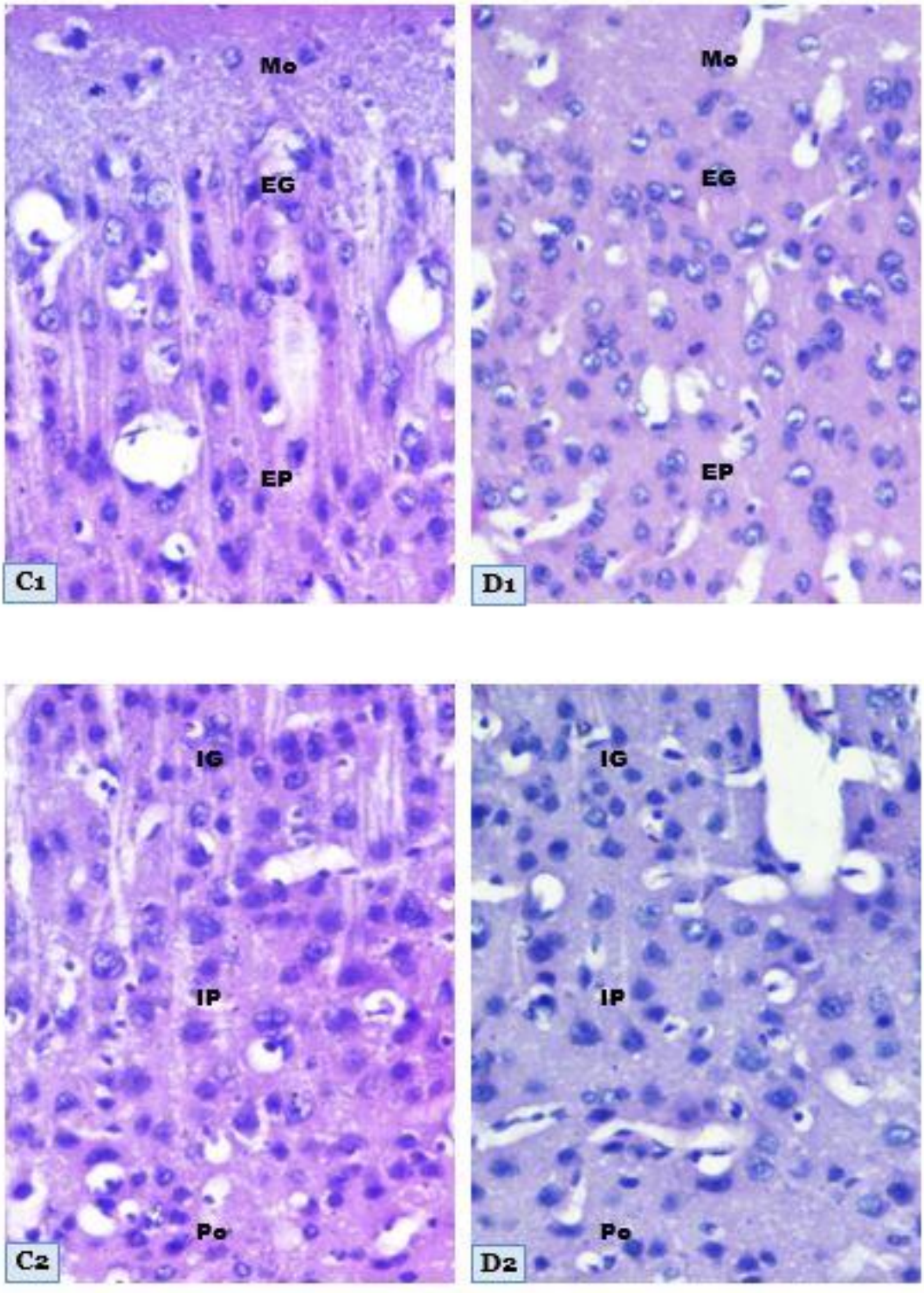

Mo
EG
EP
C1
Mo
EG
EP
D1
IG
IP
Po
C2
IG
IP
Po
D2

Fig. 73: Photomicrograph of frontal cortex of cerebrum of pups on PND 70 (H&E stain X 400)

C1&C2: Group III cerebrum showing reduced degenerative changes, increased cellular density and plenty neuroglial cells.

D1&D2: Group IV cerebrum showing different layers in the cortex.

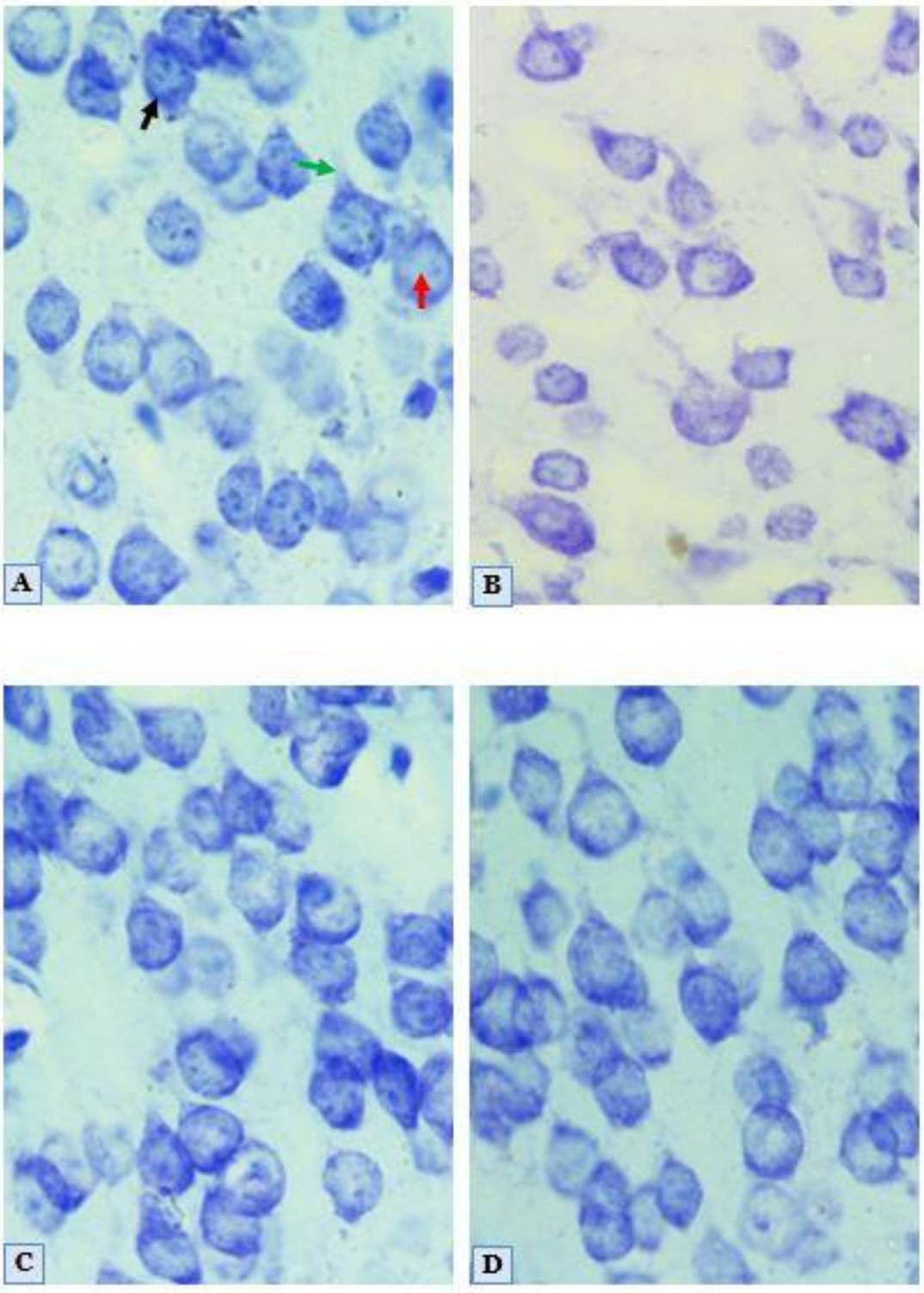

A
B
C
D

Fig. 74: Photomicrograph of layer III pyramidal cells of frontal cortex on PND 70 (Nissl's stain X 1000)

A. Group I pyramidal cells showing Nissl's granules in their cytoplasm (black arrow), pale vesicular nuclei (red arrow) and apical dendrites (green arrow).
B. Group II pyramidal cells showing decreased Nissl's granules, decreased cellular density and size.
C. Group III pyramidal cells showing increased Nissl's granules , increased cellular density and size
D. Group IV pyramidal cells showing Nissl's granules.

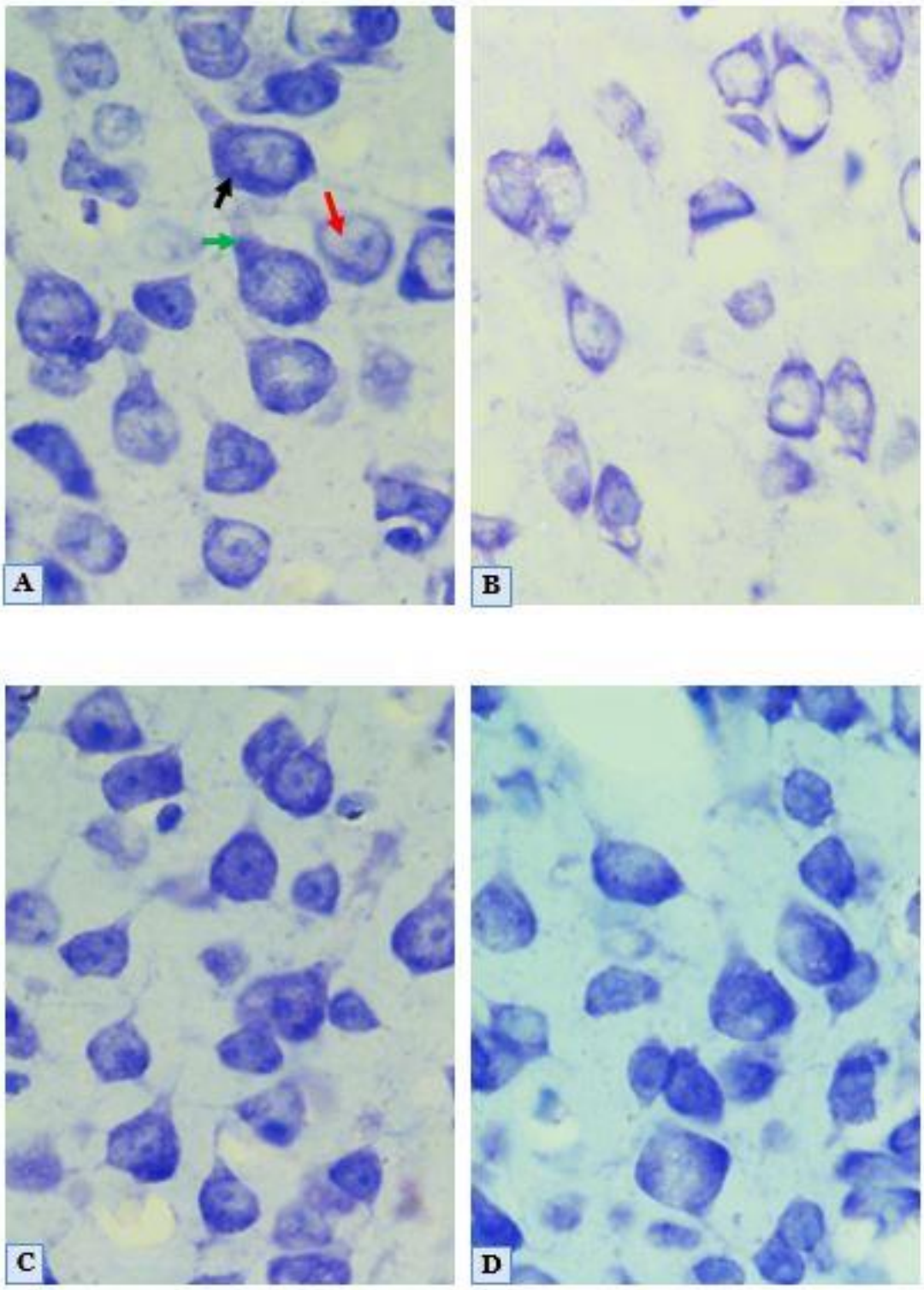

A
B
C
D

Fig. 75: Photomicrograph of layer V pyramidal cells of frontal cortex of pups on PND 70 (Nissl's stain X 1000)

A. Group I pyramidal cells showing prominent Nissl's granules in their cytoplasm (black arrrow) with pale vesicular nucleus (red arrow) and nucleolus and apical dendrites (green arrow).
B. Group II pyramidal cells showing decreased Nissl's granules, decreased cellular density and size.
C. Group III pyramidal cells showing increased Nissl's granules , increased cellular density and size
D. Group IV pyramidal cells showing Nissl's granules in their cytoplasm.

