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INFLUENCE OF ZINC AND METHIONINE ON THE EMBRYOPATHIC EFFECTS OF ETHANOL IN THE RAT

by

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ABSTRACT

Chronic ethanol consumption during pregnancy is the most prevalent cause of abnormal fetal development and mental retardation in children. The mechanism underlying the teratogenic effects of ethanol is not known. The influence of zinc, methionine, or zinc and methionine supplementation on the embryopathic effects of ethanol was investigated in vivo by the simultaneous administration of ethanol and zinc, or ethanol and methionine, or ethanol plus zinc and methionine to pregnant S/D rats from gestational days 6 through 12. The fetuses or embryos were recovered either on gestational day-20 or day-12 respectively. In the in vitro study, 9.5 days old embryos were cultured for 48 hours in a serum medium containing ethanol and various doses of S-adenosyl methionine (AdoMet). Ethanol treatment of pregnant rats resulted in a significant reduction in growth and development and in an increased incidence of developmental anomalies in the day-20 fetuses and day-12 embryos. Zinc supplementation of ethanol treated pregnant rats significantly improved fetal weight and crown-rump length, and decreased the incidence of resorptions in day-20 fetuses and in day-12 embryos, increased embryonic protein content and improved the development of somites. The growth and development of day-20 fetuses and day-12 embryos of ethanol plus methionine or those of ethanol plus zinc and methionine treated rats were not significantly different from those treated with only ethanol. In vitro exposure to ethanol also resulted in a

significant growth retardation and developmental delays in cultured whole rat embryos. Simultaneous administration of AdoMet and ethanol in vitro significantly improved growth of embryos compared to the growth observed in embryos cultured with ethanol alone. These results show that ethanol is teratogenic and embryotoxic in vivo and in vitro, respectively; in vivo zinc supplementation of ethanol treated pregnant rats and in vitro AdoMet administration partially ameliorated the embryopathic and embryotoxic effects of ethanol.

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DEDICATION

This thesis is dedicated to my son, MATTHIAS, who has given me a whole new meaning and perception of life and a new sense of direction; and who has been my source of inspiration, motivation, strength and perseverance.

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LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
DNA	deoxyribonucleic acid
FAE	fetal alcohol effects
FAS	fetal alcohol syndrome
BHMT	betaine-homocystine methyltransferase
GSH	glutathione
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
MEOS	microsomal ethanol oxidizing system
OD	optic density
RNA	ribonucleic acid
AdoMet	S-adenosyl methionine
AdoHcy	S-adenosyl homocysteine
ip	intraperitoneal
SER	smooth endoplasmic reticulum

1. INTRODUCTION

Alcohol is now recognized as the most common environmental/chemical teratogen resulting in congenital anomalies and mental retardation in man (Smith, 1979). Chronic ethanol consumption during pregnancy is the most prevalent cause of abnormal human development and mental retardation in children (Abel and Sokol, 1987). In humans, a characteristic pattern of congenital anomalies, known as the fetal alcohol syndrome, has been identified in the offspring of chronic alcoholic mothers. The fetal alcohol syndrome is an expression of the teratogenic effects of ethanol and it is characterized mainly by prenatal and postnatal growth retardation, mental retardation, microcephaly, and certain characteristic facial dysmorphism (Jones and Smith, 1973). Moreover, anomalies of the cardiovascular, skeletal, urogenital, and immune system are frequently observed in children with the syndrome.

The prevalence of the fetal alcohol syndrome is not restricted to a specific geographic area, or socio-economic status, or dietary pattern. Cases have been reported in Australia, Belgium, Brazil, Canada, Chile, Czechoslovakia, France, Germany, Hungary, Ireland, Italy, Russia, South Africa, Spain, Sweden, Switzerland and the United States (Abel, 1984). The overall incidence of the fetal alcohol syndrome world-wide varies from 0.4 per 1,000 (Sokol et al., 1980) to 3.1 per 1,000 live births (Ouellette et al., 1977). Based on these incidence rates, the average minimal incidence is 1.1 per 1,000 live births. The incidence

of fetal alcohol effects, however, is higher many folds. For example, the estimated new cases of the fetal alcohol syndrome and fetal alcohol effects in the U.S. are between 1,800 to 4,000 per year and 6,550 to 11,000 per year respectively (Abel, 1984).

The mechanism underlying the ethanol-induced teratogenesis is not clearly known. It has been proposed that the embryopathic effects of ethanol could be the result of the interaction of more than one teratogenic influence. Interactions of the indirect effects, including maternal/fetal malnutrition, impaired placental transport, fetal hypoxia, and altered endocrine function (Shenker et al., 1990) and the direct effects of ethanol on cellular function such as cell division, enzyme activity and membrane integrity, are plausible (Zajac and Abel, 1992).

In chronic alcoholics, decreased absorption (Antonson and Vanderhoof, 1983), increased urinary loss (Sullivan and Lankford, 1965) reduced serum levels (Flynn et al., 1981), and decreased placental transfer (Ghishan et al., 1982) of zinc have been reported. In addition, there is a similarity between the congenital anomalies seen in FAS and in zinc deficiency (Hurley, 1979). Chronic ethanol consumption has been shown to interfere with the metabolism of methionine. Methionine is an essential amino acid for protein synthesis, a precursor of S-adenosyl methionine, essential for DNA and phospholipid methylation, and formation of glutathione, and for maintaining hepatic integrity. Thus, it is plausible that the growth and developmental retardation

observed in the offspring of chronic alcoholic mothers could be due to ethanol-induced deficiency or alteration in the metabolism of zinc and methionine.

The objectives of the present study were to establish the teratogenic or embryotoxic effects of ethanol in vivo and in vitro, and to investigate whether or not zinc or methionine or both zinc and methionine supplementation of ethanol treated pregnant animals protects against the embryopathic effects of ethanol.

2. REVIEW OF THE LITERATURE

2.0 Pharmacology of Ethanol

2.0.0 Physical Properties

Ethanol is a relatively simple molecule (CH_3-CH_2-OH) with a molecular weight of 46. It is soluble in water and lipids and thus it readily diffuses across cell membranes. Ethanol crosses both the placental membrane and the blood-brain barrier. It is rapidly distributed throughout all body tissues in proportion to the water content. Because women have less body water (44-55%) than men (55-65%), the level of ethanol distribution is lower in women. Thus, blood ethanol concentrations will be higher in women than in men following the same amount of ethanol ingestion (Abel, 1984). This means that women may be at a relatively higher risk of developing ethanol related health problems (Abel, 1984).

2.0.1 Ethanol Intoxication

Alcohol is formed as a result of fermentation of carbohydrates by certain yeasts. The main intoxicant in alcoholic beverages is ethanol. Ethanol does not have an acceptable medical use, except that it is used as an antiseptic. It is a drug consumed by humans because of its psychoactive properties that enhance behavioural disinhibition, mood alteration and possesses sedative-hypnotic properties (Palmer, 1989). It is a central nervous system depressant;

this effect may be biphasic (Pohorecky, 1978). Ethanol at low doses could be a stimulant. Low blood alcohol concentrations (50 mg%) often results in relaxation, reduced tension and in talkativeness or sociability in the non-alcoholic. However, higher blood levels (100 mg%) impair mental and cognitive ability as well as depression of sensory-motor function. A marked impairment of sensory and cognitive functions results at blood ethanol levels exceeding 200 mg% and stupor or coma could develop at 300 mg%. In humans, the LD_{50} for ethanol is 400 mg% (Pohorecky and Brick, 1988) and blood levels of about 500 mg% could result in death as a result of respiratory depression. Lethal blood ethanol levels in rats and mice are 900 mg% and 800 mg% respectively (Abel, 1984). The degree of ethanol intoxication increases as a function of blood ethanol level and vary between individuals. Chronic consumption of ethanol and consequent tolerance also could affect signs of intoxication. Changes in the absorption, distribution or elimination of ethanol could increase or decrease the peak blood ethanol level and thus the degree of intoxication. The rate of absorption alone may affect intoxication in that the greater the degree of absorption, the greater the degree of intoxication (Conners and Maisto, 1979).

Thus, factors that affect the absorption, distribution and elimination of ethanol affect the pharmacokinetics of ethanol. For example, age affects blood flow to the liver and alters ethanol metabolism (Greenblatt et al., 1982) and also changes ethanol distribution because of variations in body water content. Hormones produced as a result of ethanol consumption use up alcohol

dehydrogenase for their degradation (Li, 1977). Stress- or ethanol-induced elevations in catecholamines increase the activity of alcohol dehydrogenase (Sze, 197) and thus increase in ethanol metabolism.

Circadian rhythms could alter sensitivity to ethanol. In rodents, a high level of sensitivity to ethanol-induced hypothermia and ethanol toxicity has been reported during the dark cycle (Brick et al., 1982).

2.0.2 Route and Method of Administration

2.0.2.0 Pulmonary Route

Ethanol, administered by vapour inhalation, could get into the body through the pulmonary epithelium, into the circulatory system (Grenant and Quinquad, 1883; Lester et al., 1951). Because the barrier, alveolar membrane, is thin and the blood flow is equivalent to the entire cardiac output, diffusion of ethanol into the circulation is enhanced. However, the intake of ethanol via the respiratory route is limited by the maximum tolerable concentration contained in the inhaled air, which is about 20 mg/l (Lester et al., 1951). Blood ethanol concentration may not exceed 50 mg/100 ml. if ethanol is not administered beyond the tolerable limit because the rate of absorption may not exceed the rate of metabolism and elimination. Higher atmospheric concentrations of ethanol, in excess of maximum tolerable levels, are quite irritating. In humans, the pulmonary route of ethanol administration may not be an efficient method of intoxicating subjects because only 62% of inhaled

ethanol is absorbed into the blood irrespective of ethanol concentration (Lester et al., 1951).

2.0.2.1 Parenteral Route

Because ethanol can diffuse across biological membranes, it is efficiently absorbed from injection sites. Sub-cutaneous, intraperitoneal and sub-arachnoid spaces serve as sites of ethanol injection. The diffusion barriers in these sites are similar, and thus rate of absorption of ethanol is determined by the concentrations which can be used without tissue damage, absorption area relative to amount of fluid injected and rate of blood flow to the site (Kalant, 1971). Ethanol administered intraperitoneally is absorbed much more rapidly compared to that injected subcutaneously. This is because of the relatively large surface area and rich blood supply of the peritoneum and, hence, peak blood ethanol levels are achieved within minutes of intraperitoneal injection (Czaja and Kalant, 1961; Fish and Nelson, 1942). However, intraperitoneal injection of ethanol is not appropriate for reproductive studies because it is possible for the amniotic sac and the fetus to be puncutred. The animal could also develop peritonitis.

2.0.2.2 Oral Route

The most common route of ethanol administration is via the oral route. In experimental animals, especially in reproductive studies, ethanol is usually

administered by gastric intubation, incorporation of ethanol in liquid diets, or by placing it in the drinking water. Gastric intubation of animals is advantageous in that control of dosage is possible (dose response relationships can be studied), time of exposure of animals can be controlled, and also relatively higher blood ethanol levels can be induced. However, this method is unlike human usage and is stressful to the animal. It could cause gastric irritation and injection of the ethanol into the lungs is possible. Furthermore, it could lead to reduction in food and fluid intake and may result in increased resorptions and abortions.