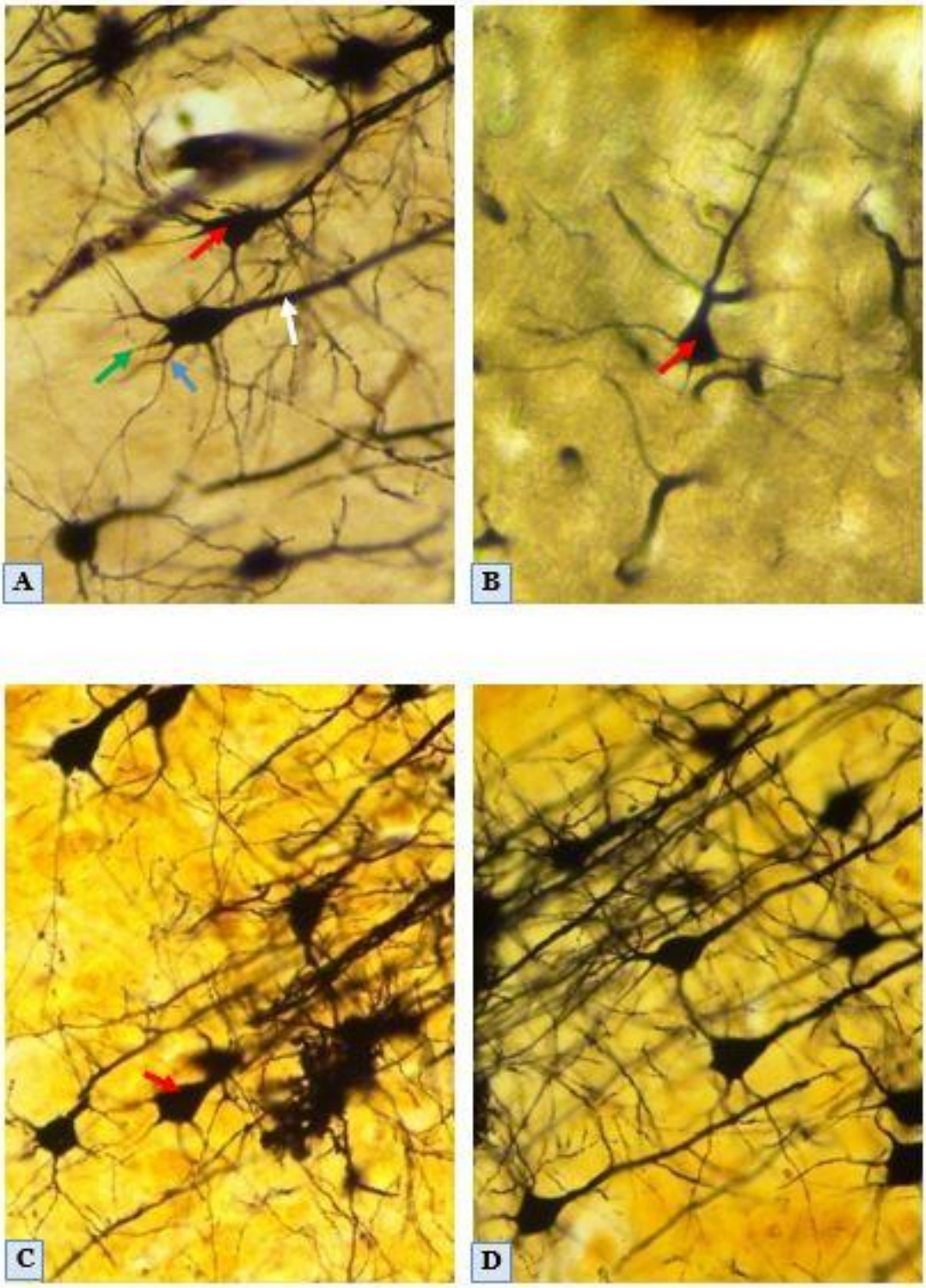

A
B
C
D

Fig. 76: Photomicrograph of layer V pyramidal cells of frontal cortex on PND 70 (Golgi stain X 400)

A. Group I pyramidal cell showing the cell body (red arrow), axon (green arrow), a apical dendrite (white arrow) and numbers of basal dendrites (blue arrow) and their arborizations.
B. Group II pyramidal cells showing decreased size of cell body (red arrow) and reduced dendritic arborizations (both apical and basal).
C. Group III pyramidal cells showing increased size of cell body (red arrow) and dendritic arborizations.
D. Group IV pyramidal cells showing extensive dendritic arborizations.

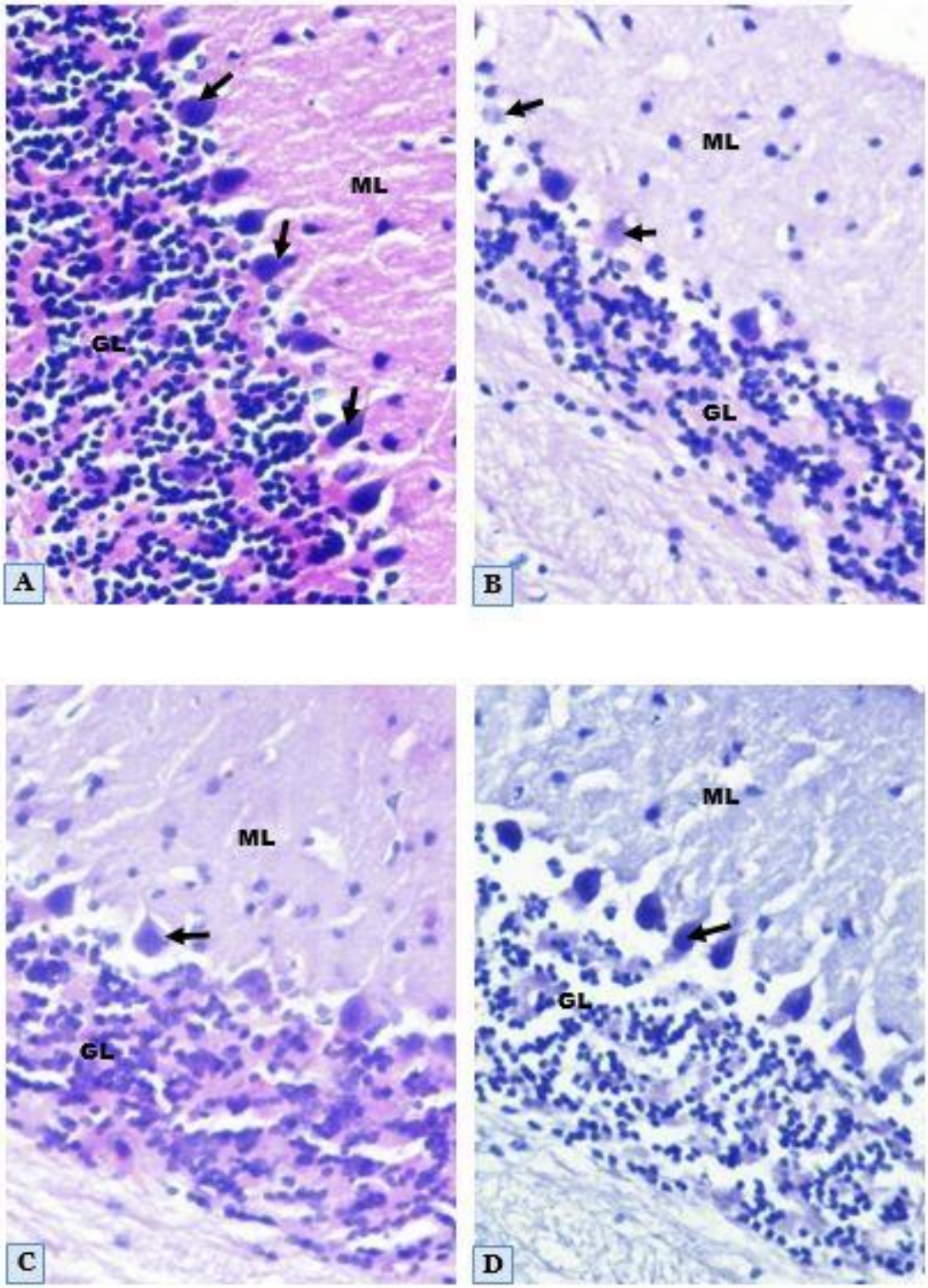

ML
GL
A
ML
GL
B
ML
GL
C
ML
GL
D

Fig. 77: Photomicrograph of cerebellum of pups on PND 70 on PND 70 (H&E stain X 400)

A. Group I cerebellar cortex showing molecular layer (ML), Purkinje cell layer (black arrow) and granular layer (GL).

B. Group II cerebellar cortex showing reduced thickness and cellular density of granular layer, reduced number of Purkinje cells and shrunken and degenerating Purkinje cells (black arrow).

C. Group III cerebellar cortex showing reduced degenerating granule cells and Purkinje cells (black arrow).

D. Group IV cerebellar cortex showing molecular layer, Purkinje cell layer (black arrow) and granular layer.

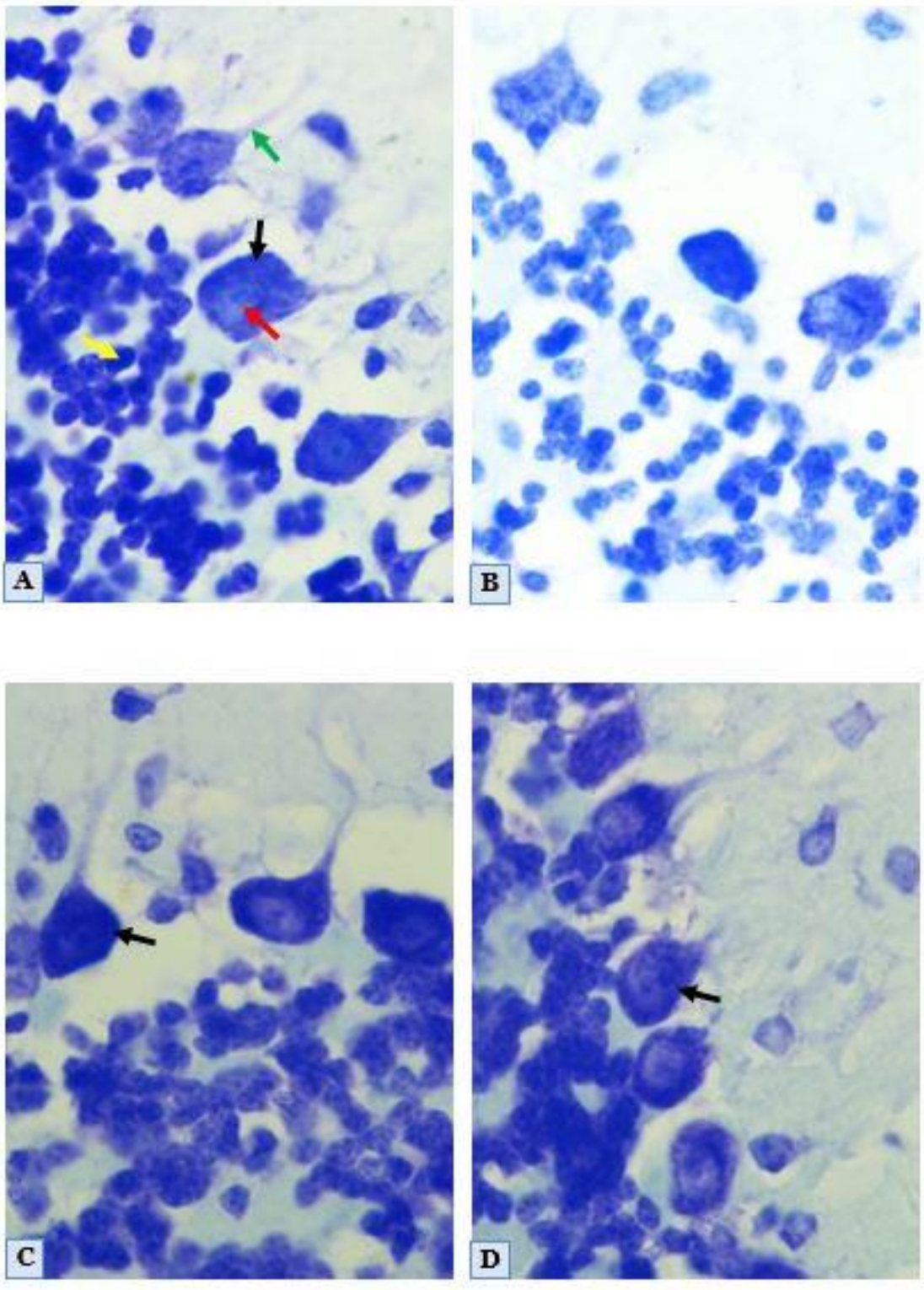

A
B
C
D

Fig. 78: Photomicrograph of cerebellum of pups on PND 70 (Nissl's stain X 1000)

A. Group I cerebellar cortex showing Nissl's granules in cytoplasm of flask shaped Purkinje cells (black arrow) and rounded granule cells (yellow arrow). Pale staining vesicular nucleus with prominent nucleolus (red arrow) as well as apical dendrites (green arrow) of Purkinje cells is also seen.
B. Group II cerebellar cortex showing decreased Nissl's granules in granule and Purkinje cells and loss of shape of Purkinje cells.
C. Group III cerebellar cortex showing increased Nissl's granules in the cytoplasm of flask shaped Purkinje cells (black arrow) and granule cells.
D. Group IV cerebellar cortex showing large number of Purkinje cells (arrow) with prominent Nissl's granules.