Administration of ethanol by way of incorporation in a liquid diet is similar to the human route of ethanol consumption and is not stressful to the animals. This method allows for pair-feeding of the animals, and steady, high blood ethanol levels can also be maintained. The animals receive a balanced diet in relation to caloric intake. However, inter-subject and intra-subject variability in food consumption is a drawback.

Incorporation of ethanol in the drinking water is another technique for ethanol administration in reproductive studies. This method is not stressful to the animals and it is also easy to carry out. Nonetheless, it is the least recommended of the three techniques (Abel, 1984) because lack of voluntary intake or drinking of ethanol, due to aversive taste, wide variability in ethanol consumption, low blood ethanol level, and reduced food and fluid consumption are associated with it.

2.0.3 Absorption of Ethanol

Because of its low molecular weight, infinite water solubility, relative solubility in lipids, and its weak charge, ethanol crosses biological membranes easily. There is no known transport mechanism for ethanol and thus absorption is by way of simple diffusion.

Ethanol is readily absorbed through the peritoneum when administered intraperitoneally. Ethanol administered by vapour inhalation and via the oral route is absorbed through the pulmonary epithelium and mucosa of the gastro-intestinal-tract respectively. Ingested ethanol is absorbed into a network of capillary beds and then into portal circulation from all regions of the gastro intestinal tract. However, rapid absorption takes place from the duodenum and jejunum. Although relatively lower, a considerable rate of absorption occurs across the mucosa of the stomach and large intestine and minimal absorption from the mouth (Harger and Fornely, 1963). For this reason, blood ethanol level increases rapidly when ethanol is placed directly into the duodenum or jejunum compared to when the same dose is placed in the stomach in the rat (Haggard and Greenberg, 1940), in the dog (Payne et al., 1966) and in man (Salvesen and Kolberg, 1958). More rapid increases and blood ethanol levels have been reported in patients with gastrectomies compared to healthy subjects following an oral dose of ethanol (Elmslie et al, 1965).

2.0.3.0 Rate of Absorption

Because of the differences in the rate of absorption of ethanol from the stomach and small intestine, under normal conditions, the speed of absorption depends on how rapidly ethanol is passed from the stomach to the small intestine. Thus, absorption times are variable even in the same subject. The delay in the passage of ethanol from the stomach to the duodenum will result in reduced peak ethanol levels and a slower rate of increase in blood ethanol levels. The range in time from the last drink to the peak blood level is often from 30-90 minutes, with the average being from 45-60 minutes. When a drink or several drinks are consumed over a relatively short period of time, the time to reach a peak ethanol level may be shorter and, if alcoholic beverages are consumed over a period of several hours, ethanol absorption could continue for several hours after drinking (Dubowski, 1963). On the other hand, peak blood ethanol level is reached within 30 minutes in subjects fasted overnight (Cocco et al., 1986).

2.0.3.1 Factors Affecting Absorption

The rate of ethanol absorption from the gastrointestinal tract is determined primarily by the concentration and dosage of ethanol. In the dog, intestinal absorption of a 10% ethanol was more rapid compared to 5%, 50% or 95% ethanol (Hanzlick and Collins, 1913). In man, a 20% ethanol solution was absorbed more rapidly than 5% ethanol which was evidenced by a faster

rise in blood ethanol level and a higher peak of the blood ethanol curve (Mellanby, 1919). Moreover, other investigators have also reported maximal rapid absorption when ethanol is consumed as a 15-30% solution and a slower absorption when the concentration of ethanol is either below 10% or above 30%.

The slower absorption of dilute ethanol solutions could be due to the lower concentrations across the mucosal surface, which results in slower diffusion. The slower rate of absorption observed with increasing concentrations of ethanol may be the result of delayed gastric emptying of ethanol due to impaired gastric mobility and inhibition of gastric peristalysis. Ethanol can cause superficial erosions and gastric mucosa hemorrhages in concentrations of 30-40% or more (Gillespie and Lucas, 1961; Dinoso et al., 1969) and paralysis of the smooth muscle of the stomach. Consequently, ethanol absorption from the stomach is impaired and ethanol is hindered from reaching the absorptive surface of the small intestine.

There have been a number of reports suggesting that blood alcohol curves of the same dose of alcohol but from different beverages are different. Thus, differences in the rate of absorption of alcohol from various beverages are not only due to differences in concentrations. The alcohol from beer is absorbed much more slowly than that from whisky (Haggard et all, 1938) and a much more rapid alcohol absorption occurs from gin than that from whisky. Alcohol absorption from both gin and whisky is slower compared to that from sweet

and red wines (Haggard et al., 1938; Kalant, 1951). The nature of various ingredients, such as the amount of carbon dioxide, found in different beverages may be responsible for the variability in the absorption rate (Kalant, 1971).

The rate of ethanol absorption could be reduced by factors that delay the passage of ethanol from the stomach to the small intestine and this will lead to a decrease in peak blood ethanol levels and a slower rise in blood ethanol levels. One of the factors that results in delay in gastric emptying and lower peak blood ethanol levels is the presence of food in the stomach, which blocks passage of ethanol (Greenberg, 1968; Santamaria, 1975). Ethanol is absorbed much more rapidly on an empty stomach than on a full stomach and hence, ethanol is tolerated more when ingested with food. The effect of food in the stomach on ethanol absorption is to delay gastric emptying and obstruct movement of ethanol. The type of food may not be important because the effect has been demonstrated with protein, carbohydrate and fat, and it makes little difference in the rate of absorption whether the meal is eaten before, with, or shortly after ethanol. However, the longer the time gap between ethanol and food ingestion, the less the effect on ethanol absorption (Serianni et al., 1953).

In addition to delaying ethanol absorption, the presence of food in the stomach could also probably affect blood ethanol levels. Wiener and coworkers (1981) reported a lower blood ethanol level in pregnant rats given an ethanol diet with a higher protein than those ingesting an ethanol diet with a lower

protein content, regardless of equivalent ethanol intakes in both groups.

Any physiological or pharmacological factors that modify gastrointestinal mobility or blood flow may affect absorption of ethanol. Physiological factors such as gastric acidity delay ethanol absorption (Chaudhury et al., 1964) whereas nausea has been reported to accelerate ethanol absorption in some individuals (Kalant, 1971). Doses of insulin capable of inducing hypoglycemia accelerated gastric emptying and increased ethanol absorption (Lolli and Greenberg, 1942). Intense mental concentration, physical exertion, reduction of body temperature by phenothiazines and immersion in cold water delayed ethanol absorption, while elevation of body temperature, by administration of dinitrophenol or by application of external heat, enhanced absorption (Kalant, 1971). The drinking of water shortly before ethanol ingestion has been reported to stimulate ethanol absorption by enhancing gastric emptying of food residues (Mellanby, 1919).

2.0.4 Distribution of Ethanol

Ethanol readily crosses the capillary wall and hence, it can move into and out of the blood circulation easily. The distribution of ethanol from the blood to body fluids and various tissues is determined by the same principle that govern its absorption into the blood circulation. Once ethanol gets into the circulation, it is distributed throughout the fluid compartments of all body tissues in proportion to their water content. The rate of distribution, however,

depends on the abundance of blood supply and the degree of capillarization of a tissue.

Thus ethanol equilibriates more rapidly in the brain, lung, kidney and liver tissue, which are highly vascular, than in bone. At equilibrium, however, ethanol is equally distributed throughout the body water (Pohorecky and Brick, 1988).

Women generally have less body water (44-55%) than men (55-65%) and also weigh less; the volume of distribution of ethanol will be lower in women. Because of the lesser amount of fluid in a woman's body, tissue ethanol equilibrates with blood ethanol at a lower tissue ethanol level. Consequently, blood ethanol level will be higher in women than in men following ingestion of the same amounts of ethanol. Therefore, women may be more likely than men to become intoxicated and experience ethanol-related health problems, despite identical levels of ethanol consumption.

The reproductive status of a woman is one of the factors which could determine blood ethanol level. Blood ethanol levels could probably be significantly higher in pregnant women than in non-pregnant women. Animal studies have shown blood ethanol levels to be considerably higher in pregnant rats compared to non-pregnant ones following administration of the same dose of ethanol (Abel, 1979).

The age of a person is another factor which could modify blood ethanol levels. Because the proportion of body water decreases with age, it is expected

that blood ethanol levels be higher in older women than in younger women, the dose of ethanol consumed being equivalent. Higher peak blood ethanol levels have been reported in older rats compared to those of younger rats, following administration of the same dose of ethanol; thus, older women may be more at risk for fetal alcohol effects (Abel, 1979). The nutritional status of a woman is another factor that would have an influence on blood ethanol levels. Because of the reduced volume of ethanol distribution in chronic undernutrition, higher blood ethanol levels are expected. A pregnant woman who is underweight because of undernutrition and also deficient in certain nutrients could give birth to an offspring with characteristics of fetal alcohol effects (Abel, 1984).

2.0.4.0 Distribution to the Fetus

Because of the highly vascular nature of the placenta, the rate of distribution of ethanol in the placenta is relatively high. Because of its small molecular weight and solubility in water and lipid, ethanol readily crosses the placental membrane, gets into the fetal circulation, and then into the fetal tissues. The transfer of ethanol from maternal circulation to fetal tissues and from the fetus back to the maternal circulation occurs by simple diffusion, depending on concentration gradients, and hence does not require energy expenditure.

In the transport of ethanol from maternal circulation to the fetus, the

rate-limiting step is the rate of placental blood flow but not the rate of diffusion. Factors that are related to the physical properties of ethanol (such as solubility, molecular weight and the degree of ionization), the placental blood flow and rate of metabolism could affect placental transfer (Mirkin and Singh, 1976). Once ethanol crosses the placental membrane, it may be localized in specific fetal tissues, fetal body compartments, or metabolized to some extent or excreted into the amniotic fluid, or it may be transferred back to the maternal circulation.

Many investigators have demonstrated, in humans and animals, that ethanol is transferred from maternal circulation and distributed to the body of the fetus. Ethanol is distributed to all tissues of the fetus, however, the liver, pancreas, kidney, lung, thymus, heart and brain may receive the most (Akesson, 1974; Ho et al., 1972). The observation that suggested that ethanol rapidly appears in fetal circulation at a concentration similar to that of maternal circulation, was reported by Nicloux as early as 1900.

In a more recent investigation involving pregnant women, ethanol was detected in the fetal circulation, only at 30 minutes after an i.v. infusion of ethanol, and equilibrium between maternal and fetal circulation was achieved at 60 minutes (Idanpaan-Heikkila et al., 1972). In addition, ethanol that has reached the fetal circulation and fetal tissue, is distributed to the amniotic fluid, and thus, there could be exchange between fetal body and amniotic fluid. The appearance of ethanol in the amniotic fluid, after an oral administration

of ethanol to four month pregnant women, has been documented (Brien et al., 1983). The authors reported that there was a time gap between the appearance of ethanol in the amniotic fluid and in the maternal venous blood such that appearance in the amniotic fluid was slower. The peak maternal venous blood ethanol concentration occurred earlier and was twice the peak amniotic fluid ethanol concentration.