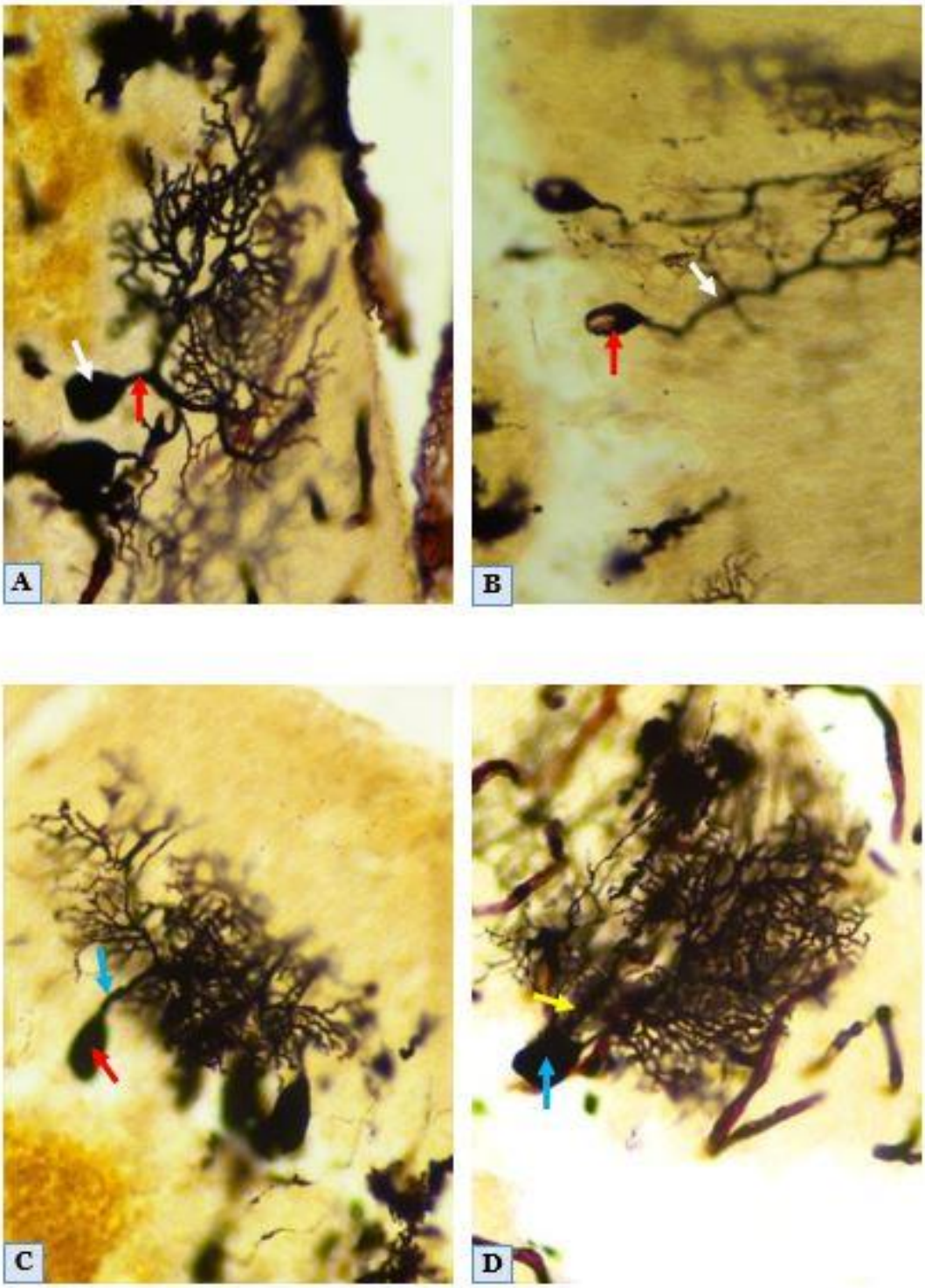

A
B
C
D

Fig. 79: Photomicrograph of cerebellum of pups on PND 70 (Golgi stain X 400)

A. Group I Purkinje cell showing the cell body (white arrow) and the arborization of apical dendrite (red arrow).
B. Group II Purkinje cells showing shrunken and vacuolated cell body (red arrow) and reduced dendritic arborization (white arrow).
C. Group III Purkinje cells showing increased size of cell body (red arrow) and improved dendritic arborizations (blue arrow).
D. Group IV Purkinje cells (blue arrow) showing extensive dendritic arborization (yellow arrow)

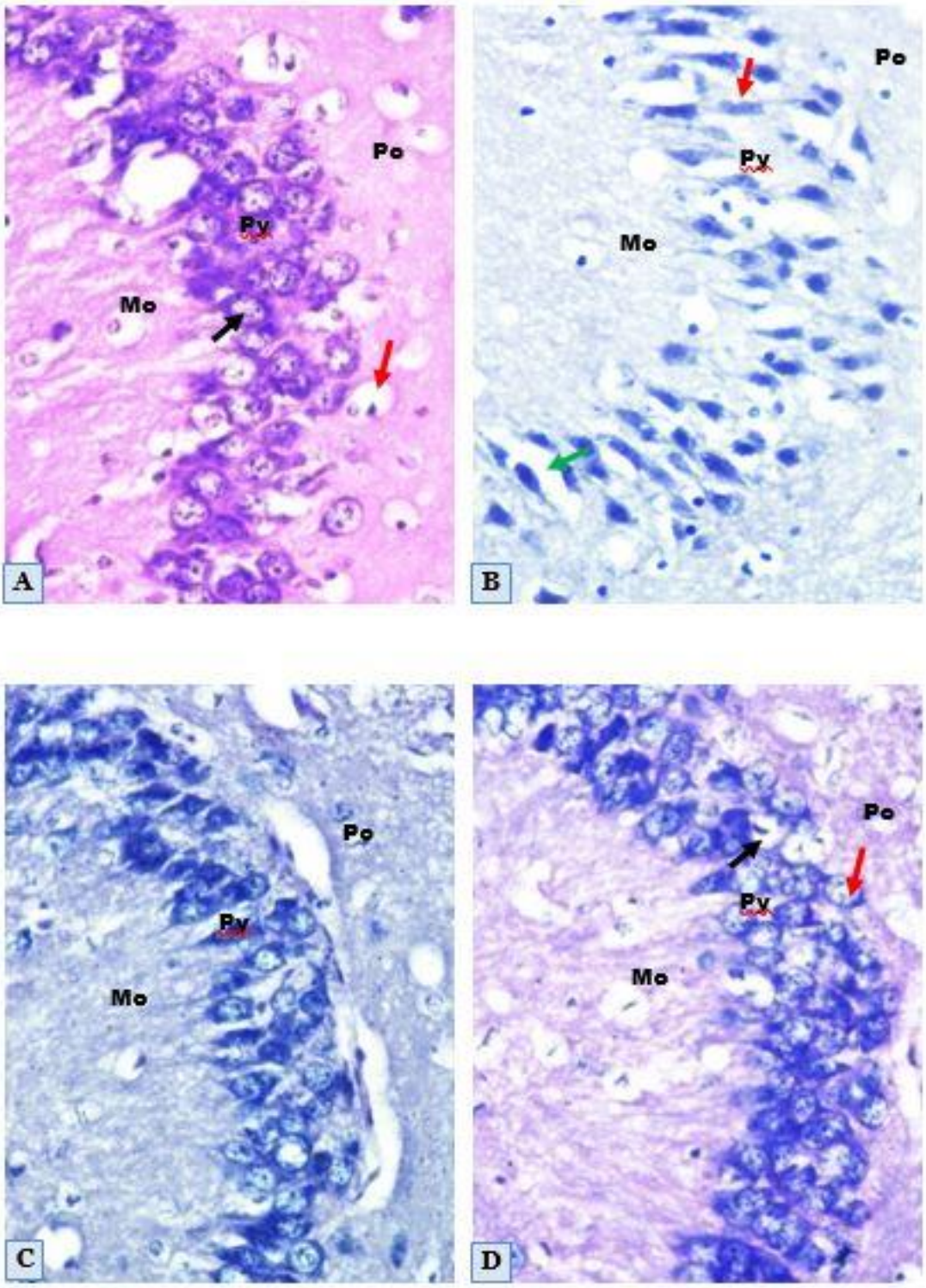

Po
Py
Mo
A
Po
Py
Mo
B
Po
Py
Mo
C
Po
Py
Mo
D

Fig. 80: Photomicrograph of CA3 region of hippocampus of pups on PND 70 (H&E stain X 400)

A. Group I CA3 region showing molecular layer (Mo), pyramidal cell layer (Py) and polymorphic layer (Po). Many pyramidal cells are seen in pyramidal cell layer (black arrow). Few glial cells are also seen (red arrow).
B. Group II CA3 region showing degenerating cells (red arrow) and increased pericellular space (green arrow).
C. Group III CA3 region showing reduced degenerating cells.
D. Group IV CA3 region showing neurons (red arrow) and glial cells (black arrow) in different layers.

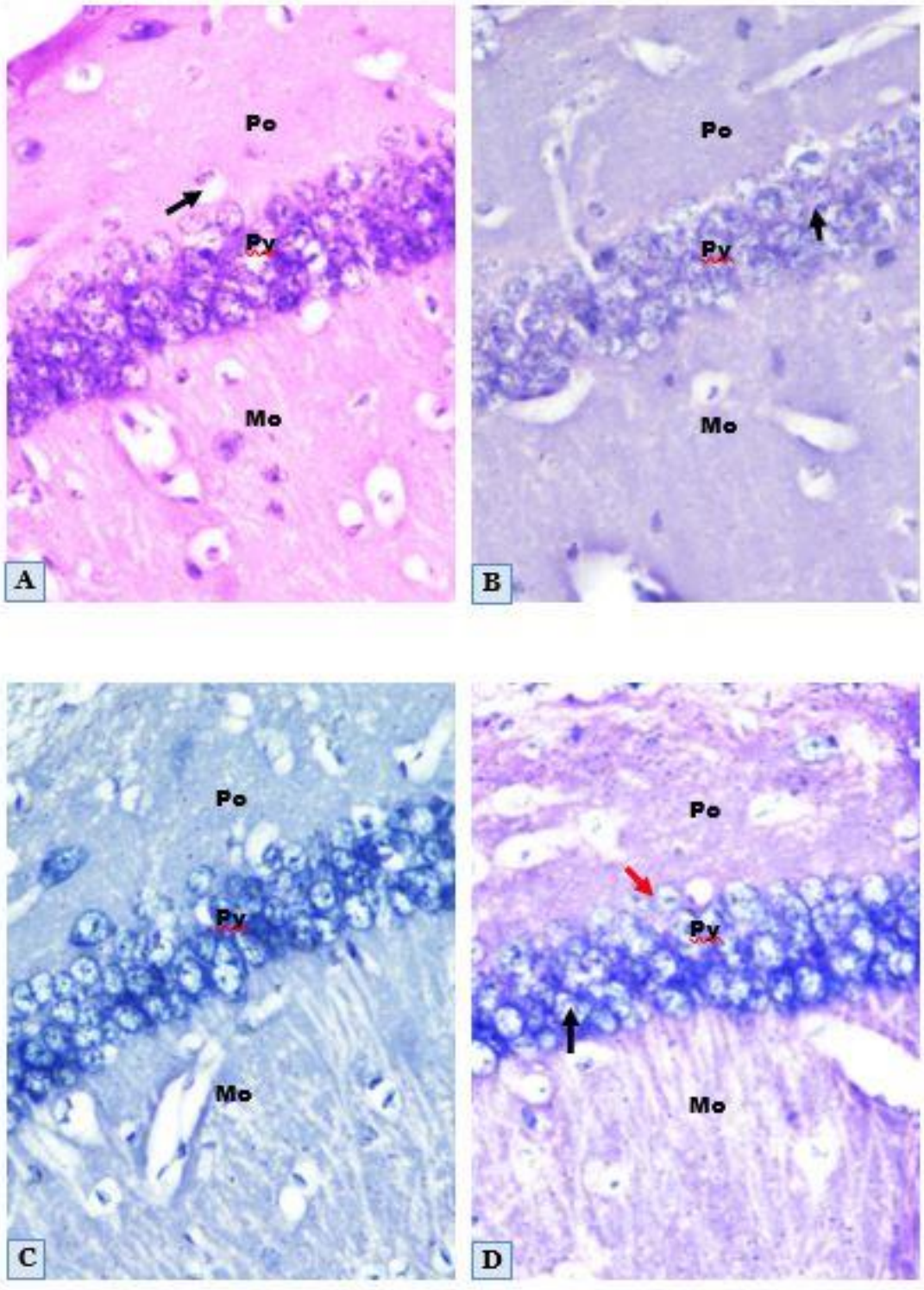

Po
Py
Mo
A
Po
Py
Mo
B
Po
Py
Mo
C
Po
Py
Mo
D

Fig. 81: Photomicrograph of CA1 region of hippocampus of pups on PND 70 (H&E stain X 400)

A. Group I CA1 region showing molecular layer (Mo), pyramidal cell layer (Py) and polymorphic layer (Po). Few glial cells are also seen (black arrow).
B. Group II CA1 region showing reduced thickness of pyramidal cell layer and degenerating pyramidal cells (black arrow) in pyramidal cell layer.
C. Group III CA1 region showing increased thickness of pyramidal cell layer and reduced degenerating pyramidal cells.
D. Group IV CA1 region showing neurons (black arrow) and glial cells (red arrow) in different layers.