The rate of ethanol elimination from the amniotic fluid was about half the elimination rate from maternal venous blood. At 3.5 hours, ethanol was still present in the amniotic fluid while there was no measurable ethanol in maternal venous blood. Thus, the amniotic fluid could act as a reservoir for ethanol following maternal ingestion and the fetus would be exposed to ethanol for a longer period of time. Therefore, during binge-drinking, the fetus could be exposed to a high ethanol concentration for an extended period of time because of the reservoir effect of the amniotic fluid (Brien et al., 1983). A similar lag period in the appearance of ethanol in the amniotic fluid, compared to its appearance in the maternal blood, also was observed in animals. After an oral dose of ethanol administered to pregnant mice, peak ethanol levels were recorded first in the maternal blood, then in the fetus and shortly after in the amniotic fluid (Kaufman and Woollam, 1981).

The pregnant woman readily metabolizes ethanol. As a result, her blood ethanol level will continuously drop until there is virtually none. However, the fetal liver does not possess a well developed ethanol metabolizing enzyme

system (alcohol dehydrogen base) and thus, the fetus is dependent on maternal metabolism. The fetal ethanol level remains elevated until ethanol disappears from maternal circulation (Sepala et al., 1971; Waltman et al., 1972). Thus, the peak fetal blood ethanol concentrations are of a longer duration and the fetus is exposed to peak ethanol levels for a longer time, until fetal blood ethanol diffuses into the maternal circulation because of concentration gradient differences.

2.1 Ethanol Metabolism

Following ethanol ingestion, ethanol rapidly diffuses from the stomach and the small intestine into the blood circulation and is then transported to the liver by the portal vein. The metabolism and elimination of ethanol begins before the completion of its absorption. About 90-98% of the absorbed ethanol is metabolized in the body and only 2-10% is eliminated unchanged through the lungs, kidneys and the skin, in the breath, urine and perspiration (Lieber, 1993).

A number of investigations in different animal species have demonstrated that the liver is the principal site of ethanol metabolism (Hawkins and Kalant, 1972) although small amounts of ethanol are oxidized in the stomach, kidney, lung and intestine. Hepatic metabolism accounts for at least 75% of the absorbed ethanol (Larsen, 1959; Lundquist, 1970; Khanna and Israel, 1980) however, other reports suggest that extrahepatic metabolism of ethanol could

reach as high as 40% (Utne and Winkler, 1980; Berstein, 1982).

In the adult human, the rate of metabolism of ethanol averages about 15 mg/dl/hr. In the rat and mouse, widely used animal species for ethanol associated investigations, the rate of elimination is about three times and five and a half times faster, respectively (Wallgren and Bary, 1970). The rate of ethanol elimination is not altered by pregnancy (Abel, 1979) and the rate of metabolism in the newborn human is about half of that of the adult (Wagner et al., 1970; Seppala et al., 1971; Gartner and Ryden, 1972).

Ethanol metabolism takes place in two steps. The first step involves the oxidation of ethanol to acetaldehyde, mainly by the catalytic action of the enzyme alcohol dehydrogenase (ADH). In the second step, acetaldehyde is metabolized to acetate in the liver by aldehyde dehydrogenase. Each step involves the reduction of the co-enzyme nicotinamide adenine dinucleotide (NAD) to NADH. The acetate produced as a result of acetaldehyde oxidation, is then metabolized to carbon dioxide and water through Kreb's cycle.

There are three enzymatic pathways in the hepatocytes for the oxidation of ethanol. These are the alcohol dehydrogenase (ADH) pathway, which is located in the cytosol of the cell, the microsomal ethanol oxidizing system (MEOS), located in the endoplasmic reticulum, and the catalase pathway in the peroxisomes. However, some minor hepatic pathways such as glucuronide sulfate conjugation, fatty acid esterification, and extrahepatic pathways, could have small contributions to the total metabolism and elimination of ethanol in

the body (Hawkins and Kalant, 1972; Li, 1977). At the levels of ethanol achieved in the blood, extrahepatic enzymes are inactive and thus, extrahepatic metabolism of ethanol is negligible with the exception of gastric metabolism (Lieber, 1993).

2.1.0 The Alcohole Dehydrogenase (ADH) Pathway

2.1.0.0 Enzymatic Properties

The alcohol dehydrogenase pathway is the principal pathway of ethanol metabolism in hepatocytes (Lieber, 1977; Pohorecky and Brick, 1988; Palmer, 1989). Alcohol dehydrogenase is an enzyme located in the cytosol of hepatocytes and catalyzes the conversion of ethanol to acetaldehyde. In addition to metabolizing ethanol, ADH from many mammalian species also catalyzes the oxidation of other alcohols, aliphatic and aromatic alcohols and thus has a broad substrate specificity (Von Wartburg et al., 1964; Von Wartburg and Papenberg, 1966; Lieber, 1977).

In the alcohol dehydrogenase pathway, ethanol is metabolized by ADH to acetaldehyde, ethanol's primary metabolite. Acetaldehyde is oxidized to acetate by the catalytic action of aldehyde dehydrogenase (ALDH). In the process, the co-factor NAD^+ (nicotinamide adenine dinucleotide) acts as a hydrogen acceptor and is reduced to NADH. Acetate is readily metabolized to carbon dioxide and water in the Kreb's cycle. The metabolism of ethanol via the ADH pathway occurs as follows:

$$C_2H_5OH + NAD^+ \xrightarrow{ADH} \underset{acetaldehyde}{CH_3CHO} + NADH + H^+$$

$$CH_3CHO + NAD^+ + H_2O \xrightarrow{ALDH} \underset{acetate}{CH_3COOH} + NADH + H^+$$

Net reaction: $C_2H_5OH + 2NAD^+ + H_2O \longrightarrow CH_3COOH + 2NADH + 2H^+$

ADH is located in the cytosol of the cell and ALDH, on the other hand, is present both in the cytosol and in the mitochondria. Ethanol metabolism via the ADH pathway generates an excess of reducing equivalents in the form of NADH in the cytosolic and mitochondrial compartments of hepatocytes. This altered redox state is one mechanism by which ethanol causes abnormalities in hepatic metabolism, such as inhibition of gluconeogenesis which results in hypoglycemia, and inhibition of fatty acid oxidation, which results in accumulation of fat in the liver (Palmer, 1989); Lieber, 1977). The rate of ethanol oxidation depends on NADH oxidation in the mitochondria and the supply of NAD. The speed in the initial step of acetaldehyde formation may depend on ADH activity and not on NAD supply (Crow et al., 1977; Peters, 1982).

2.1.0.1 Development of ADH

The development of hepatic ADH is characterized by a typical pattern of progression during human development. During fetal life the presence of only one form of ADH has been reported compared to four forms (Pikkarainen and Raiha, 1969), and seven to nine forms (Li, 1977) in the adult human. In the early fetus, the alpha, alpha-polypeptide chain form, in more mature fetuses an additional alpha-beta form, and in the neonate, the alpha-alpha, alpha-beta and beta-beta forms were identified (Smith et al., 1973). The 10-16 week old fetus is incapable of metabolizing ethanol (Pikkarainen, 1971), and in two-month old fetal liver, ADH activity was only about three to four percent of adult activity (Pikkarainen and Raiha, 1967). ADH activity increased linearly with gestational age and adult level of activity was achieved at five years of age. In human liver, ADH activity of 30% and 40% of the adult activity has been reported in the fetus and in infants less than one year old respectively (Smith et al., 1971). In newborn rats and guinea pigs, activity levels of 25% and 20% respectively, have been observed (Raiha et al., 1967). In neonatal lambs, ADH activity of only 7% of that of the adult sheep was reported (Cumming et al., 1985).

2.1.0.2 Physico-chemical Properties

Human, rhesus-monkey and horse liver alcohol dehydrogenase are zinc-metalloenzymes (Kalant, 1971). Zinc, being an essential component of alcohol

dehydrogenase plays a role in both catalysis and stabilizing the enzyme, or in maintaining the structural integrity of the enzyme. Zinc is bound to the catalytic site of ADH by the amino acids glutamic acid and histidine and to the structural site by cysteine and histidine (O'Dell, 1992). Thus, zinc appears to participate in ethanol metabolism. Significantly lower levels of ADH and slower rates of ethanol elimination have been reported in rats maintained on a zinc deficient diet than in pair fed controls (Das et al., 1984). Chronic ethanol consumption has been reported to decrease serum/plasma zinc levels in animals (Das et al., 1984; Ghishan et al., 1982) and in humans (Sullivan and Lankford, 1965; Flynn et al., 1981).

The activity of ADH increases at higher pH. In man, the optimal pH for ethanol oxidation via ADH is about 10.8 and ADH activity decreases to 40% and zero at pH 7.0 and less than 6.0 respectively (Von Wartburg et al., 1964). ADH also has a broad substrate specificity and thus competitive inhibition between two substrates is possible and the one with a higher affinity to ADH will be metabolized first.

2.1.1 Microsomal Ethanol Oxidizing System (MEOS)

The second pathway of hepatic ethanol metabolism is known as the microsomal ethanol oxidizing system (MEOS) and it is located in the smooth endoplasmic reticulum which is part of the microsomal fraction of the cell (Orme-Johnson and Ziegler, 1965; Lieber and DeCarli, 1968; Roach et al., 1969;

Palmer, 1989). The existence of an interaction of ethanol with hepatocyte microsome was first provided by a morphological observation of proliferation of the smooth endoplasmic reticulum (SER) in ethanol-fed rats (Iseri et al., 1964; 1966). The MEOS is dependent on a special cytochrome, Cytochrome P_{450}, and requires NADPH (nicotinamide adenine-dinucleotide phosphate, the reduced co-enzyme) and molecular oxygen. MEOS is fully active at physiological pH. It is similar to the microsomal drug detoxifying system (Lieber and DeCarli, 1968) and its presence has been demonstrated in rat liver as well as in normal and alcoholic human liver (Lieber and DeCarli, 1968b).

The MEOS also generates acetaldehyde and ethanol oxidation via this pathway occurs as follows:

$$C_2H_5OH + NADPH + H^+ + O_2 \xrightarrow[\text{acetaldehyde}]{MEOS} CH_3CHO^+ + 2H_20$$

The acetaldehyde formed is further oxidized to acetate by ALDH. Ethanol's oxidation via the MEOS is attributed to enzymes of the mixed function oxidase system but not to ADH or catalase (Lieber and DeCarli, 1970).

The MEOS is inducible by chronic exposure to ethanol. The activity of this system is increased during chronic ethanol consumption, and is associated with proliferation of SER. The MEOS has a k_m of 8 to 10 mM compared to 0.26 to 2.0 mM for ADH (Reynier, 1969; Lindros et al., 1974). The oxidative capacity of the MEOS among moderate drinkers is relatively low. However,

among heavy drinkers, this pathway could contribute to as much as 25% (Lieber and DeCarli, 1973) and 50% (Rosset and Weiner, 1984) of ethanol oxidation in the liver. Thus, habitual heavy drinkers have an increased capacity to metabolize ethanol mainly due to the induction of MEOS. Because ethanol can induce the MEOS and many other drug metabolizing enzyme systems (Rubin and Lieber, 1968) which are capable of oxidizing other sedative drugs, alcoholics may develop a resistance or a cross-tolerance to various sedatives (Rubin et al., 1968).

The mixed-function oxidase system, induceable by ethanol, is involved not only in oxidizing ethanol to acetaldehyde, but also in the metabolism of xenobiotics. Thus, there is a direct competition between ethanol and xenobiotics for the MEOS which leads to acute inhibition of hepatic xenobiotic oxidation and transformation. Chronic ethanol consumption results in the proliferation of SER and induction of the mixed-function oxidase system, including the MEOS. The induction and enhanced activity of the MEOS increases the capacity of the liver to metabolize ethanol and leads to metabolic adaptation to chronic heavy drinking. Moreover, the induction of the mixed-function oxidase system in the sober state increases the capacity of the liver to metabolize xenobiotics, certain vitamins, and steroids. Therefore, the sober or recovering alcoholic requires a much higher than normal dose of certain drugs to achieve therapeutic levels because drug oxidation in these individuals is very rapid (Palmer, 1988).