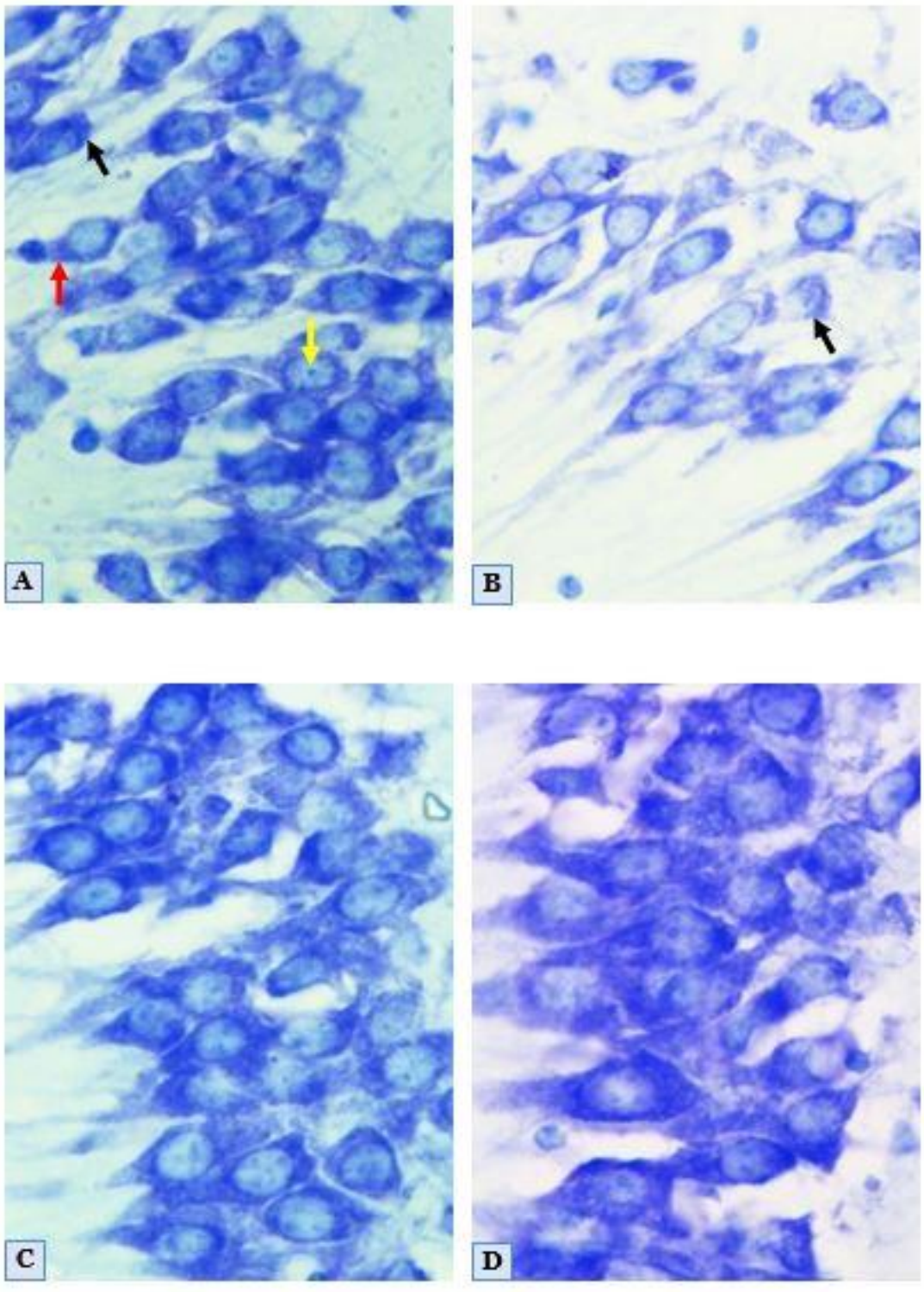

A
B
C
D

Fig. 82: Photomicrograph of CA3 region of hippocampus of pups on PND 70 (Nissl's stain X 1000)

A. Group I pyramidal cell layer showing Nissl's granules (black arrow) in the cytoplasm of pyramidal cells. Faintly stained nucleus (yellow arrow) with prominent nucleolus is also seen. Apical dendrites (red arrow) of the cells are also distinguished.
B. Group II CA3 region showing decreased number of pyramidal cells with reduced Nissl's granules and degenerated cells (black arrow).
C. Group III CA3 region showing increased number of pyramidal cells and increased Nissl's granules in their cytoplasm as compared to group II.
D. Group IV CA3 region showing larger pyramidal cells with abundant Nissl's granules in their cytoplasm.

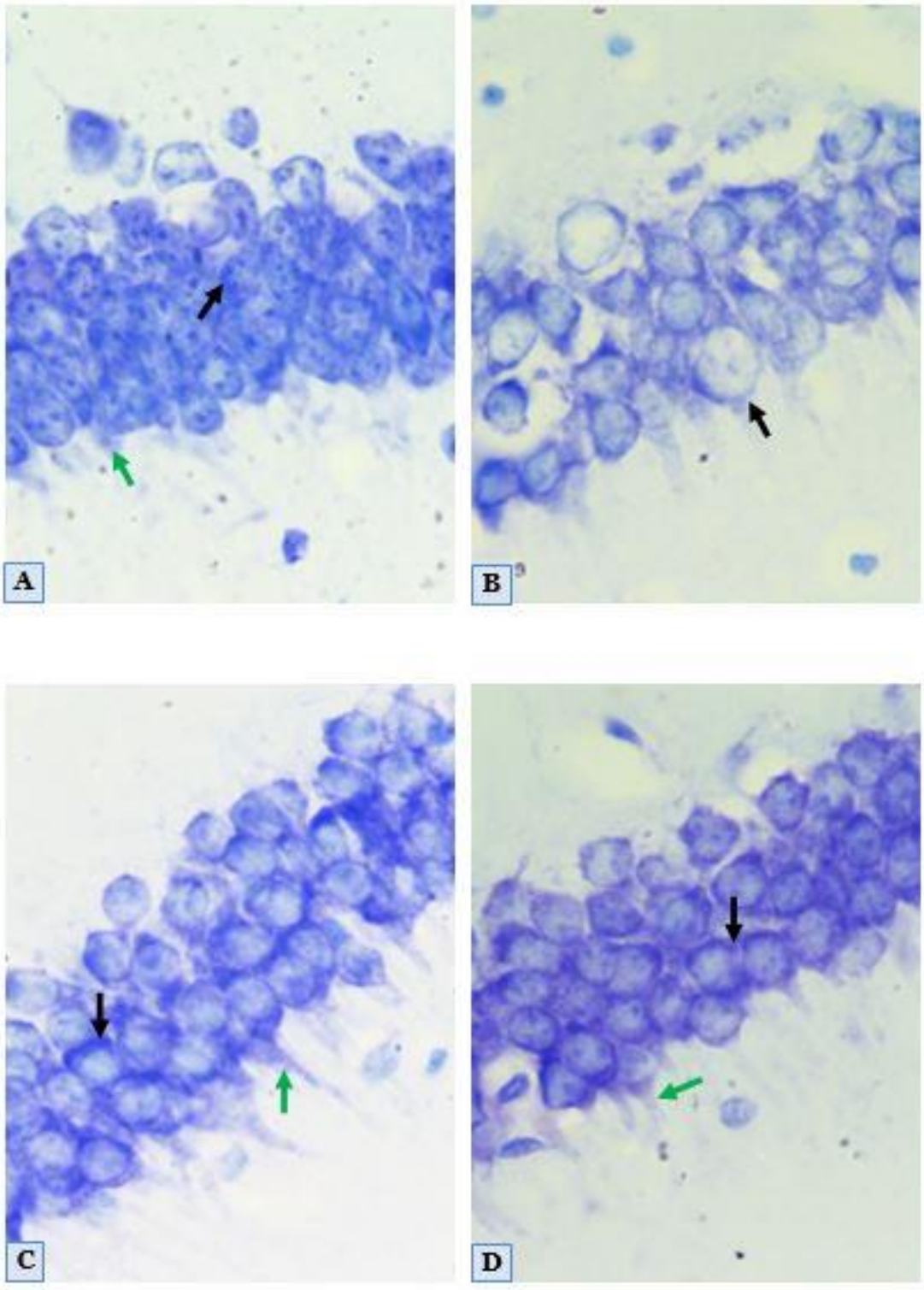

A
B
C
D

Fig. 83: Photomicrograph of CA1 region of hippocampus of pups on PND 70 (Nissl's stain X 1000)

A. Group I CA1 region showing pyramidal cells with apical dendrites (green arrow) and Nissl's granules in their cytoplasm (black arrow).
B. Group II CA1 region showing decreased Nissl's granules in the cytoplasm of pyramidal cells. Vacuolated cells are also seen (black arrow).
C. Group III CA1 region showing increased Nissl's granules in the cytoplasm of pyramidal cells (black arrow). The cellular density and thickness of pyramidal cell layer is increased. Apical dendrites (green arrow) are also seen.
D. Group IV CA1 region showing prominent Nissl's granules in the cytoplasm of pyramidal cells (black arrow). Apical dendrites are also prominent (green arrow).

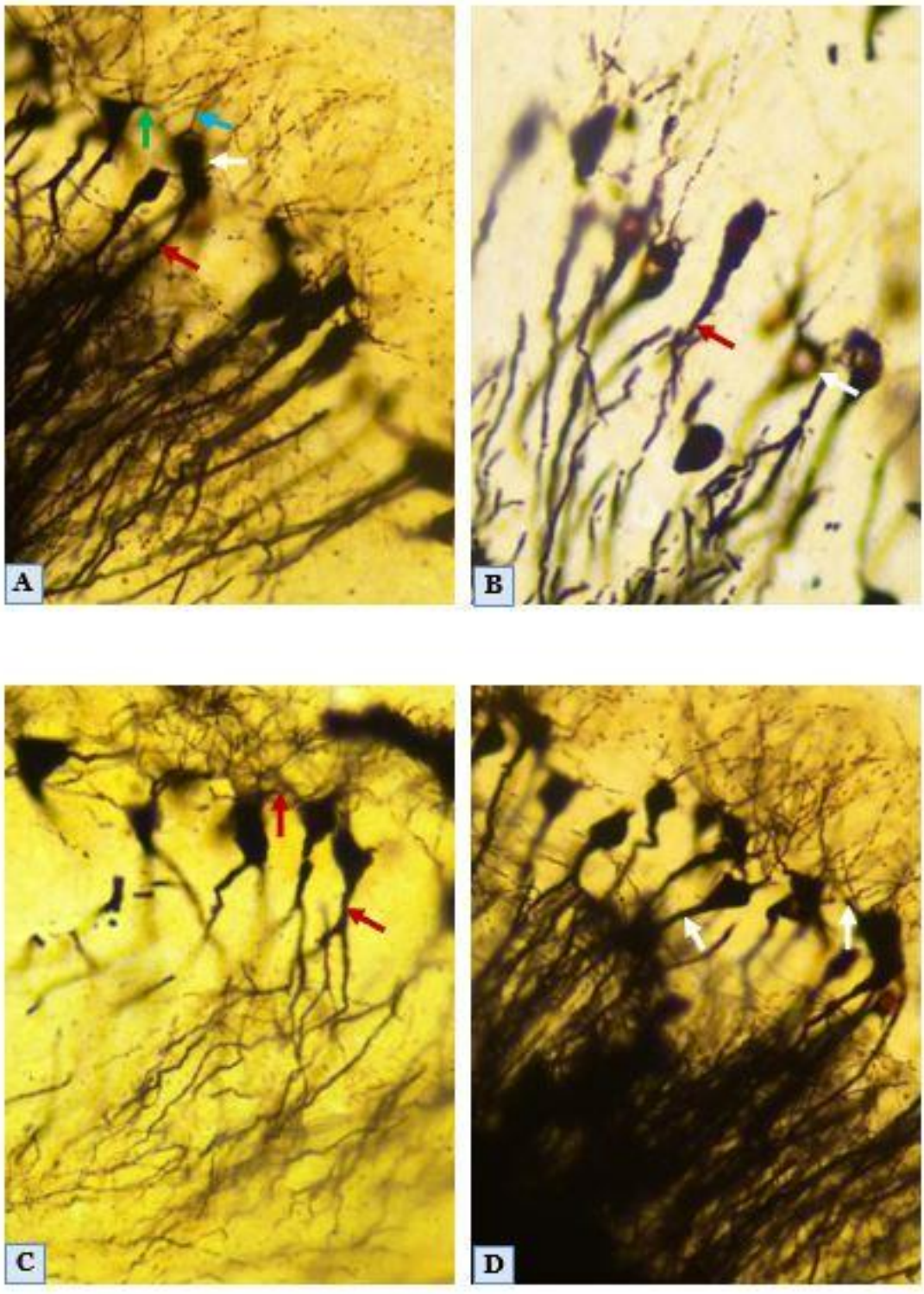

A
B
C
D

Fig. 84: Photomicrograph of CA3 region of hippocampus of pups on PND 70 (Golgi stain X 400)

A. Group I CA3 region showing cell body of pyramidal cells (white arrow), their axons (blue arrow) and the arborization of basal (green arrow) and apical (red arrow) dendrites.
B. Group II CA3 region showing vacuoles (white arrow) in cell body of pyramidal cells and decreased dendritic arborizations (red arrow).
C. Group III CA3 region showing increased dendritic arborizations of pyramidal cells (red arrows).
D. Group IV CA3 region showing extensive arborizations of dendrites of pyramidal cells (white arrows).