Because the MEOS uses molecular oxygen, the supply of oxygen to the liver is decreased (French et al., 1984; French, 1989) and this enhances the development of hypoxia in hepatocytes without ATP production (Lieber and DeCarli, 1968). Moreover, the ethanol inducible cytochrome P_{450} is known to metabolically activate many hepatotoxins (Ingelman-Sundberg et al., 1988).

2.1.2 Catalase

The third, relatively minor, pathway of hepatic ethanol oxidation involves the enzyme catalase, located primarily in the peroxisomes and mitochondria and small amounts of it are found as contaminants in the microsomes of hepatocytes (Redman et al., 1972). Catalase is capable of oxidizing ethanol in vitro in the presence of an independent system that generates hydrogen peroxide (H_2O_2) (Keilin and Hartree, 1945; Palmer, 1989). The oxidation of ethanol via this pathway is limited by the rate of peroxisomal H_2O_2 generation rather than by the amount of catalase present (Oshino et al., 1975). Its contribution to the total ethanol metabolism in vivo or under physiologic conditions is small and cannot account quantitatively for the ADH-independent pathway of ethanol metabolism.

In the presence of sufficient H_2O_2, ethanol oxidation by catalase occurs as follows:

$$C_2H_5OH + H_2O_2 \xrightarrow{catalase} \underset{acetaldehyde}{CH_3CHO} + 2H_20$$

The abundance of catalase and the existence of various peroxide-generating systems add to the plausibility of ethanol oxidation via the catalase pathway.

2.1.3 Ethanol Metabolic Rates

In the adult human, the rate of ethanol metabolism averages about 15 mg/dl/hr. The rate of ethanol elimination in the newborn human is about half of that of the adult. It has been reported that the mean metabolic rate of ethanol in five premature newborn infants was 7.4 mg/dl/hr (Wagner et al., 1970). Similar ethanol metabolic rates have been observed by others. Seppala et al. (1971) reported metabolic rates of 7.0 and 8.0 mg/dl/hr in twins born after an unsuccessful attempt to prevent premature labour in the mother by administrating ethanol. In another investigation, involving six new born infants, a mean ethanol elimination rate of 7.7 mg/dl/hr has been obtained (Idanpaan-Heikkila et al., 1972). On the other hand, the same investigators reported a metabolic rate of 14.0 mg/dl/hr in the mother, which was twice that of the new born infant.

The rate of ethanol elimination in the adult rat is about three times and in the mouse five and a half times faster than that in the adult human (Wallgren and Bary, 1970).

In the new born lamb, Cumming and coworkers (1985) reported a metabolic rate of 3.6 mg/dl/hr which is about 17% of the rate reported in the near-term pregnant sheep (Cumming et al., 1984). In neonatal monkeys,

delivered two hours after maternal infusion of ethanol, Hill and coworkers (1983) reported an ethanol elimination rate of 3.9 mg/dl/hr which was about 27% of that reported for the adult monkey. According to these reports, it is evident that there is a faster ethanol metabolic rate in the new born human than that in the new born monkey or lamb.

2.2 Fetal Alcohol Syndrome

The fetal alcohol syndrome (FAS) is a characteristic pattern of growth and developmental anomalies observed in the offspring of chronic alcoholic mothers. It is an expression of the teratogenic effects of ethanol. The growth and developmental abnormalities observed in FAS include prenatal and postnatal growth retardation, microcephaly, mental retardation, characteristic facial dysmorphism as well as various non-specific malformations (Jones and Smith, 1973; Persaud, 1988). Moreover, anomalies of the cardiovascular, skeletal, urogenital, and immune systems are often observed in FAS. According to the Fetal Alcohol Study Group of the Research Society on Alcoholism (Rosett, 1980a), diagnosis of FAS should be made only when the patient has signs in each of the following categories:

1. Prenatal and/or postnatal growth retardation (weight, length and/or head circumference below the 10th percentile).
2. Central nervous system dysfunction (signs of neurological abnormality, developmental delay, or intellectual impairment).

3. Characteristic facial dysmorphology with at least two of the following three signs:
   (a) microcephaly (head circumference below the 3rd percentile);
   (b) microphthalmia and/or short palpebral fissures;
   (c) poorly developed philtrum, thin upper lip, and/or maxillary hypoplasia.

The term fetal alcohol effects (FAE) has been recommended by the Fetal Alcohol Study Group if all the three categories of growth and developmental anomalies are not detected. Prenatal and postnatal growth retardation in weight, length and head circumference is the most prevalent sign of FAS (Rosett and Weiner, 1984).

Signs of FAS/FAE persist during infancy as well as adulthood, and mental retardation is the most serious sign. Fetal alcohol exposure is now recognized as the principal cause of mental retardation in the Western world (Abel and Sokol, 1987). FAS, however, is associated with very heavy maternal alcohol consumption.

2.2.0 Historical Background

The production of alcoholic beverages via the process of fermentation is one of man's earliest achievements (Keller, 1979). The history of alcohol use by virtually every tribe or population, as part of the diet or for medicinal or religious purposes, goes back to the dawn of civilization. For example, beer

and wines were commonly used in ancient Egypt, before 3,400 B.C. (Persaud, 1988). Although the dangers of drinking alcohol during pregnancy appeared to have been known in the ancient world (Jones and Smith, 1973), appreciation of the link between drinking during pregnancy and fetal alcohol effects did not surface until the late 19th century (Abel, 1984).

The biblical quotation in Judges 13:3-4 which states: "Behold, thou shalt conceive and bear a son, and now drink no wine or strong drink" suggests that drinking alcohol during pregnancy was condemned even in biblical times. During the Greek and Roman era, people believed that Vulcan, the god of fire, was lame, deformed and ugly because his parents were intoxicated at the time of conception. Aristotle, the Greek philosopher and scientist, in his "Problemata," wrote that "foolish, drunken or harebrain women, for the most part bring forth children like unto themselves, morosos et languidos (morose and languid)" (cited in Burton, 1621). In ancient Rome, bridal couples were forbidden, by Carthaginian law, to drink wine on their wedding night for fear of producing a defective offspring (Haggard and Jellineck, 1942).

During the gin epidemic (early 18th century) in England, the harmful consequences of maternal alcohol consumption during pregnancy were recognized. The College of Physicians (in 1726) urged the British Parliament to control the production and distribution of the liquor because "gin was a cause of weak, feeble and distempered children" (George, 1965).

During the proclamation of the Alcohol Licensure Act of 1834, the

government of England characterized the offspring of alcoholic mothers as having a "starved, shrivelled and imperfect look" (Jones et al., 1973; Persaud, 1988). In 1899 increased frequency of infant mortality and stillbirths (2.5 times that of the control population) was reported in the offspring of alcoholic mothers in Liverpool prison (Sullivan, 1899).

After the introduction of the Prohibition Act in 1920 in the U.S., the interest in research on the relationship between alcohol consumption during pregnancy and the development of the fetus was lost. Interest in this area of thought remained dormant until the Lemoine report in 1968.

Lemoine et al. (1968) carried out a study of 127 children of chronic alcoholic families. The results revealed that 25 of the children had a distinctive pattern of anomalies consisting of characteristic facial features, prenatal growth retardation, increased frequency of organ malformations and impaired psychomotor and language development (Lemoine et al., 1968). A similar characteristic pattern of growth and developmental anomalies was later reported by Jones et al. (1973) which brought the teratogenic potential of alcohol to international attention once again. In order to describe the characteristic pattern of anomalies observed in the offspring of alcoholic mothers, Jones et al. (1973) introduced the term "Fetal Alcohol Syndrome."

2.2.1 Incidence

The prevalence of FAS may be difficult to establish with certainty because of differences in the definition of cases and because retrospective diagnosis of FAS is not conducted. According to Smith (1979), there have been over 600 cases of FAS as of 1979. Cases have been reported in Australia, Belgium, Brazil, Canada, Chile, Czechoslovakia, France, Germany, Hungary, Ireland, Italy, USSR, South Africa, Spain, Sweden, Switzerland, and the United States (Abel, 1984), suggesting that cases are not restricted to a specific geographic area, socio-economic status, or dietary pattern.

The fetal alcohol syndrome is not expressed in all of the offspring of alcoholic mothers and the incidence rate is variable depending on the study population and criteria used for estimation. The overall incidence of FAS worldwide varies from 0.4 per 1,000 (Sokol et al., 1980) to 1.3 (Hanson et al., 1978) to 1.4 (Dehaene et al., 1981) to 1.6 (Olegard et al., 1979) to 3.1 per 1000 live births (Ouellette et al., 1977). Based on these incidence rates, the average minimal incidence is 1.1 per 1,000 live births (Abel, 1984). If birth rate is 1,000,000 per year, there will be 1,100 (1,000,000 x 0.0011) new cases of FAS based on this average minimal incidence rate. The incidence of FAS in the offspring of alcoholic women in the U.S., however, is higher; that is, 24 per 1,000 live births (Ouellette et al., 1977), 25 per 1000 (Sokol et al., 1980) and 29 per 1,000 live births (Hanson et al., 1978). The average minimal incidence is 25 per 1,000 live births. However, incidence rates as high as 40% (Jones et

al., 1974; Majewski, 1981), 33% (Dehaene et al., 1977b) and 10% (Rosett et al., 1983b) have been reported in children of alcoholic mothers.

The estimated incidence rate of fetal alcohol effects (FAE) is, however, higher than for FAS. The estimated average minimal incidence for the total population, based on a more conservative estimate, is 3.1 per 1,000 live births.

In 1980 there were approximately 3,598,000 newborns in the U.S. and it is estimated that about 2% of women are in the category of "heavy" drinkers. Therefore, the estimated new cases of FAS and FAE in the U.S. are between 1,800 to 4,000 per year and 6,550 to 11,000 per year respectively (Abel, 1984).

2.3 Fetal Alcohol Effects (FAE)

Alcohol is now recognized as the most common environmental/chemical teratogen resulting in congenital anomalies and mental retardation in man (Smith, 1979). Chronic ethanol consumption during pregnancy is the most prevalent cause of abnormal human development (Abel and Sokol, 1987) and the third major cause of mental retardation in children (Wright, 1983). In humans a characteristic pattern of congenital anomalies, known as the fetal alcohol syndrome, has been identified in the offspring of chronic alcoholic mothers. Growth and developmental abnormalities, as a result of chronic ethanol consumption, have been demonstrated in various animal models as well. FAS is associated with very high levels of ethanol consumption/heavy drinking. However, even moderate drinking, about two drinks a day, can have

adverse effects on the fetus ranging from spontaneous abortions (in humans) or resorptions (in animals) to prenatal growth retardation. Lower levels of ethanol consumption during pregnancy still pose an unknown risk to the fetus.

2.3.0 Prenatal Growth Retardation

One of the most common characteristic abnormalities associated with intrauterine exposure to ethanol is prenatal growth retardation. Birth weight reduction is not restricted to offspring of chronic alcoholic women but also occurs in the children of moderate drinkers.

In a prospective study involving primarily middle class pregnant women, Little (1977) reported the occurrence of low birth weight in the infants of moderate drinkers. In another prospective study, Ouellette and coworkers (1977) also reported a relatively higher incidence of low birth weight among offspring of mothers who drank five or more drinks per occasion. Other large prospective studies also observed significant relationships between ethanol consumption and birth weight where pregnant mothers consumed at least two or three drinks per day (Kaminski et al., 1976; Streissguth et al., 1981; Olson et al., 1983).

Decreased birth weight, related to ethanol consumption, also has been reported in various strains of rats (Abel, 1978; Leichter and Lee, 1979; Detering et al., 1979; Persaud, 1982), in guinea pigs (Napara-Nicholson and Telford, 1957), mice (Yani and Ginsberg, 1977) and in miniature swine (Dexter

et al., 1980). Moreover, reduced crown-rump length and a smaller head circumference were observed in infants born to drinking mothers during pregnancy (Streissguth et al., 1981; Smith et al., 1986; Russell and Skinner, 1988).

Postnatal growth retardation was also observed in the children of women who consumed ethanol during pregnancy, but who mostly were not heavy drinkers (Plant, 1987). Postnatal weight deficits were also reported in animals (Martin et al., 1979).

2.3.1 Central Nervous System Defects

Chronic ethanol consumption during pregnancy is the third major cause of mental retardation (Wright, 1983) and mental retardation is the most serious consequence of ethanol teratogenicity. Majewski and Bierich (1978) have reported structural alterations in the brain of infants exposed to alcohol in utero. Clarren et al. (1978) observed microcephaly, hydrocephaly, aberrant neuronal and glial cells of the brain surface, and cerebral, cerebellar and brain stem disorganization during autopsy of brains of infants exposed to ethanol in utero. Incomplete cortical development, abnormal neuronal and glial migration, absence or poor development of the corpus callosum and anterior commissure have also been documented (Clarren et al., 1978). Neural tube defect in infants of mothers who consumed ethanol during pregnancy has also been reported (Majewski, 1981; Smith et al., 1981; Friedman, 1982).

In addition to structural alterations in the central nervous system, behavioural deficits such as mental retardation, attention deficits, and sleep disturbances are commonly seen in children of alcoholic mothers. Hanson and coworkers (1976) reported that 107 of the 126 children (85%) exposed to ethanol in utero scored more than two standard deviations below the mean. An IQ score of 70 or less was reported, in in utero ethanol exposed children, in France (Lemoine, 1968), Sweden (Olegard, 1979), Germany (Majewski, 1981) and in the United States (Jones et al., 1973; Streissguth et al., 1978). A follow-up study by Streissguth et al. (1978) indicated that the deficits in IQ scores remained the same over a one to four year period in most of the patients suggesting that these deficits may be long lasting.

The relationship between chronic ethanol consumption and central nervous system disorder has also been demonstrated in animals. In rats, reduced brain weight, incomplete development of cortical neurons, and delayed postnatal cerebellar development (Reyes et al., 1983) in mice, exencephaly (Chernoff, 1977; Kronick, 1976), hydrocephaly (Chernolf, 1977; Randall and Taylor, 1979), absence of corpus callosum (Chernoff, 1977); and in the developing chick embryo, neural tube defects (Sandor, 1968) have been reported.

2.3.2 Other Fetal Alcohol Effects

Disorders of bodily organs, such as anomalies of the heart, kidney and

urogenital tract, liver, limbs and the skeleton were also observed in humans and animals as a result of chronic ethanol consumption and often these defects are associated with FAS. In human infants exposed to ethanol in utero, cardiac or ventricular septal defect (Dupis et al., 1978; Smith et al., 1981), kidney and urogenital anomalies (Qazi et al., 1979; Sokol et al., 1980), skeletal defects (Herman et al., 1980; Smith et al., 1981) and liver anomalies (Peiffer et al., 1979; Newmann et al., 1979) have been reported. Chronic ethanol consumption of pregnant rats has also been found to retard fetal skeletal maturity (Leichter and Lee, 1979).

Although there are insufficient studies to establish a threshold, ethanol consumption during pregnancy is associated with abnormal pregnancy outcome. A significant association between ethanol consumption and spontaneous abortion even at low levels of one (Harlap and Shiono, 1981) or two drinks per day (Kline et al., 1980) has been reported. Dose related effect of ethanol was also observed with fetal wastage (Anokute et al., 1986) and with stillbirths (Kaminiski et al., 1976). Little et al. (1986) have found a significantly higher number of pre-term deliveries at an ethanol consumption level of 1.3 drinks per day.

2.4 Mechanisms of Action

Chronic ethanol consumption during pregnancy has been reported to cause prenatal and postnatal growth retardation, mental retardation, anomalies of

bodily organs, as well as abnormal pregnancy outcome in both humans and animals. The mechanism underlying ethanol induced teratogenesis is not clearly known. However, there is growing evidence regarding possible general mechanisms of the teratogenic effects of ethanol.

Altered cellular function is considered, by many investigators, as a requisite for ethanol induced teratogenic manifestation (Adickes, 1990). The embryo-pathic effects of ethanol may be the result of the interaction of more than one teratogenic influence (McClain and Su, 1983; Majewski, 1981). Interactions of the indirect or maternally mediated effects, including maternal malnutrition, impaired placental transport of nutrients, hypoxia and altered endocrine function (Schenker et al., 1990), and the direct effects of ethanol on cellular function such as cell division, enzyme activity, membrane integrity, and chemoreceptor or neurotransmitter activities are possible (Zajac and Abel, 1992).

2.4.0 Indirect Effects of Ethanol

2.4.0.1 Ethanol Induced Nutrient Deficiencies

Chronic ethanol consumption affects the absorption, transport, metabolic activation, excretion and storage of nutrients (Lieber, 1983). This will reduce nutrient availability to the fetus and may result in vitamin and mineral deficiencies.

In chronic alcoholics, decreased absorption (Antonson and Vanderhoof,

1983), increased urinary excretion (Sullivan and Lankford, 1965) and reduced serum levels (Flynn et al., 1981) of zinc have been reported. Zinc deprivation, even for a short period during pregnancy, could affect the developing central nervous system (Hurley and Shrader, 1972), and ethanol related zinc deficiency may contribute to ethanol induced growth and developmental anomalies (Mandelson and Huber, 1980).

Ethanol could impair prostaglandin metabolism (Horrobin, 1980). Ethanol affects the synthesis of gamma-linoleic, the precursor of prostaglandins, by inhibiting the activity of delta-desaturase, the enzyme that converts linoleic acid to gamma-linoleic acid (Persaud, 1988). In an investigation by Varma and Persaud (1982), efamol (9% gamma-linoleic acid and 72% linoleic acid), administration (i.g.) to ethanol treated pregnant rats, resulted in lower growth retardation and dysmorphogenesis in the embryos compared to those in the embryos of ethanol alone treated rats.

2.4.0.2 Altered Amino Acid Metabolism and Placental Transport

Impaired incorporation into protein, decreased tissue uptake (Fisher et al., 1981) and altered metabolism of amino acids (Barak et al., 1987) have been documented in chronic ethanol consumption. Such effects of ethanol may result in impaired protein and DNA synthesis and thus cell size and number, respectively, could be affected.

For the normal growth and development of the fetus, the normal

functioning of the placenta is essential. Abnormal placental size and cellular structure have been reported in humans (Halmesmaki et al., 1987) and in rodents (Eguchi et al., 1989) as a result of chronic ethanol consumption. In addition, chronic ethanol feeding of rats during gestation has been reported to decrease placental blood flow (Jones et al., 1981), fetal amino acid uptake and concentration (Gordon et al., 1985). Thus the capacity of the placenta to transport nutrients to the fetus is hindered, reducing amino acid, glucose and other nutrient availability (Fisher et al., 1986; Kennedy et al., 1986).

In rodents, both acute and chronic exposure to ethanol results in a reduction in placental transfer of various amino acids (Fisher et al., 1982; Henderson et al., 1982), zinc (Ghishan et al., 1982) and glucose (Snyder et al., 1986) to the fetal circulation. Inhibitory effects of chronic ethanol consumption on the transfer of amino acids by the placenta also has been reported in monkeys (Fisher et al., 1983; Schenker et al., 1989).

2.4.0.2 Ethanol Induced Hypoxia

Ethanol induced hypoxia could produce the structural, physiological and biological defects associated with fetal alcohol exposure by reducing the oxygen supply to the cells during cellular development (Abel, 1984).

Ethanol induced fetal hypoxia can occur because of impaired umbilical blood flow (Mukherjee and Hodgen, 1982). Savoy-Moore and coworkers (1989) have reported a dose-dependent vasoconstrictive effect of ethanol on isolated

human umbilical artery preparations in vitro. Prenatal growth retardation has been associated with increased umbilical arterial resistance (Zajac and Abel, 1992). Chronic ethanol consumption increases the rate of oxygen consumption in hepatocytes by 100% (Ugarte and Valenzuela, 1971). This could deprive other tissue of the required oxygen, especially tissues with limited blood supply, and these tissues could become hypoxic, functionally impaired or necrotic.

Acute maternal ethanol intoxication has been reported to significantly reduce cerebral blood flow, cerebral oxygen consumption, cerebral glucose consumption and to result in significant fetal hypoglycemia in term fetal sheep (Richardson et al., 1985). These adverse effects may contribute to ethanol-induced teratogenesis especially during the early part of gestation. Man and coworkers (1975) also have observed a considerably reduced blood oxygen level and acidosis in fetal sheep 30 minutes following maternal ethanol infusion. Ethanol-induced hypoxia could also result in intrauterine growth retardation by inhibiting synthesis and activity of ATP.

2.4.1 Direct Effects of Ethanol

The direct embryopathic effect of ethanol, without the involvement of confounding maternal factors such as altered maternal function, or nutrition or metabolism, has been demonstrated unequivocally. Brown and coworkers (1979) cultured 9.5 day old whole rat embryos for 48 hours in ethanol

containing medium and observed growth and developmental retardation. The crown-rump length, total DNA, total protein, head length, number of somites and morphological score were significantly reduced in the ethanol treated embryos (300 mg%) compared to control values. A similar investigation by Wynter and coworkers (1983) also has demonstrated a marked growth retardation even when embryos were cultured with lower ethanol concentrations (200 mg%). A higher concentration of ethanol (800 mg%) produced neural tube defect.

The direct effect of ethanol could be on cell functions, such as enzyme activities and division, as well as on membrane integrity.

Prenatal ethanol exposure has been reported to decrease fetal organ or embryonic DNA and impair protein synthesis (Henderson et al., 1980; Rewat 1976; Brown et al., 1979). Ethanol also alters membrane properties by modifying the nature and activities of several proteins (Chin and Goldstein, 1981). The activity of cell membrane enzymes are reduced in ethanol exposed animals. These enzymes play an important role in controlling the cell's internal environment and maintaining the viability of the cell. Any impairment in such enzymatic activity could affect normal developmental processes (Zajac and Abel, 1992).

Ethanol-induced malformation of the craniofacial structures may be explained by impaired neural crest cells migration and disorganized actin cyto-skeleton (Hassler and Moran, 1986b). Also, some of the neuronal impairment

observed following in utero ethanol exposure in humans and animals may be explained by the effect of ethanol on enzyme and neurotransmitter activities or on enzymes involved in free radical removal (Zajac and Abel, 1992).