Chapter-6

Discussion

In general population studies throughout the world indicate that men are more often drinker and consume more alcohol as compared to women (Wilsnack *et al.*, 2005). However, with the modernization of social living the alcohol drinking by women is increasing progressively. It is reported that about 1.6% of pregnant women frequently drinking during pregnancy but an even greater percentage, about 12.5% women are reported to consume some amount alcohol during pregnancy though irregularly. It was observed that less than half of all women of reproductive age abstain from alcohol use (Tsai *et al.*, 2007). This showed that exposure rates may be higher during early pregnancy as pregnancy is not confirmed until approximately 6^{th} to 8^{th} weeks of gestation. The effect of ethanol is more deleterious during the period of organogenesis, which corresponds to the period from the 6-15^{th} gestational days in mouse and from third to eighth week of pregnancy in humans. This period of development represents a stage particularly sensitive to a teratogenic insult because it is marked by the progressive subdivision of germinative layers, resulting in the beginning of organ differentiation (Becker, 1996). Maternal ethanol consumption during pregnancy can produce several birth defects in the developing fetus that manifest as congenital malformations in postnatal life which may be structural, functional or behavioral. Oxidative stress, resulting from an imbalance between the formation and degradation of ROS, appears to play a major role in ethanol-induced toxicity in a number of organ systems. The

present study was carried out to observe the ameliorative effect of folic acid on alcohol induced congenital malformation as well as behavioral impairments in mice as folic acid is essential for the synthesis of proteins, RNA and DNA which is important for rapidly dividing cells and also reduces oxidative stress.

In the present study the dose of ethanol used (6g/kg/day) seemed appropriate to induce developmental toxicity in Swiss albino mice. Oral route was chosen in the present study since it is more relevant to human ethanol exposure, and may be less stressful to the pregnant mice for daily exposure for 10 days period. Other investigators have used intraperitoneal (i.p.) injection, inhalation, intravenous infusion and voluntary drinking methods (Riley, 1984). Since, intraperitoneal injection, inhalation and intravenous infusion methods may be fairly stressful and prenatal stress is known to adversely affect fetal development (Kapoor & Matthews, 2005) these routes were avoided. In voluntary drinking it is difficult to monitor the dose of the ethanol used and most rodents find the taste of ethanol aversive, so their fluid and food intake in response to ethanol in the drinking water is reduced (Riley & Meyer, 1984). Since, Fetal alcohol syndrome is more common among babies born of alcoholic mothers (who consume alcohol throughout pregnancy) the mice in our experiments were exposed to alcohol from GD 6-15 as it reflects the gestational exposure for human fetal alcohol syndrome.

The weight gain during pregnancy in alcohol exposed dams was severely affected as compared to remaining groups. In our experiment the dams were provided food and water ad libitum during the whole test period to mimic the human situation. The food and water consumed in alcohol exposed mice was equivalent to those in other groups. Alcohol can interfere with digestion of ingested food and their absorption including those of micronutrients and vitamins like folic acid (Marin *et al.*, 1973). The dose related decrease in maternal weight gain during pregnancy in ethanol treated mice was also

observed by Wang *et al*. (2009). The dose of folic acid that was given in group III mice along with alcohol reestablished the weight loss due to alcohol in our study. Chronic alcohol exposure is known to decrease the serum folic acid level by decreasing its intestinal absorption and increasing the urinary excretion (McGuffin *et al*, 1975). Folic acid deficiency may lead to occurrence of anemia, by impairing erythropoesis, hence inducing hypoxia which might result in decreased weight gain in alcohol exposed dams (Hibbard, 1964).

The MDA level in maternal serum was severely increased after exposure to alcohol for 10 days as compared to control. This is due to generation of free radicals or reactive oxygen species during oxidative metabolism of alcohol which can inflict damage on all classes of cellular macromolecules (e.g. mitochondria, endoplasmic reticulum etc) leading to increased cell death (Bergamini, 2004). During the metabolism of alcohol, acetaldehyde and fatty acids are formed which generate a large amount of reactive oxygen species (ROS) by the microsomal enzyme CYP2E1. The oxidation of acetaldehyde also produces superoxide free radicals that are able to react with hydrogen peroxide to from other types of free radicals, further increasing the ROS. Furthermore, chronic alcohol exposure decreases serum folate level, as ethanol has a negative impact on folate absorption, distribution and metabolism in the body and increased urinary excretion (Gloria *et al.,* 1997; Halsted *et al.,* 2002; Muldoon and McMartin, 1994). Because folate helps to maintain normal concentrations of homocysteine, methionine, and S-adeonosylmethionine (SAM), its deficiency can disturb methionine metabolism, leading to hyperhomocysteinemia and SAM depletion. Elevated homocysteine increases H_2O_2 production (Mattson *et al.,* 2002) and affects antioxidant defense systems (Blundell, 1996) further increasing the ROS species. The unpaired electron residing within the structure of ROS is the driving force behind their destructive nature which makes them unstable and highly reactive. These free radicals degrade the phospholipids of cellular membranes through the process

of lipid peroxidation that increases the level of malondialdehyde as it's by product (Millan-Plano *et al.,* 2003 and Topal, 2004). This was similarly seen in the present study in alcohol exposed dams. The GSH level was significantly decreased in the alcohol exposed dams in our study. The conversion of GSH to its oxidized form (GSSG) generates an electron for use in stabilizing free radicals. So, whenever the free radical production increases, conjugated decrease in GSH level occurs. Decreases in GSH level due to alcohol exposure have been reported by many authors (Casteele *et al.,* 2002). Folic acid administration along with alcohol in group III increased the GSH level and decreased the MDA level as compared to that in alcohol exposed dams of group II. This might be due to free radical scavenging activity of folic acid (Nakano *et al.*, 2001 and Joshi *et al.,* 2001) which reduced the alcohol induced free radical generation. It had been reported that folic acid can protect biomolecules from free radical damage by competition and also inhibit lipid peroxidation although it is water soluble molecule. This was mainly due to scavenging and repair of thiyl radicals by folic acid (Joshi *et al.,* 2001). Folic acid supplementation also reduces homocysteine level which was again a factor for the reduction of oxidative stress in folic acid supplemented mice as indicated by reduced MDA and increased GSH levels.

In the present study, the number of implantations did not differ significantly between the groups because the treatments were started from GD 6 onwards at which implantation was already completed. The number of live fetuses was significantly decreased and the number of resorptions and dead fetuses was increased in alcohol exposed group. Similarly, ethanol exposure during organogenesis resulted in developmental retardation of fetuses as shown by decreased weight, crown rump length and tail length. Similar findings were reported by other investigators also (Day *et al.,* 2002; Hannigan *et al.,* 1993; Keiver and Weinberg, 2004). Co-administration of folic acid along with alcohol dramatically decreased the deleterious effect of alcohol on pregnancy

outcome by increasing the number and growth of live fetuses and decreasing the number of dead/resorbed. It has been reported that folic acid deficiency is a common feature in pregnancy and becomes more severe in alcoholics during pregnancy (Lin, 1991) which might be due to increased urinary excretion and decreased intestinal absorption as discussed earlier. It has also been reported that ethanol may impair transport of folic acid across placenta by decreasing expression of folate transport proteins (Janine *et al,* 2012). So, the adverse pregnancy outcome in the present study might be due to deficiency of folic acid induced by alcohol consumption which was reversed by the administration of folic acid which neutralizes the effect of ethanol on the pregnancy outcome. Folic acid deficiency also causes elevated serum homocysteine (HCY) in animals and humans (Carmel and James, 2002). Elevated HCY during pregnancy may result in different developmental defects (Xu *et al.,* 2006). So the increased number of fetuses with congenital malformations in alcohol exposed mice that was observed in the present study might be due to elevated HCY level. Folic acid supplementation decreases the homocysteine level. This may be the reason of lowering the number of malformations that was seen in folic acid supplemented group in our study. Folic acid is an important cofactor for enzymes that are essential in DNA and RNA synthesis that is involved in the transfer of methyl groups in the amino acid methylation cycle. It is an essential step in the recycling of homocysteine back to methionine. The demand for folic acid is increased during pregnancy as the synthesis of the nucleic acids and proteins increases during rapid embryonic and fetal growth during development. The function of specific proteins, lipids, or even myelin might be impaired when the methylation cycle is inhibited due to deficiency of folic acid. These mechanisms might explain the adverse pregnancy outcomes and increased congenital malformations resulting from folic acid deficiency (Hibbard, 1964; Wagner, 1995) due to alcohol exposure in utero. Xu *et al,* (2006) also observed that birth defects induced by ethanol are reduced by

maternal folic acid and Vit B12 supplementation. But the ethanol dose that was used in that study was lower than in the present study. Wang *et al,* (2009) also observed that teratogenic effects of ethanol can be suppressed by folic acid supplementation.

Several skeletal malformations were also observed in prenatally alcohol exposed fetuses of group II. Many fetuses were lightly stained with alizarin red indicating decreased calcium deposition in the developing bone. The enhanced skeletal malformations in the prenatally alcohol exposed fetus might be due to oxidative stress induced by alcohol and defect in methionine metabolism during their development. Decrease in calcium deposition might be due to facilitatory effect of ethanol on calcium inhibitors which are family of inorganic phosphates, phosphonates and diphosphonates. The calcium inhibitors normally act to prevent calcium deposition in soft tissues (Anderson, 1978). Pyrophosphate enzymes, which are normally secreted in vesicles, destroy the inorganic calcium inhibitors. Ethanol suppresses these pyrophosphates enzyme activities due to which calcium inhibitors are activated resulting in delayed bone mineralization (Friday & Howard, 1991). Furthermore, alcohol consumption also reduced osteoblast activity which inhibited the synthesis of matrix during ossification (Klein & Fausti-Carlos, 1996). Chronic alcohol consumption also decreased folic acid level in the body as described earlier due to which the homocysteine level increases. Elevated homocysteine levels also leads to defective bone matrix by interfering with the proper formation of collagen, the main protein in the bone. It is believed to interfere with cross links of newly formed collagen and, consequently, with bone strength and bone mineralization (Bozkurt *et al.,* 2009). These could explain the poor calcification of the developing bones. Decreased bone mineralization and bone density due to chronic alcohol exposure was also reported by other studies (Turner *et al.,* 1991). Folic acid supplementation along with alcohol reduced the skeletal malformations as well as increased the

calcium deposition in the developing bones in group III as compared to those in alcohol exposed fetuses of group II. This might be due to decrease in homocysteine level after folic acid administration. The decrease in oxidative stress might also increase osteoblast activity, inducing bone mineralization.

In the present study alcohol exposure to pregnant dams during the period of organogenesis significantly decreased the placental weight as well as its thickness and diameter. The finding is supported by many other studies (Kennedy, 1984; Greizerstein and Aldrich, 1983; Garban *et al.,* 1985). In human also placental weight as well as size was decreased in dose dependent manner (Burd, 2007). However, there are some conflicting reports also (Akay and Kockaya, 2005, Eguchi *et al,* 1989, Lui *et al,* 2014) where increased placental weight after alcohol intoxication has been reported while others didn't find any change (Chernoff, 1977; Nelson *et al.,* 1985). The variations in the results by different authors might be due to differences in the experimental conditions, duration and dose of exposure and stage of pregnancy. Exposure usually during first trimester of pregnancy disturbs the development of placenta leading to placental dysfunction and the organism reacts through compensatory mechanisms causing its hyperplasia and cellular hypertrophy, thereby, inducing an increase in placental mass in order to restore its integrity and functions, while severe exposure during latter period of pregnancy results in decrease in placental weight probably due to alcohol induced malnutrition due to disturbance in digestion and absorption of nutrients (Aufrere and Lebourhis, 1987). As previously discussed chronic alcohol consumption could lead to folic acid deficiency. The rapidly developing placenta showed retarded growth due to folic acid deficiency resulting to decreased placental weight, thickness and diameter as well as placental insufficiency as observed in the present study. The reduced thickness of the placenta could also be explained in part by increased necrosis, due to ischemic infarction, as evidenced by the presence of congested maternal sinusoids and increased number of degenerating cells

within the labyrinth and basal zones of ethanol-exposed placentas. The labyrinth zone also showed thickening of placental barrier affecting the exchange of materials between mother and fetus occurs. In the present study, it might be responsible for resorptions, fetal death and fetal growth retardation. When folic acid was administered along with alcohol the placental weight and its dimension was increased significantly as compared to those in alcohol exposed group. The degenerative changes observed in the labyrinth zone as well as basal zone were also much reduced when compared to ethanol exposed placentas. The placental barrier was also comparatively decreased in its thickness. This might be due to increased cell proliferation, DNA replication and antioxidant protection in folic acid supplemented group where also the fetal lethality, retardation in growth and congenital malformations induced by alcohol were decreased.