It is also possible that acetaldehyde, the primary and more toxic metabolite of ethanol, may be responsible for the teratogenic properties of ethanol. O'Shea and Kaufman (1979) injected pregnant mice (i.v.) with 1% or 2% acetaldehyde on gestation days 7 to 9 and observed significant growth retardation and increased resorptions. The weight was reduced in day-19 fetuses and protein content also was significantly reduced in day-12 embryos of acetaldehyde treated mice. Thus, although ethanol is the proximate teratogen, a possible role for acetaldehyde in FAS cannot be ruled out.

2.5 Pharmacology of Zinc

2.5.0 Absorption, Transport and Metabolism of Zinc

The absorption of zinc takes place from the small intestine and it is homeostatically controlled. Zinc absorption is dependent on zinc concentration in the intestinal lumen; thus, absorption increases in zinc deficiency or when luminal zinc concentration is low and decreases when luminal concentration is high (Smith and Cousins, 1980).

Zinc uptake across the brush border surface occurs by both passive diffusion and carrier-mediated processes (Menard and Cousins, 1983). The mediated or saturable component accounts for a major portion of the

absorption in zinc depletion and thus, this transport component responds homeostatically to the dietary zinc supply.

Regulation of zinc absorption and homeostasis takes place at cellular levels and through endogenous secretion. Endogenous secretion of zinc via pancreatic and biliary secretions increases and decreases at zinc intakes above the dietary requirement and below the dietary requirement, respectively. In addition, the carrier-mediated mechanism of zinc absorption is activated at low luminal zinc concentration.

Another mechanism for the regulation of zinc absorption involves intestinal metallothionein. Increased zinc administration induces metallothionein biosynthesis, and increased metallothionein results in a reduction in the absorption of zinc. There was little metallothionein in the intestines of zinc deficient rats, while dietary repletion of zinc increased metallothionein concentration dose-dependently (Richards and Cousins, 1976). Therefore, the inducible nature of intestinal metallothionein suggests that this protein is an important regulatory mechanism for zinc absorption. The intracellular zinc binding property of metallothionein which limits the availability of zinc could be a defensive mechanism to protect against zinc toxicity.

Intestinal zinc absorption is affected also by dietary factors. High dietary protein, ascorbic acid, prostaglandin and low dietary iron enhance zinc absorption, whereas high dietary fiber, phytate, calcium and iron decrease

absorption.

Absorbed zinc is transferred to the portal circulation and it is transported bound to plasma albumin. Plasma is a metabolically active transport compartment for zinc and most of the plasma zinc, usually about two-thirds, is bound to albumin. The zinc bound to albumin is loosely bound zinc and thus the ability of albumin to give up zinc could be a requirement in the exchange of zinc between plasma and tissues. The association constant of the zinc-albumin complex is relatively lower (10^6) compared to that for zinc-macroglobulin, the other zinc binding protein ($>10^{10}$). Albumin is also the major zinc-binding protein in human amniotic fluid (Gardner et al., 1982).

The macroglobulin and a histidine-rich glycoprotein transport the non-albumin bound zinc tightly bound zinc. Thus, their ability to donate zinc to cells, under physiological conditions, may be difficult (Cousins, 1985).

Zinc uptake into hepatocytes occurs via a carrier-mediated, energy-dependant process. Cellular hepatocyte zinc is then distributed among various organelles, metalloproteins, membranes, and certain macromolecules and this represents the labile zinc pool (accumulated zinc) and a metalloprotein zinc pool which accounts for exchange (Cousins, 1985). Efflux of zinc from hepatocytes depends on the availability of zinc-binding ligands. Most of the newly accumulated zinc, however, is not readily available for exchange or efflux into plasma and hepatocyte induction of metallothionein could favour hepatocyte zinc accumulation (Failla and Cousins,1 978). Functional zinc

deficiency has been reported in hepatocytes cultured in zinc-deficient medium (Guzelian et al., 1982) suggesting that a constant supply of zinc is necessary to maintain adequate levels of cellular zinc. During infection or physical stress, immediate responses include reduction in plasma zinc, increased uptake by the liver and redistribution within hepatocytes.

The largest zinc pools are bone and muscle. Transfer of zinc from bone to plasma may not occur except when bone is mobilized to maintain calcium homeostasis, and increased efflux from muscle may encourage urinary zinc loss. Studies of zinc deficiency in the rat during pregnancy suggest that the zinc in the bone is not readily available even in the presence of teratogenic zinc deficiency in the fetuses. In addition, the occurrence of rapid and severe adverse effects of even short duration zinc deficiency on fetal development, suggest that a pregnant rat is not able to mobilize enough zinc from body stores to fulfil the needs of the developing embryo (Hurley, 1981).

2.5.1 General Functions and Deficiency of Zinc

Zinc has a multiplicity of functions and the variety of signs observed in zinc deficient animals is a reflection of this property. Zinc provides and maintains the structural integrity of the more than 100 zinc metalloenzymes and also participates directly in catalysis. A majority of these zinc-metalloenzymes are found in the liver and include DNA and RNA nucleotide transferases, DNA polymerase, alcohol dehydrogenase and -aminolevulinic

acid dehydratase (Dixon and Webb, 1979).

Zinc is also involved in bone formation, cell-mediated immunity, generalized host defence, wound healing, tissue growth and in maintaining membrane integrity.

There is considerable evidence which suggests that zinc deficiency is teratogenic in both humans and animals. The observation that zinc deficiency results in congenital malformations in mammals was first reported in 1966 (Hurley and Swenerton, 1966). In one study, female rats were fed a marginally zinc deficient diet (9 ppm zinc) from weaning until maturity and another zinc deficient diet (1 ppm zinc) from day 1 to 21 of gestation. The results showed that the rats maintained on the zinc deficient diets lost weight during pregnancy. These animals had an offspring mortality rate of more than 50%, smaller litter size, and the pups were less than half the normal body weight, compared to controls. Congenital malformations were observed in 98% of fetuses of zinc deficient rats compared to only 2-4% of implantation sites affected in the adequate zinc group. Gross malformations observed in the fetuses include tail malformation (83%), fused or missing digits (80%), scoliosis or kyphosis (47%), cleft palate (34%), clubbed forefeet (34%) and micrognathia (28%). There were also short or entirely missing long bones, missing ossification centres, doming of the skull and poor ossification of the cranial bones. Malformations of the internal organs included anomalies of the brain (hydrocephalus, exencephalus, anencephalus), heart, lung and the urogenital

system (Hurley and Swenerton, 1966; Hurley, 1981).

Periods of zinc deficiencies shorter than the whole gestation period and also marginal maternal zinc deprivation in the rat have been reported to result in growth retardation and congenital malformations (Hurley et al., 1971). For example, gross congenital malformations were observed in almost 50% of the offspring when pregnant rats were restricted to the zinc deficient diet from day 6 to day 14 of gestation.

Teratogenic effect of zinc deficiency has also been reported in humans. Low serum/plasma zinc levels could be an indicator of zinc deficiency and subsequent adverse maternal and fetal effects. An inverse relationship between maternal plasma zinc concentration and the expression of congenital malformations in the infant has been observed (Flynn et al., 1981). Spontaneous abortion and congenital malformations of the skeletal and nervous systems, which are very similar to the anomalies observed in the offspring of zinc deficient rats, have been reported in human fetuses (Hambridge et al., 1975).

2.5.2 The Relationship of Ethanol and Zinc

Because ethanol ingestion interferes with the normal metabolism of essential trace elements such as zinc, it is plausible that the growth and developmental abnormalities observed in the offspring of chronic alcoholic mothers or in FAS cases, could be due to ethanol-induced zinc deficiency or

impaired metabolism of zinc.

Laboratory experiments and clinical studies have demonstrated the relationship between chronic ethanol consumption and zinc deficiency in laboratory animals and humans respectively. Chronic administration of ethanol in laboratory animals has been reported to interfere with zinc metabolism (Antonson and Vanderhoof, 1983; Assadi and Ziai, 1986) and zinc deficiency could impair ethanol metabolism (Das et al., 1984). Chronic ethanol consumption has been associated with decreased absorption, lower serum levels, increased urinary loss of zinc (McClain and Su, 1983). Sullivan and Lankford (1965) investigated in human subjects of chronic alcoholism in relationship to zinc excretion and serum zinc levels. The subjects were 124 chronic or acute alcoholic patients and 24 staff physicians served as controls. An excess urinary zinc excretion, and decreased serum zinc levels were observed in the alcoholic patients. About 42% of the alcoholic patients excreted zinc more than the normal mean and two standard deviations; the mean serum zinc levels were about 63 mg% in the alcoholics compared to 94 mg% in the controls. Another investigation, by Flynn et al. (1981), involving 25 alcoholic and 25 non-alcoholic pregnant women, investigated the link between ethanol consumption and zinc deficiency in the occurrence of gross congenital anomalies. The results revealed that maternal plasma and fetal cord-plasma zinc levels were significantly lower in the alcoholics compared to those of non-alcoholic women. There was also an inverse relationship between maternal

plasma zinc concentrations and the expression of fetal developmental anomalies. Chronic alcohol consumption has also been reported to reduce serum/plasma zinc levels in laboratory animals (Das et al., 1984; Ghishan et al., 1982). In addition, lower levels of hepatic zinc and decreased hepatic level or activity of alcohol-dehydrogenase, the major ethanol metabolizing enzyme, have been documented (Sullivan, 1962; Prasad et al., 1967). Moreover, inverse relationships between maternal alcohol dehydrogenase activity and maternal blood ethanol level or frequency of fetal abnormality (Chernoff, 1980), and also between maternal plasma zinc concentration and the incidence of fetal anomalies similar to FAS (Flynn et al., 1981), have been reported.

One of the possible mechanisms by which ethanol becomes teratogenic may be through inhibition of placental transport of nutrients. The effect of long-term, as well as short-term, ethanol feeding on the placental transport of zinc has been investigated (Ghishan et al., 1982). In this experiment, S/D rats were fed a 5% liquid ethanol diet from gestational days 4 through 12 (long-term) and a 25% ethanol diet on day 20 of gestation (short-term). Both long-term as well as short-term ethanol feeding resulted in a significant reduction in placental (40%) and fetal (30%) uptake of zinc. In addition, fetal body, placental and maternal serum zinc levels were significantly depressed in the long-term ethanol group. Thus, the effects of ethanol consumption during pregnancy are not restricted to only maternal zinc status. Assadi and Ziai (1986) have reported significantly lower plasma zinc concentrations and

increased urinary loss of zinc in infants with FAS.

It has been reported that there is a similarity between the congenital malformations in FAS and in zinc deficiency (Hurley, 1979). Thus, the hypothesis that zinc deficiency acts as a co-teratogen in FAS has been investigated in mice (Keppen et al., 1985). The results revealed a higher resorption frequency, smaller fetuses and increased number of external malformations in the fetuses of mice fed a low zinc diet and ethanol compared to the offspring of mice given an ethanol diet containing sufficient zinc. The authors concluded that zinc deficiency potentiated the teratogenic effects of ethanol, and thus nutritional intervention and zinc supplementation for alcoholic women during pregnancy may ameliorate the frequency and severity of FAS.

A synergistic effect of organic zinc salts and thiols in protecting mice against acute ethanol toxicity also has been reported (Floersheim, 1987). The survival of mice injected with a lethal dose of ethanol increased significantly when organic zinc salts were administered together with thiols (sulfhydril compounds), such as glutathione.

2.6 Pharmacology of Methionine

2.6.0 Absorption and Transport

The digestion of proteins is completed in the small intestine principally by pancreatic proteolytic enzymes. The end products of protein hydrolysis are

amino acids, including methionine and peptides.

Methionine is absorbed, by the brush border membrane of intestinal mucosa as free methionine, as well as methionine-methionine peptide. The peptide form is hydrolized to the free form in the intestinal mucosal cells. The methionine that enters the portal blood is almost entirely the free methionine and this is evidenced by the appearance of a large concentration of the amino-acid in portal plasma after protein feeding (Elwyn et al., 1968). The transport of methionine and other amino acids across the enterocytes is characterized by (1) absorption from the gut across the brush-border membrane, (2) diffusion through enterocyte cytoplasm, and (3) efflux to the portal circulation across the basolateral membrane.

Methionine, like the other amino acids, is transported by intestinal epithelium, enterocytes, by simple diffusion, facilitated (carrier-mediated) diffusion, and active transport mechanisms (Stevens et al., 1984). Quantitatively, however, the most important transport process is considered to be the active transport. These same three transport mechanisms also are responsible for the efflux of methionine and other amino acids from enterocytes, via enterocyte basolateral membrane, into the portal circulation. Methionine and phenylalanine have an additional specific carrier pathway for transport across the brush-border. Hepatocyte transport of methionine is very similar to the basolateral membrane transport mechanisms of enterocytes (Stevens et al., 1984).

It has been reported that caloric restriction increases amino acid absorption in animal intestine (Kershaw et al., 1960; Steiner and Gray, 1969) and decreases it in human intestine (Adibi and Allen, 1970; Steiner et al., 1969). Lis and coworkers (1972) have reported that methionine absorption from rat small intestine was increased by short term (10 days) dietary restriction and by short-term high protein or high methionine feeding. The site of maximal or rapid absorption is the upper jejunum in humans (Adibi, 1969) and the distal half of the intestine in animals (Nathans et al., 1960). The intestinal absorption of L-methionine is much more rapid than the D-isomer.

2.6.1 Methionine Metabolism

The principal pathway for methionine metabolism in mammalian liver is the transmethylation-transsulfuration reaction process. The first reaction in the pathway involves the enzyme methionine adenosyltransferase which catalyzes the synthesis of S-adenosyl methionine (AdoMet) from methionine in the presence of ATP. The liver contains the isoenzyme with the high K_m, and it is able to adapt promptly to high levels of methionine (Baldessarini, 1966). Fetal liver and extrahepatic tissues contain the low K_m isoenzyme.

S-adenosyl methionine (AdoMet), the active form of methionine, is the principal methylating agent in virtually all mammalian systems and the tissue AdoMet content depends on availability of methionine (Finkelstein, 1990). During AdoMet-dependent transmethylation, methyl acceptors are methylated

and molecules such as carnitine, choline, and creatinine are synthesized and AdoMet is converted to S-adenosyl homocysteine (AdoHcy) which is a general inhibitor of transmethylation reactions. S-adenosyl-methionine is also decarboxylated by the enzyme S-adenosyl methionine decarboxylase and produce polyamines, such as spermidine or spermine which are involved in promoting growth. Adenosyl homocysteine is metabolized to homocysteine, in a reversible reaction, by the enzyme adenosyl homocysteinase.

Homocysteine is irreversibly metabolized to cystathionine by cystathionine-ß-synthase and to methionine by betaine-homocysteine-methyltransferase (BHMT) and by methylfolate-homocysteine methyl-transferase. The conversion of homocysteine to methionine is highly important because methionine is conserved, AdoMet is produced and also homocysteine is detoxified. In the reaction catalyzed by BHMT, a methyl group is transferred from betaine to homocysteine to form methionine. In the reaction catalyzed by the enzyme methylfolate-homocysteine methyl-transferase, a methyl group is transferred from N^5-methyltetrahydrofolate to Vitamin B_{12} (Coablamine) and from methylcobalamine to homocysteine to form methionine (Barak and Beckenhauer, 1988).

In the metabolism of homocysteine to cystathionine by cystathionine-ß-synthase, homocysteine is used up in the transsulfuration pathway. Thus, cysteine cannot serve as a precursor of methionine because the cystathionine-ß-synthase reaction is irreversible. The formation of cysteine from cystathionine

is catalyzed by cystathionase, another pyridoxal phosphate enzyme, and this is the final reaction in the transsulfuration pathway. Cysteine serves as a precursor for glutathione (the primary cellular antioxidant), taurine and other molecules important in biological systems (Finkelstein, 1990).

Regulation of the metabolism of methionine exists and may allow for adaptation to alteration in dietary availability of methionine, cysteine, choline, folate and cobalamine (B_{12}). During inadequate methionine supply, dietary cysteine replaces methionine in the synthesis of cysteine and its derivations. This methionine sparing effect allows for increased conservation and reutilization of methionine. Thus, the distribution of homocysteine between re-methylation to methionine and cystathionine synthesis represents a major regulatory mechanism (Finkelstein, 1970). Increased availability of methionine and protein is associated with enhanced synthesis of cystathionine. Another possible regulatory site is the competition between protein synthesis and AdoMet production for available methionine (Young, 1987).

In human fetal liver or brain, cystathionase activity does not exist and that of cystathionine-synthase is smaller compared to the activity in the mature organ (Sturman et al., 1970). Cystathionine synthase and cystathionase, enzymes of the transsulfuration pathway, are not active during fetal life. Thus, methionine is conserved by the remethylation of homocysteine instead of becoming lost via the transsulfuration pathway. There is also an increased synthesis of polyamines (spermine or spermidine), which promote

growth. In addition, the activity of methyltetrahydrofolate-homocysteine-methyltransferase is higher in fetal liver than in mature human liver. The methyltetrahydrofolate remethylation pathway is important in the synthesis of DNA. Therefore, during fetal life, it was suggested that the transsulfuration pathway of methionine metabolism is turned off in favour of increased DNA, protein and polyamine synthesis which are essential for the rapid cellular proliferation and growth that is taking place (Gaull et al., 1973; Sturman and Gaull, 1974).

Methionine is transferred from maternal blood to the fetus even against concentration gradients suggesting that the placental transport of methionine is both an active and a passive process. It has been reported that methionine was transferred from maternal to fetal plasma against a two to threefold initial concentration gradient after an intravenous administration (Gaull et al., 1973).

2.6.2 Function of Methionine

Methionine is involved in a number of vital biological processes including protein synthesis, transmethylation, DNA, phosphotidyl choline, carnitine and polyamine synthesis; formation of cysteine and glutathione; and it is the source of homocysteine required for the metabolism of folate and choline. Thus, methionine is essential for the normal growth and development of mammalian tissue (Finkelstein, 1990).

S-adenosyl methionine (AdoMet), the active form of methionine, is

important in transmethylation reactions and contributes to the synthesis, activation, and metabolism of nucleic acids, proteins, phospholipids, hormones, neurotransmitters, and drugs. The involvement of AdoMet in DNA and phospholipid synthesis reflects its importance in tissue growth and maintenance of membrane structure respectively (Friedel et al., 1989). The role of AdoMet in the transmethylation and biosynthesis of membrane phospholipids allows for the regulation of important activities within the cell membrane including enzymatic and bioelectrical activities (Stramentinoli, 1987a). The biosynthesis of phosphotidylcholine is reduced in chronic liver disease because of alteration in methionine metabolism which could be due to a decrease in AdoMet-synthetase activity (Ortiz et al., 1987). Administration of AdoMet has been reported to protect against such alterations in membrane biochemistry (Cimino et al., 1984).

S-adenosyl methionine is a precursor of cysteine which in turn is a precursor for glutathione, the major cellular antioxidant responsible for detoxification of various compounds. Like the transmethylation pathway, the transsulfuration pathway also is impaired in chronic liver disease, and administration of AdoMet to patients with chronic liver disease results in increased plasma sulfurated compounds such as glutathione (Friedel et al., 1989).

Because AdoMet is an initiator of fundamental metabolic pathways such as transmethylation, transsulfuration and polyamine synthesis (Stramentinoli,

1987b), many of the variety of functions of methionine could be attributed to AdoMet.

2.6.3 Relationship of Ethanol and Methionine

One of the nutrients affected by chronic ethanol consumption is methionine, an essential amino acid for protein synthesis, methylation of DNA and membrane phosopholipids, formation of polyamines as well as glutathione. Methionine is also a lipotrope, a substance that protects against the formation of fatty liver, and plays a role in maintaining hepatic integrity.

Chronic ethanol consumption has been shown to interfere with methionine metabolism. In Sprague-Dawley rats, maintained on a protein-restricted diet, ethanol feeding for 3 to 10 days resulted in a decrease in methionine synthetase activity and increases in the activities of AdoMet-synthetase, betaine-homocysteine methyltransferase and cystathionine synthase (Finkelstein et al., 1974). As a result, increased amounts of methionine are lost via the transsulfuration pathway and homocysteine is not remethylated back to methionine, a reaction essential for the conservation of methionine. Thus, alcohol increases the dietary lipotrope (methionine) requirement by inducing impairment in methionine conservation (methionine wasting effect) and by depleting available methionine. A decrease in the activity of methionine synthetase has been shown also by Barak and coworkers (1987) in the rat after 30 days of ethanol feeding. The hepatic AdoMet level,

however, did not decrease significantly until after 90 days of ethanol treatment. Methionine administered (i.p.) to ethanol treated mice and rats significantly reduced both circulating and hepatic levels of acetaldehyde (Tabakoff et al., 1989).

Methionine is a precursor of AdoMet, its activated form. AdoMet is a universal methyl donor for many biological reactions involving trans-methylation. Transmethylation reactions are important for the synthesis, activation or metabolism of nucleic acids, proteins and phospholipids (Friedel et al., 1989). AdoMet is also an activator of transsulfuration reactions which result in the biosynthesis of glutathione, the major cellular antioxidant, responsible for the detoxification of toxic compounds (Ross, 1988). Ethanol consumption has been shown to alter hepatic levels of AdoMet and glutathione (GSH). Both acute (Speisky et al., 1985) and chronic (Pierson and Mitchell, 1986) administration of ethanol in mammals decrease liver levels of glutathione. Exposure to ethanol has also been reported to deplete intracellular glutathione concentrations in human (Pondosa et al., 1991) and rat (Vina et al., 1980) hepatocytes, respectively.

In a study reported by Lieber et al. (1990), baboons were fed a liquid ethanol diet (50% of their calories as ethanol) for 18 to 36 months. A group of the ethanol fed baboons was supplemented with AdoMet (400 mg/1000 calories). The results revealed that ethanol feeding resulted in a significant depletion in hepatic levels of AdoMet and GSH. AdoMet supplementation,

however, significantly improved AdoMet and GSH concentrations, resulting in attenuation of some ethanol induced hepatic injury.