Reduction in weight of fetal kidney was observed in prenatal alcohol exposed fetuses. Histopathological study revealed severe glomerular necrosis and degeneration of epithelial lining of renal tubules, Bowman's capsule and collecting ducts. Such detrimental effect of alcohol on the kidney might impair their ability to regulate the volume and composition of fluid and electrolyte balance in the body (Epstein, 1997). The observed dilation of urinary space, lumen of renal tubules and collecting ducts might be due to impaired renal function. Morphometric analysis showed that the nephrogenic zone was significantly thicker in the kidney of alcohol exposed fetuses indicating delayed nephrogenesis. Similarly, the number of renal corpuscles was significantly decreased and concomitant increase in size of the renal corpuscles was observed in alcohol exposed fetuses. The number of collecting ducts was also reduced in the medulla of those kidneys. Decreased number of renal corpuscles and collecting ducts might be due to partial attenuation of ureteric branching induced by alcohol and increased size of renal corpuscles was presumed to indicate their compensatory hypertrophy. Similar observations were made by

Gray *et al.* (2010) and Eluwa *et al.* (2008). According to Gray *et al.* (2010) the changes induced in kidney due to prenatal alcohol exposure also affect adult cardiovascular as well as renal function. Another mechanism for decreased nephron number might be due to ethanol induced increased apoptosis of nephrogenic mesenchyme which may result in a lower number of nephrogenic condensates, leading to a reduction in nephron number. Apoptosis is a critical process in metanephric development, and increased apoptosis has been shown to reduce the nephron number (Burrow, 2000, Saikumar *et al.,* 2003). Administration of folic acid along with alcohol decreased the degenerative changes induced by alcohol. It also decreased nephrogenic zone thickness, increased the number of renal corpuscles as well as decreased their size. This might be again due to cell proliferation, DNA replication and antioxidative property of folic acid which lowered the detrimental effect of alcohol on developing kidney.

The weight of fetal liver was also significantly decreased in the prenatally alcohol exposed fetuses. Histopathological examination revealed marked degenerative cellular changes resulting into disorganization and distortion of hepatic laminae. Appearance of large lacunar spaces without any evidence of developing sinusoids and central veins was a common feature. Decreased cellular density and increased fibrosis along the lineages of developing hepatoblasts and haematopoietic cells was also observed. Decreased PAS positive material and increased megakaryocytes throughout the sections were observed in PAS stained sections. The observation of decrease in fetal liver weight corroborated with many other reports also (Henderson *et al.,* 1979; Pullen *et al.,* 1988; Buts *et al.,* 1992). The degenerative changes observed in the liver of alcohol exposed fetuses might be due to alcohol induced oxidative stress. Oxidative stress induces apoptosis and necrosis of cells. Alcohol induced injury of the cells is associated with membrane damage caused by lipid peroxidation, altered cell glutathione homeostasis and deranged

mitochondrial structure and function (Devi *et al.,* 1993). Alcohol also causes reduction in the activity of alcohol dehydrogenase and cytochrome P-450 in the liver that are involved in alcohol metabolism. Although the alcohol is primarily metabolized by maternal liver during pregnancy, the reduced activity of these enzymes in fetal liver leads to accumulation of alcohol and subsequent death of cells due to the toxic effect of alcohol (Guerri & Rosa, 1986). As already described chronic alcohol consumption decreases the folic acid level resulting into folic acid deficiency which disturbs hepatic methionine metabolism and this promotes liver injury (Halsted *et al.,* 2002). The reduced number of hepatoblasts and haemopoetic cells might be due alcohol induced decrease in DNA synthesis affecting their multiplications (Devi *et al.,* 1993). The increased number of megakaryocytes might be due to alcohol induced vascular damage in developing liver as also reported by Kockaya *et al.,* (2006). During intrauterine life the liver is the primary site of haematopoiesis. The histopathological changes observed in alcohol group may be due to reason that intoxication might affect haematopoietic cell trafficking from the liver to other sites which affect the growth of other organs as well as the fetus as a whole. These findings are in line with the results of a study in rats by Gallo and Weinberg (1986). Folic acid co-administration along with alcohol significantly increased the liver weight and reduced the degenerative changes induced by alcohol in the fetal liver. The number of hepatoblasts and haematopoietic cells was also increased. The PAS-positive material was also increased in the developing liver. This might be due to antioxidant and cell proliferative property of folic acid. Folic acid decreases oxidative stress by scavenging free radicals which might reduce the alcohol induced degenerative changes in the liver. It also promotes cell division by the synthesis of cellular proteins, RNA and DNA that might result in increased cell proliferation in the liver of folic acid exposed fetuses. It also promotes hepatic methionine metabolism which decreased the alcohol induced liver injury (Halsted *et al.,* 2002).

In the present study, the fetal brain weight as well as relative weight of brain on GD 18 was significantly decreased in fetuses that were exposed to alcohol as compared to that of control. The finding was supported by many other studies (Jones and Smith, 1973; Wisniewski *et al.,* 1983). The reduced brain weight in alcohol treated fetus might be due to negative impact of alcohol on the development of neural tube which results into nervous system anomlaies. The delay or failure of proper proliferation and migration of neuroepithelial cells induced by alcohol may underlie the cause of decrease in neural tissue resulting into decreased brain weights (Zhou *et al.,* 2011).This may be the reason of decrease in relative brain weight.

The oxidative stress was severely increased in the brain of alcohol exposed fetuses as evidenced by increased MDA and decreased GSH levels. This increase in oxidative stress was reduced when supplemented with folic acid along with alcohol due to antioxidative property of folic acid as indicated by decreased MDA and increased GSH levels. Singh *et al.* (2011) also observed reduction of oxidative stress by folic acid supplementation in the brain of rats. Ethanol readily crosses the placenta and reaches concentration in the fetus which is similar to that in the maternal blood (Waltman and Iniqez, 1972). Due to low activity of hepatic dehydrogenase, the fetus has limited ability to metabolize alcohol. Therefore, the elimination of alcohol from the fetus is through passive diffusion of alcohol across placenta followed by maternal elimination. In addition, the rate of diffusion of alcohol from amniotic fluid is slow, resulting into relatively high alcohol concentration in amniotic fluid even when alcohol level is low or almost nil in the maternal blood. So, the amniotic fluid acts as reservoir for alcohol and the fetus is actually exposed to it for a longer period than assumed on the basis of maternal alcohol concentration (Brien *et al.,* 1983). This circulating alcohol in the fetus increases the oxidative stress increasing free radicals within the cells. Typically, the

levels of ROS and other free radicals are controlled by various scavenger molecules, known as antioxidants. But, alcohol also reduces antioxidant levels, further increasing the oxidative stress that may contribute to alcohol-induced cell damage and cell death in the fetus (Guerri, 1998; Henderson *et al.*, 1995; Kotch *et al.*, 1995). Furthermore, brain tissue is characterized by a low content of natural antioxidants (Surai *et al.*, 1996) and generates a greater amount of free radicals per gram of tissue than any other organ (Reiter, 1995) in response to alcohol exposure.

Histopathological study of fetal cerebrum revealed, decreased thickness of whole cerebrum as well as that of cortical plate, but the thickness of ventricular zone was increased in prenatally alcohol-exposed brains. Degenerating cells were common in ventricular zone as well as in the cortical plate. The cellular density in the cortical plate was also decreased. The decreased thickness of cerebrum might be due to smaller brain. The increased ventricular zone thickness and decreased cortical plate thickness might be due to defect in neuronal migration as well as alterations in the proliferation of neuronal precursor cells. As described already all the neurons in the cortical plate are formed by the proliferation and migration of the precursor cells from the ventricular zone. The migrations of developing neuroblasts are guided by the radial glia which serves as elongated cellular tracks that direct neurons to their appropriate destinations. Once all neurons have migrated to their final positions, the radial glial cells normally change into astrocytes. Ethanol exposure alters the radial glia and disturbs the proliferation, generation and migration of the neuroblasts. Due to alcohol, radial glia may become astrocytes prematurely (Miller and Robertson, 1993) stopping the migration of developing neurons. In the fetal hippocampus also degenerative changes were observed in pyramidal layer of CA3 region in the alcohol exposed fetus. The number of developing neuroblasts was also much reduced. Large number of vacuolated

cells as well as cells with ruptured membrane and pyknotic nuclei were also observed. Similarly in fetal cerebellum, cellular number was quite decreased in inner granular layer. The cells were enlarged in size showing ballooning degeneration. The cells migrating from the external granular layer had lost their rounded shape, became much smaller and clumped together. At some places, vacuolated cells were also seen showing the signs of degeneration. The degenerative changes observed in the developing cerebrum, hippocampus and cerebellum might be due to deleterious effect of alcohol due to increased oxidative stress in the developing brain. The developing brain, which has only a fraction of the antioxidant enzyme activity of the adult brain, is perhaps even more vulnerable to the neurotoxic effects of oxidative stress than the adult brain (Henderson *et al.,* 1999). Increased oxidative stress induces necrosis and apoptosis in the developing brain. Since the cells of developing brain have lower threshold for alcohol than other parts of the developing fetus, the developing brain is more vulnerable to prenatal alcohol insult. Increased cellular death can affect production, migration and differentiation of future cell lines. The alcohol induced formation of excess levels of ROS also can also damage cells and induce cell death by interfering with the function of mitochondria. Besides generating most of the cellular energy the mitochondria also serves an additional important function of storing calcium in the cell and maintains calcium channels, which is particularly critical in neurons. To ensure accurate neuronal function, calcium levels inside the neurons must be properly regulated (Choi, 1995). Therefore, the ability of the mitochondria to actively sequester calcium is vital for maintaining neuronal function and survival. Alcohol-induced formation of excess ROS levels leads to disturbed mitochondrial function including the mitochondria's ability to regulate intracellular calcium levels which can further lead to neuronal necrosis and apoptosis (Kroemer *et al.,* 1997). Folic acid supplementation along with alcohol increased the thickness of cerebrum where the thickness of cortical

plate was increased and the thickness of ventricular zone was decreased as compared to those of alcohol exposed fetuses. The cellular density in the cortical plate of cerebrum as well as in developing hippocampus and cerebellum was also increased. The degenerative changes in the developing cerebrum, cerebellum and the hippocampus induced by alcohol was also much reduced. This might be due to the antioxidative and cell proliferative property of folic acid which reduced the deleterious effect of alcohol on those regions of developing brain. As described already folic acid deficiency is a common feature in pregnancy and becomes more severe in alcoholics during pregnancy (Lin, 1991) that might be due to increased urinary excretion and decreased intestinal absorption. Ethanol also impairs transport of folic acid across placenta by decreasing expression of folate transport proteins (Janine *et al,* 2012). This results in the decreased availability of folic acid for the proliferating and differentiating cells of the developing brain resulting into degenerative changes as observed in the present study which were reduced when supplemented with folic acid.

Postnatal growth of the offspring of different experimental animals was assessed from the time of their birth until postnatal day 70. The weight of pups that were prenatally exposed to alcohol tended to be significantly low throughout the study period. Similar observations were reported by many authors (Day *et al.,* 2004; Geva *et al.,* 1993; Klug *et al.,* 2003; Leichter and Lee, 1979, Dursun *et al.,* 2006, Wang *et al.,* 2009) also. This result may be attributed solely to the effect of alcohol which alters the digestion and nutrient absorption including micronutrients resulting in malnutrition. That is the reason that prenatally undernourished mice undergo prolonged catch-up growth (Cogswell *et al.,* 2003 and Dreotsi, 1993). The weight of pups of group I, III and IV were comparable during the growth period till PND 70. This showed that folic acid induced catch up growth in the offspring.

In the present study prenatal ethanol administration at the dose of 6g/kg/day from GD6 to GD15 to pregnant dams significantly decreased their locomotor activity as assessed by open field test in terms of decreased ambulation, rearing and increased freezing time in alcohol exposed mice. Several authors have also reported decreased locomotor activity in offspring after alcohol intoxication during pregnancy as compared to control (Dursun *et al.,* 2006 and Carneiro *et al.,* 2005). The folic acid administration along with alcohol significantly increased the locomotor activity. Decreased open field activity in folate deficient animals has also been reported by Ferguson *et al.* (2005).

In elevated plus maze test, the ethanol exposed mice spent significantly less time in open arm and more time in closed arm as compared to controls. This observation shows lower ambulation score in this group as compared to controls in open field test suggesting increased level of anxiety in alcohol pretreated mice as also reported earlier (Dursun *et al.,* 2006). Elevated anxiety in alcohol treated mice offspring as assessed by elevated plus maze test is consistent with earlier reports by several authors (Dursun *et al.,* 2006; Ogilvie and Rivier, 1997; Osborn *et al.*, 1998). According to them the animals exposed to alcohol in utero are typically hyper responsive to stressors in adulthood as indicated by increased activation of the hypothalamic-pituitary-adrenal axis. It is also consistent with the report that prenatal exposure to alcohol decrease sensitivity to GABA receptor's allosteric modulators such as endogenous neurosteroid allopregnanolone which is thought to act as an endogenous anti-anxiety agent in novel or stressful situations (Zimmerberg *et al.,* 1995). In the present experiments when folic acid was administered along with ethanol, the open arm duration was significantly increased and closed arm duration significantly decreased as compared to alcohol exposed group. There was no significant difference in open arm and closed arm duration among the groups I, III and IV. The results showed that prenatal alcohol exposure significantly

increased the anxiety level in mice which was subsequently decreased by folic acid administration. In folic acid deficient mice decreased open arm duration and increased closed arm duration has also been reported by Ferguson *et al.* (2005).

In both place acquisition and reversed acquisition task of Morris water maze test, the ethanol exposed pups took longer time to locate the hidden platform than the controls i.e. escape latency. However, folic acid administration dramatically decreases the escape latency. Although during the initial days of both place acquisition and reversed acquisition experiments, pups of each group showed similar latency to locate the hidden platform, during follow up days of the folic acid supplemented pups located the platform more quickly than the alcohol exposed group. In the probe trial experiment also the pups of the folic acid supplemented group remained in the quadrant, which initially contained the platform, for longer duration. However during cued version of Morris water maze test the escape latency for each group did not differ significantly indicating that the difficulty in locating the hidden platform by alcohol treated mice was not due to visual impairment or physical deformity but due to learning and memory deficits. Our findings corroborated with those of Wang *et al.* (2009) who also observed significant differences between alcohol exposed group and controls in both place acquisition and probe trial of Morris water maze test. The only difference being that the dose of ethanol given in that study was slightly less than in the present study and the behavior experiment was done around PND40. Similarly Kim *et al.* (1997) also reported longer escape latencies in offspring of rats who were given free access to ethanol throughout gestation and the offspring were tested at 16-17 month of age. Westergren *et al.* (1996) also reported tendency towards a lower acquisition of place learning in the Morris water maze in rats prenatally exposed to ethanol. Cronise et al (2001), reported that offspring of alcohol exposed rats demonstrated longer latencies during place learning in Morris

water maze when juveniles, however, this deficit was not observed when the rats became adult. Similarly, Dursun et al (2006) and Abel (1979) didn't find any significant difference in Morris water maze test between offspring of alcohol exposed group and controls. The variation in the result might be due to the difference in severity of the behavioral deficits which depends upon the interaction of several factors such as the amount of consumed alcohol, duration and pattern of alcohol consumption, degree of difficulty of the employed behavioral task and eventually age at testing, gender and even the strain of the tested animals. As already described chronic alcohol consumption decreases hepatic and serum folic acid level leading to folic acid deficiency by increasing urinary excretion and decreasing intestinal absorption. It also decreases placental transfer of folic acid by decreasing expression of folate transport proteins. Folate (vitamin B9) deficiency can decrease methylation reactions due to S-adenosyl-methionine depletion (Mattson, 2003; Ulrey *et al.,* 2005) and elevate plasma homocysteine level (Bottiglieri, 2005). In neural tissue, excessive HCY promotes excitotoxicity via stimulation of glutamate-NMDA receptors, synaptic dysfunction, DNA damage, and activation of apoptosis (Ho *et al.,* 2002; Kruman *et al.,* 2004). The homocysteine converting gene is highly expressed in hippocampus and cerebellum. So the elevated homocysteine may affect spatial learning and motor performance. It has been reported that rats born of folate deficient dams exhibit diminished learning capacity and electro-encephalogram (EEG) abnormalities (Whitley, 1951). The learning deficits observed in folate deficient rats was reversed by the administration of folate or thiamine (Bachevalier, 1981). So in the present study severe learning and memory deficits that were observed in fetal alcohol mice might be due to deficiency of folic acid during gestation and when folic acid was given along with alcohol the deficit was reduced.

In behavior despair test as well as in the tail suspension test initially mice in all groups exhibited gross motor movements which were indicative of

escape behavior, then eventually became relatively immobile or made only small motor movements (twitching and kicking). The total immobility time during test period for the fetal alcohol mice was significantly higher as compared to other groups which clearly showed signs of depression. Caldwell *et al.* (2008) obtained similar results and found that brain-derived neurotrophic factor is decreased in fetal alcohol mice. Carneiro *et al.* (2005) also reported dose dependent increase in immobility time in behavior despair test in fetal alcohol rats as compared to controls. FASD in combination with depression has been reported in many studies (Famy *et al.*, 1998; O'Connor and Kasari, 2000; Roebuck *et al.*, 1999). When folic acid was administered along with alcohol there was marked reduction in immobility time indicating reduction of depression induced by prenatal alcohol administration by folic acid co-treatment. Several clinical studies have reported association between depressive disorder and low folic acid levels (Reynolds, 2002; Abou-Saleh and Coppen, 2006). Additionally, a deficiency of folate causes elevated homocysteine concentrations, which may contribute to the pathogenesis of major depression (Bottiglieri, 2005; Coppen and Bolander-Gouaile, 2005).

. The present study showed attenuation of postnatal brain growth due to prenatal exposure of alcohol during the period of organogenesis. Numerous human and animal studies have also shown reduction in brain weight induced by prenatal alcohol exposure (Tran *et al.*, 2000; Maier *et al.*, 1999; Qiang *et al.*, 2002). This might be due to oxidative stress induced by alcohol during the development. Furthermore alcohol may interfere with growth factors necessary for normal brain development. This has been shown in several studies on insulin-like growth factors (IGF) I and II (Singh *et al.*, 1996). Ordinarily, IGF I and II bind with an IGF receptor on the neuron and a message is sent to stimulate cell division. In the presence of alcohol, the receptor site becomes dysfunctional and the message is never sent. Furthermore, for non-dividing nerve cells, IGF I and II receptors are important for maintenance of cell life.

Their function is impaired in the presence of alcohol (Singh *et al.,* 1996). Folic acid co-treatment along with alcohol significantly increased the brain weight and was comparable with that of control. This might be due to antioxidative and cell proliferative property of folic acid.

Histopathological study of the cerebrum of the prenatally alcohol exposed pups showed numerous degenerative changes in each layer of the frontal cortex. The laminar pattern of the cortex was also disorganized. Cellular density was decreased in each layer. Moreover the diameter of pyramidal cells of layers III and V was also significantly decreased on histomorphometric study. Prenatal ethanol exposure depresses the proliferation of the cortical neurons generated which might result into decreased number of neurons in different layers of the frontal cortex. It also induces abnormal migration of the neurons resulting in disruption of the lamination of the cortex (Guerri, 1998) as observed in the present study. Alterations in neuronal migration and in cell death may be the result of either change in radial glia guiding fibres or alterations in the production of glial factors in response to alcohol that are essential to neuronal migration or survival (Guerri, 1998). Therefore, alterations in the radial glia might be an underlying mechanism in the ethanol induced disorganization of laminar pattern and degenerative changes in the frontal cortex as observed in the present study. Ethanol also triggers widespread neuronal death in forebrain by blocking the N-methyl-D-aspartate (NMDA)-glutamate receptor and activating GABA receptors (Ikonomidou *et al.,* 2000). When folic acid was given along with alcohol the degenerative changes in the frontal cortex were much reduced, the laminar pattern was more organized and the cellular density was increased in each layer. The diameter of pyramidal cells of layer III and V was also increased as compared to prenatally alcohol exposed pups. The antioxidative and cell proliferative property of folic acid might reduce deleterious effects of ethanol on cerebrum.

In the hippocampus, degenerative changes were observed in the pyramidal layer of CA1 and CA3 regions in prenatally alcohol exposed pups. The thickness of both the layers was observed to be reduced as compared to controls. Frequent shrinkage of pyramidal cells with pyknotic nuclei in CA3 region was also observed. In some of the cells, pyknotic nuclei as well as vacuolation were seen. Pericellular lacunar spaces were also present which may be due to edematous infiltration that was responsible for compression of the cells. Histomorphometric analysis revealed marked reduction in cellular density in both the CA1 and CA3 regions as compared to control. Similar observations have been reported by other workers also (Barnes and Walker, 1981 and Perez *et al.,* 1991; Ba *et al.,* 1996). Morphological studies using unbiased stereological procedures indicate that reduced neuronal populations in the hippocampus following prenatal alcohol exposure are regionally selective and are due to decrease in neuronal generation or proliferation, rather than cell death or changes in density due to changes in area or volume (Berman and Hannigan, 2000). Folic acid administration along with alcohol reduced the degenerative changes in the CA1 and CA3 regions of the hippocampus. The regions were thicker and cellular density was also increased as compared to alcohol exposed pups. This might be due to antioxidative and cell proliferative property of folic acid.

The degenerative changes were also observed in the cerebellum of pups that were prenatally exposed to alcohol. The thickness of folia, cortex, molecular layer as well as granular layer was also decreased. The cellular density in the granular layer and molecular layer was severely affected. The linear density of Purkinje cells and its diameter was also reduced as compared to those in controls. The Purkinje cells also showed degenerative changes such as shrinkage and rupture of the cell. Similar degenerative changes were also observed in granule cells. The reduction in thickness of different layers might be due to smaller brain by the action of alcohol (Maier *et al.,* 1997; Sulik,

2005). The decreased neuronal density in the molecular and granular layer might be due to alcohol induced decrease in neuronal generation and proliferation (Berman and Hannigan, 2000). The Purkinje cells were also affected in the similar way. Similarly the degenerative changes observed in Purkinje cells and granule cells might be due to oxidative stress and mitochondrial dysfunction induced by alcohol during their development (Heaton *et al*., 2000). Similar results were reported by others (Phillips and Cragg, 1982). Behav & Hoffmann (1997) and Heaton *et al.* (2000) reported that alcohol may induce apoptosis in granular cells of cerebellum. Moreover, Tavares and Paula-Barbosa (1986) counted the number of granular cells per unit volume in both the normal and ethanol treated rats and found that alcohol exposure induced granular cell loss. The width of this layer in the treated groups was narrow reflecting restricted brain development (Luke, 1990; Sulik, 2005). Some studies (Thomas *et al.,* 1998) have shown that the Purkinje cells are more vulnerable to damage by alcohol during differentiation than during neurogenesis, but no comparison can be made in the current study. Folic acid administration along with alcohol decreased the degenerative changes induced by alcohol in group II. It also increased the thickness of different layers as well as cellular density in molecular and granular layers. The linear density of Purkinje cells as well as its diameter was also increased. Moreover, the degenerating changes in Purkinje and granule cells were also greatly reduced. This might be due to antioxidative and cell proliferative property of folic acid.

On Nissl's staining the pyramidal cells in layer III and V were faintly stained indicating decreased Nissl's granules in their cytoplasm. Similar effect was observed in pyramidal cells of CA1 and CA3 regions of hippocampus as well as in Purkinje cells of cerebellum. The intensity of Nissl's granules in the neurons referred to the high metabolic activity of these neurons (Stevens and Lowe, 1997). In the control, the intensity of Nissl granules in the pyramidal cells as well as Purkinje cells was high when compared to alcohol exposed

group. Alcohol impairs cell metabolism (Hu *et al.*, 1995) and leads to protein deficiency (Luke, 1990) so the intensity of Nissl granules was low in prenatally alcohol exposed group. Heaton *et al.* (2000) also mentioned that alcohol causes disturbance in the metabolism and leads to cell dysfunction. The current findings were in accordance with other studies (Allam and Abdul-Hamid, 2013). Folic acid administration along with alcohol increased the Nissl's granules within the pyramidal cells of frontal cortex, pyramidal cells of hippocampus and the Purkinje cells of cerebellum to the extent that it was comparable to controls. This showed that prenatal folic acid administration along with alcohol reduced the deleterious effects of alcohol on those neurons to a certain extent due to which the metabolic activity of the neurons was improved during postnatal life.

On Golgi staining of the frontal cortex, the arborizations of the layer V pyramidal cells was observed to be severely affected in prenatally alcohol exposed pups. The dendritic spines were also much reduced as compared to controls. Similar features were observed in pyramidal cells of the hippocampus and the Purkinje cells of the cerebellum. Decreased dendritic arborizations, dysmorphic dendritic spines and altered synaptic development in cerebral cortex of prenatally exposed rats have been reported by other studies (Guerri, 1998). Both apical and basilar dendritic arborization as well as spine density was reduced in pyramidal cells of the hippocampus. Similar observations were made by other studies also (Abel *et al.*, 1983, Perez *et al.*, 1991). Decreased arborization of the dendrites and reduced spine density in the Purkinje cells of the cerebellum have also been reported (Allam & Abdul-Hamid, 2013). The decreased dendritic arborizations and reduced spine density might be due to ethanol induced delay or retardation of neuronal maturation (Hammer & Scheibel, 1981; Abel, 1984). Alcohol impairs the placenta and the mammary glands functions leading to fetus malnutrition which has indirect effect on the dendrites of the neurons (Luke, 1990; Luisa *et al.*, 2001). Fetal malnutrition

also reduced the size of arborizations and synaptic connectivity (Griffin *et al.*, 1977). Folic acid co-treatment along with alcohol significantly increased the dendritic arborization and dendritic spines. Folic acid is required for cell proliferation and differentiation as it is required for synthesis of cellular proteins, DNA and RNA. Folic acid is also required for the formation of red blood cells. Supplementation of folic acid had also observed to increase the absorption of other micronutrients (Milne, 1984) decreasing malnutrition due to alcohol. As described earlier folic acid deficiency is a common feature in pregnancy and becomes more severe in alcoholics by increasing urinary excretion, decreasing intestinal absorption and hepatic uptake and increased oxidative cleavage of folate molecule. The increased dendritic arborization and dendritic spine density in folic acid supplemented pups might be due to folic acid induced cell proliferation, differentiation, increased absorption of micronutrients and decreased oxidative stress.

Therefore, in the present study the behavior of the experimental animals correlate with the cellular morphology of their brain. Alcohol induced behavioral alterations that were observed in the present study might be due to degenerative changes in their brain. The study also showed that the degenerative changes that were observed in fetal life also persisted till adulthood. So, the degenerative changes observed in brain of fetus due to alcohol exposure were permanent. Similar observation was made by other studies (Barnes & Walker, 1981; Bonthius & West, 1991). It has been reported that alcohol exposure can permanently disturb adult brain plasticity and adult neurogenesis in the hippocampus. The study observed that there was no difference in the number of new hippocampal neurons produced but their survival for an extended period of time was altered in the alcohol exposed group in comparison to the controls (Choi *et al.*, 2005). Thus, the fetal alcohol exposure had a negative impact on the survival of neurons produced through adult neurogenesis. These observations suggest that the impact of fetal alcohol

exposure extends long into adulthood, affecting ability to respond to behavioral or drug treatments. Thus, early exposure impairs the brain's later ability to remodel itself, closing off functions that provide the healthy brain architecture with suitable adult refinements and repairs (Choi *et al.*, 2005).

Ethanol may deplete neurotrophic support by either decreasing neurotrophic factors (NTFs) or other molecules important for cell migration or by altering NTF receptors leading to alterations in response to the trophic factors. Moreover, ethanol induced impairment of astroglial cells leads to a decrease in the release of nerve growth factor (NGF) and changes in the astroglial NGF receptor. A deficient neurotrophic support, which may be produced by moderate ethanol, would alter neural survival and differentiation leading to some functional and behavioral deficits that may occur in the absence of physical abnormalities (Guerri, 1998) as observed in the present study. Alterations in NTF or other type of receptors (i.e., neurotransmitters, hormones) may also be produced by ethanol-induced free radical generation. The present study confirmed that the prenatal alcohol exposure increased free radical generation and lipid peroxidation in fetal brain. Free radical generation and lipid peroxidation have been associated with cell and membrane damage. Heavy ethanol consumption may also induce other types of damage, such as hypoxia/ ischemia in some areas of the brain (such as hippocampus, cerebellum) that may induce a cascade of events that precipitate in structural and morphological brain damage leading to severe CNS dysfunctions as observed in present study. The nutrition of the mother is also important, because malnutrition (i.e., intestinal malabsorption) associated with ethanol intake would decrease the availability of essential nutrients, trophic factors, and antioxidants to the fetal tissues (Guerri, 1998).

The formation of memory has been traditionally associated with the hippocampus (Guerri, 1998). So, the learning and memory deficits that were

observed in prenatally alcohol exposed mice in the present study correlates with the hippocampal damage and the cell loss. One critical neurochemical factor during hippocampal formation and the integration of the learning and memory processes is glutamate-mediated neurotransmission. Prenatal exposure to ethanol has been shown to depress glutamate release, decrease glutamate binding, diminish long-term potentiation of synaptically activated population of spines in CA1 and in N-methyl-D-aspartate (NMDA) binding site density, assessed in adult rats at 45 days of age (Savage *et al.*, 1991). Glutamate is known to play an important neurotrophic role in neuronal development and synaptic plasticity, and to be involved in the long-term potentiation. Moreover, exposure to ethanol during hippocampal formation significantly decreases the number of specific NMDA receptors, with no change in their affinity. A recent study demonstrates that the sensitivity of NMDA receptor-mediated activity to ethanol is greater in the immature than in the mature hippocampus (Guerri, 1998). Similarly, the impairments in locomotor activity, anxiety, exploration and depression that was observed in present study after prenatal alcohol exposure was supported by the degenerative changes, decreased cellular density, decreased metabolic activity and decreased dendritic arborizations in different regions of the brain such as cerebellum and the cerebrum. In Human study also it was found that prenatal alcohol exposure is associated with wide range of neuropsychological deficits including impairments in overall IQ, memory, language, attention, reaction time, visuospatial abilities, executive functioning, fine and gross motor skills, and social and adaptive functioning (Riley & McGee, 2005). Folic acid induced recovery of the different regions of the brain might be responsible for the improved learning and memory, increased locomotor activity and exploration, decrease anxiety and decreased depression behavior in the experimental animals. Such effect was not reported by any other studies. Folic acid is an essential vitamin involved in donation of methyl group in numerous biochemical pathways, including neurotransmitter

synthesis, DNA biosynthesis, regulation of gene expression, amino acid synthesis and metabolism and myelin synthesis and repair. It is thought that the participation of folate (specifically, 5 methyl tetrahydrofolate) in neurotransmitter synthesis is most responsible for its effects on mood and cognition. Furthermore it also reduces oxidative stress and homocysteine level which are detrimental to developing brain.

Chapter-7

Summary and Conclusion

Exposure to ethanol in utero is known to produce many congenital malformations in developing fetus. Ethanol depletes folic acid from the body which is essential for synthesis of DNA, RNA and protein during cell division and its requirement is increased during pregnancy. The present study was carried out to observe ameliorative effect of folic acid on alcohol induced congenital malformations in mice. For this the pregnant dams were divided into four different groups. Group I was termed as control which received distilled water orally from gestational day (GD) 6 to 15. Group II mice was given alcohol at the dose of 6 g/kg/day and group III mice were given alcohol (6 g/kg/day) and folic acid (60 mg/kg/day) during same gestational days while group IV mice received folic acid (60 mg/kg/day) only from GD 6 to 15 intragastrically through oral gavage needle. On GD 18, 60% of the dams were sacrificed. Blood was collected from orbital sinus for biochemical study. The uterine horns were observed for dead/resorbed and live fetuses. The fetuses were collected and observed for malformations They were weighed, CRL and tail length was measured. Two fetuses from each dam were kept in 70% alcohol for alizarin red staining and head of two fetuses was kept in Phosphate buffer saline (pH: 7.4; 0.02M) for biochemical study. Remaining fetuses and placenta were preserved in 10% neutral formalin for histological study. Forty percentages of the dams were allowed to deliver naturally for behavioral study of the offspring. The findings of the study were summarized as follows:

1. Folic acid co-treatment along with alcohol increased the maternal weight gain during pregnancy which was observed to be reduced in alcohol treated dams.
2. Oxidative stress in maternal blood was reduced in group III as indicated by decreased MDA level and increased GSH level in comparison to that in group II. This showed that folic acid decreased the oxidative stress by scavenging free radicals.
3. The adverse pregnancy outcome was reduced in group III by increasing the number of live fetuses and decreasing the number of resorbed/dead fetuses as compared to those in group II. This established that folic decreased the adverse pregnancy outcome induced by alcohol.
4. The fetal growth was increased in group III as shown by increased fetal weight, crown-rump length and tail length which was reduced in alcohol exposed group. This indicated that folic acid increased the fetal growth by reducing the toxic effect of alcohol on fetal growth.
5. The frequency of external malformations as well as skeletal malformations was reduced in group III as compared to those in group II showing folic acid decreased the congenital malformations induced by in utero exposure to alcohol.
6. The placental weight, diameter and thickness were increased by folic acid co-administration along with alcohol as compared to those in alcohol treated group. The histological study of placenta of group II showed marked degenerative changes in labyrinth zone such as congested maternal sinusoids, thickened placental barrier and degenerating cells and basal zone such as degenerating spongiotrophoblast and giant trophoblast cells in both H/E and Mallory stained sections. Folic acid reduced the degenerative changes in placenta and also decreased the thickness of placental barrier. This pointed that alcohol had negative impact on placental development and function that

resulted in increased fetal demise and malformations while folic acid reduced the toxic effect of alcohol and improved the fetal growth and decreased the fetal demise and malformations.

7. The fetal liver weight was increased in group III as compared to that in group II. Histological study of liver of group II showed marked degenerative changes such as disorganization and disruption of hepatic lamina, appearance of acidophilic mass and fibrosis along the lineages of hepatic lamina, appearance of large lacunar spaces with distortions of developing central veins and sinusoids and decreased hepatoblasts and haematopoietic cells in H&E stained slides. PAS staining also revealed increased megakaryocytes and reduced PAS positive material. In group III, the degenerative changes were much reduced. The cellular density was increased and PAS positive material was also increased. The number of megakaryocytes was also reduced as compared to that in group II. This showed that folic acid reduced the alcohol induced degenerative changes in liver and also reduced the alcohol induced weight loss of the fetal liver.

8. The weight of fetal kidney was also increased in group III as compared to that in group II. Histological study showed that alcohol induced degenerative changes in fetal kidney such as glomerular necrosis, degeneration and disruption of epithelial lining of Bowman's capsule, renal tubules and collecting ducts. Similarly dilation of urinary space, renal tubules and collecting ducts was also observed. Alcohol also decreased the number of renal corpuscles and collecting ducts. It also decreased the cortical and medullary thickness while increased the nephrogenic zone thickness and diameter of renal corpuscles. These deleterious effects of alcohol were reduced by folic acid. This proved that folic acid had protective role in alcohol induced degenerative changes in fetal kidney.

9. The weight of fetal brain was increased in group III as compared to that in group II. The fetal brain MDA level was decreased and GSH level was increased in group III as compared to that in group II. This showed that folic acid reduced the alcohol induced weight loss of fetal brain and also decreased the oxidative stress induced by alcohol in fetal brain. This might be due to antioxidative property of folic acid.
10. Alcohol induced degenerative changes in developing cerebrum, hippocampus as well as in developing cerebellum. It also decreased the neuronal density in those regions of the brain. Folic acid co-administration decreased the degenerative changes and also increased the cellular density in those regions. This indicated that folic acid had neuroprotective role which reduced the alcohol induced neurotoxicity.
11. Prenatal alcohol exposure delayed the postnatal growth of the pups. This delay in growth was accelerated by folic acid. This showed that prenatal alcohol exposure affects the postnatal growth of offspring. This effect was reduced by folic acid.
12. Prenatal alcohol exposure decreased the locomotor activity, decreased the exploration, increased anxiety, decreased learning and memory and increased depression in offspring. Folic acid administration along with alcohol increased the locomotor activity, increased exploration, decreased anxiety, increased learning and memory and decreased depression in the experimental animals indicating folic acid reduced the prenatal alcohol induced behavioral impairments in mice.
13. Prenatal alcohol exposure reduced the weight of the brain of the pups and induced degenerative changes in frontal cortex of cerebrum, cerebellar cortex and CA1 & CA3 region of hippocampus. It also reduced thickness of frontal cortex, decreased cellular density in different layers of frontal cortex, reduced the diameter of layer III and layer V pyramidal cells. In cerebellum it decreased the thickness of

folia, cerebellar cortex and molecular & granular layers. It also decreased the cellular density in molecular and granular layer and also decreased the linear density of Purkinje cells and its diameter. In hippocampus it decreased the thickness of pyramidal layer of CA1 and CA3 region and cellular density of those regions. Folic acid treatment increased the weight of the brain and reduced those degenerative changes, increased the cellular density and improved the neuronal architecture of those regions. This indicated that folic acid had neuroprotective role which was long lasting that persisted till adulthood.

14. On Nissl's staining, prenatal alcohol exposure decreased the Nissl's granules in pyramidal cells of cerebrum, Purkinje cells of cerebellum and pyramidal cells of CA1 and CA3 region of hippocampus. Folic acid administration along with alcohol increased the Nissl's granules in those neurons. This showed that prenatal alcohol administration decreased the metabolic activity of neurons which was improved by folic acid co-administration.

15. On Golgi staining, prenatal alcohol exposure decreased the dendritic arborization in layer V pyramidal cells of frontal cortex, pyramidal cells of CA3 region of hippocampus and Purkinje cells of cerebellum. Folic administration along with alcohol increased the dendritic arborizations of those neurons. This indicated that prenatal alcohol had deleterious effect on postnatal maturation of neurons and these deleterious effects of alcohol was reduced by folic acid.

So the present study confirmed that alcohol exposure during pregnancy was detrimental to developing fetus. It also produced degenerative changes in placenta, developing liver, kidney as well as brain of the fetus. The degenerative changes in brain were long lasting affecting postnatal behavior of the exposed pups. So, the consumption of alcohol during pregnancy is seriously discouraged. Folic acid supplementation reduced the congenital malformations

as well as improved behavioral impairments in the offspring but to a certain level only as it didn't reach the normal level. Usually folic acid is recommended to pregnant women before and during the first trimester of pregnancy due to its role in preventing neural tube defects. Our study suggests that pregnant women should take adequate folic acid throughout pregnancy as that affects brain development. This is very important for alcoholic pregnant mothers. A high dose of folic acid (60 mg/kg/day) was used in the present study to reduce the deleterious effect of alcohol. Folic acid is a water soluble vitamin which is excreted through urine when consumed in excess. There are some reports that high folic acid causes developmental defects in mice (Pickell *et al.,* 2011). So the adequate experimentation in human is required to determine the appropriate dose of folic acid in known alcoholic pregnant mothers.

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LIST OF ACCEPTED / PUBLISHED PAPERS

1. Title : "Effect of Alcohol and Folic Acid on Pregnancy Outcome of Swiss Albino Mice"

Candidate : **Uttam Shrestha**

Supervisor : **Prof. Mandavi Singh**

International Journal of Therapeutic Application (IJTA), Volume 12, 2013, 16-22, ISSN 2320-138X.

2. Title : "Effect of Folic Acid in Prenatal Alcohol Induced Behavioral Impairment in Swiss Albino Mice"

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Annals of Neurosciences, Volume 20, Number 4, 2013, 134-138, ISSN 0972 7531 eISSN 0976-3260.

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