Administration of exogenous AdoMet has also been reported to prevent depletion of GSH, production of acetaldehyde and deposition of fat in the liver of rats maintained on chronic ethanol diets (Feo et al., 1986; Pascale et al., 1989). Depletion of intracellular GSH was prevented by AdoMet administration in ethanol exposed human hepatocytes (Pondosa et al., 1991). AdoMet supplementation of ethanol treated female Wistar rats, improved hepatic AdoMet and GSH levels and significantly decreased both liver and blood acetaldehyde levels, and liver triglyceride concentrations (Feo et al., 1986). Simultaneous administration of AdoMet and ethanol in healthy humans has also been shown to lower plasma ethanol and acetaldehyde concentrations (DePadova et al., 1984). Moreover, it has been demonstrated that administration of thiols, such as GSH, together with organic zinc salts, increased significantly and synergistically the survival of mice poisoned with a lethal dose of ethanol (Floersheim, 1987). Therefore, ethanol induced alteration in methionine metabolism and subsequent depletion of methionine or its derivatives may be another mechanism involved in fetal alcohol effects.

3. MATERIALS AND METHODS

3.0 Experimental Animals

Throughout the course of this investigation, nulliparous female albino Sprague-Dawley rats (220-250 g) and sexually mature males of proven fertility were used. The rats were obtained from the Central Animal Care Facility located in the Basic Medical Sciences Building of the University of Manitoba.

3.0.0 Housing and Animal Care

Animals were housed in suspended stainless steel cages in an environmentally controlled room (22-23°C; relative humidity of 50%±10). A cycle of 12 hours of light from 0800 to 2000 hours, and dark from 2000 to 800 hours was maintained at all times.

During the period of adaptation, all the animals received food (Wayne F6 Rodent Blox Pellets) and tap water ad libitum.

3.0.1 Breeding

After about five days of adaptation period, the animals were mated overnight by placing a male albino Sprague-Dawley rat into a cage containing two nulliparous female rats. The male rat was introduced into the cage at about 1700 hours. Following removal of respective male rats, at 0900 hours the following morning, vaginal smears were taken.

3.0.2 Determination of Pregnancy

After an overnight mating, female rats were inspected for the presence of copulatory plug the following morning and vaginal smears were taken for microscopic determination of the presence of sperm. The presence of spermatozoa in the vaginal smear was considered as day-1 of gestation.

3.1 Experimental Design

Both in vivo and in vitro experiments were carried out to investigate the effects of the various treatments (ethanol, zinc, methionine and zinc and methionine).

3.1.0 In Vivo Experiments

The in vivo experiments were designed to establish the teratogenic effects of ethanol and to investigate whether or not zinc, methionine or zinc + methionine supplementation of ethanol treated pregnant rats protects against the embryopathic effects of ethanol. These experiments were carried out in (1) day-20 fetuses (Day-20 Experiment) and (2) in day-12 embryos (Day-12 Experiment).

The treatment period of the animals was from day-6 through day-12 of gestation. During the treatment period, the animals were fed a Lieber-DeCarli (1982) liquid diet (Bio-Serv, Inc., Frenchtown, NJ) which is based on the high-

protein (25%) formulation recommended for use in reproductive studies. In addition, animals were treated with ethanol, or zinc, or methionine or any combination of these treatments. The liquid diet and solutions of a zinc salt and methionine were prepared fresh every day.

The weight of pregnant animals was recorded either on days 1, 6, 12 and 20 of gestation (Day-20 Experiment) or on days 1, 6, and 12 of gestation (Day-12 Experiment).

3.1.0.0 Day-20 Zinc Experiment

This experiment was designed to establish possible teratogenic effects of ethanol and investigate whether or not zinc supplementation of ethanol-fed pregnant animals protects against ethanol embryopathy in near-term fetuses.

Once pregnancy was confirmed, animals were randomly assigned to ad libitum control, pair-fed control, zinc control, ethanol, zinc supplemented, and ethanol + zinc groups. Each group consisted of 10 pregnant animals.

The ethanol group received the liquid diet containing ethanol ad libitum, and the pair-fed control group received an isocaloric control liquid diet, with maltose-dextrin substituted for ethanol. Each rat in the pair-fed control group was paired to a rat fed the liquid ethanol diet and also was fed the amount consumed by the respective pair-mate the previous day. The inclusion of such a pair-fed (restricted-fed) group was important because ethanol feeding during pregnancy has been reported to result in reduced food intake (Lieber and

DeCarli, 1982). Pure 100% ethanol (Canadian Industrial Alcohols and Chemicals Limited), diluted to 95%, was used to make the ethanol liquid diet. The liquid ethanol diet contained 6.4% ethanol and the animals obtained about 36% of their calories from ethanol. The control diet was isocalorically balanced with maltose-dextrin.

The ethanol + zinc group received the liquid ethanol diet which was supplemented with zinc sulfate. Zinc sulfate ($ZnSO_4$, Molec. wt. 161.44, Sigma Chemical Co.) was administered intraperitoneally (i.p.) at a dose of 15 mg/kg. body weight. Granular zinc-sulfate, 750 mg, was dissolved in 100 ml. of physiological saline and a volume of 0.5 ml. was injected i.p. every morning throughout the treatment period. The zinc supplemented group was given the control liquid diet and zinc (15 mg/kg) also was administered i.p. This group was included in order to determine whether or not the given zinc dose has any adverse effects on embryonic growth and development. Another group which received the control liquid diet, was injected with 0.5 ml. of saline and served as a control for the zinc supplemented group.

The ad libitum control group received the control liquid diet unrestricted and served as a standard comparison group. Groups and treatment schedule are presented in Table 1.

Table 1. Treatment schedule: day-20 zinc experiment.

Treatment Groups	Number of Animals	Treatment
Ad libitum control	10	Maltose-dextrin control liquid diet (unrestricted)
Pair-fed control	10	Maltose-dextrin control liquid diet (restricted)
Ethanol	10	Ethanol-liquid diet
Zinc-supplemented	10	Maltose-dextrin control liquid diet and zinc
Ethanol + zinc	10	Ethanol liquid diet and zinc
Zinc control	10	Maltose-dextrin liquid diet and saline

Pure ethanol was incorporated in the liquid ethanol diet (6.4%) and maltose-dextrin substituted the calories derived from ethanol in the control (maltose-dextrin) liquid diet. Zinc, in the form of $ZnSO_4$ was administered i.p. (15 mg/kg).

During the entire treatment period the liquid diet was the only source of nutrients and water for the animals. The daily food intake of each animal was recorded every morning and animals were weighed and weight gain was recorded on days 1, 6, 12 and 20 of gestation.

On gestational day 20, gravid females were killed by an overdose of ether, the uterine horns were exposed and examined intact. The number of implantation sites was determined by counting the metrial glands which are yellowish nodules located along the mesometrial margin of the uterine horns. The metrial nodules which are not occupied by living or recently dead fetuses represent the number of prior resorptions. The number of live or dead fetuses was determined by applying a gentle pressure on them.

The uterine horns were incised along the antimesometrial border to reveal the fetuses, fetal membranes and the placenta. Fetuses were then recovered and were dissected free of the placenta and all fetal membranes and weighed to the nearest 0.01 gram. The crown-rump length, and placental weight were also recorded. Following these measurements, 2 to 3 fetuses from each animal (20-30 fetuses from each group) were randomly selected and placed in 95% ethanol for subsequent skeletal development examination. The rest of the fetuses were fixed in Bouins solution (aqueous saturated solution of picric acid 75%, formalin 25%, and glacial acetic acid 5%) for gross external and visceral examination. Following examination, from head to tail, for gross external developmental defects, fetuses were investigated for visceral

anomalies using Wilson's free hand razor blade technique (Wilson, 1965).

For determining serum levels of ethanol, zinc and copper an additional three groups of pregnant animals were used. These groups of animals were treated identically to the ethanol, pair-fed control and to the ethanol + zinc groups, respectively. On gestational day 12, the animals were lightly anesthetized with ether and blood was collected by inserting a 21 gauge needle at the bifurcation of the abdominal aorta. The blood was immediately centrifuged and serum was obtained. The ethanol levels in the serum were determined by capillary gas chromatography (Smith, 1984) and zinc and copper levels by flame atomic absorption spectrophotometry (Delves, 1987).

3.1.0.0.1 External Evaluation

Head to tail examination of fixed fetuses was carried out under a dissecting microscope for gross external malfunctions.

The parameters assessed were:

(i) craniofacial development (exencephaly, anencephaly, microphthalmia and anophthalmia)

(ii) development of the limbs (syndactyly, adactyly, polydactyly)

(iii) vertebral column (neural tube defect, kyphosis, scoliosis)

(iv) tail development (missing tail)

(v) external genitalia.

3.1.0.0.2 Visceral Examination

Fetuses fixed in Bouin's solution were examined for gross visceral anomalies using Wilson's razor blade technique (Wilson, 1965). Whole fetuses were sectioned in a cranio-caudal direction, at an interval of about 3-5 mm, and the slices were examined under a dissecting microscope.

The organs assessed were:

(i) Brain (hydrocephalus, dilation of ventricles)

(ii) Craniofacial (nasal septum, palate)

(iii) Thoracic region (lungs: lobes, heart: septal defect)

(iv) Abdominal area (liver, stomach, and gut)

(v) Pelvic region (kidneys: agenesis, ectopic, and hydronephrosis; gonads: testes, ovaries).

3.1.0.0.3 Skeletal Staining

Skeletal staining was performed by using the method of Dawson (1926). Depending on the litter size, 2 to 3 fetuses per litter were totally eviscerated by way of a small midline incision in the anterior abdominal wall. Eviscerated fetuses (2-3) were then placed in a small bottle containing 95% alcohol and dehydrated for at least one week. Following dehydration, the specimens were cleared in a solution of 1% potassium hydroxide until the bones were clearly visible, usually for two days. The specimens were then transferred to a fresh solution of 1% potassium hydroxide (KOH) and were stained with a few drops

of (0.4 ml.) alizarin red. The staining continued overnight and overstaining was corrected by storing the specimens in Mall's solution (79% distilled water, 20% glycerine, and 1% KOH). The specimens were then passed through increasing concentrations of glycerine (20%, 40%, 60%, 80%) for a period of about one week in each concentration, and were finally stored in 100% glycerine for evaluation. A small thymol crystal was added to prevent fungal growth and contamination during storage in pure glycerine.

3.1.0.0.4 Skeletal Evaluation

Skeletal assessment was performed by using a skeletal scoring chart, designed by Nash and Persaud (1989), which is a modification of the scoring system reported by Aliverti et al. (1979). This scoring chart is shown in Appendix 1.

Ossification of the hyoid, sternum, metacarpal, metatarsal, and the thoracic bones were examined under the microscope and the number of ossified centers were counted. The degree of ossification of the sternebrae, metacarpal, metatarsal and sacro-coccygeal bones has been reported to be the primary indices of skeletal development in the rat (Aliverti et al., 1979).

3.1.0.0.5 Placental Histology

Six to seven placentae from each of the six groups VI) were randomly selected. These Bouin's fixed placentae were transferred into 70% ethyl alcohol

and were left immersed overnight. Following routine processing for light microscopy, the placentae were blocked in paraffin and cut into sections of 4μm thickness using a Sorval-JB-4 microtome. The sections were then mounted on glass slides, stained with haematoxylin and eosin (H&E) and cover-slipped for microscopic examination.

The sections were examined for evidence of structural and vascular alterations using a Nikon binocular light microscope. The following structures were examined and were used as indices of functional as well as structural changes in the placenta:

(i) Basal zone of the placenta

(ii) Labyrinthine zone

(iii) Intervillous spaces

(iv) Giant cells, and trophoblasts

3.1.0.0.6 Serum Ethanol Determination

The serum ethanol levels of samples from the ethanol and ethanol + zinc treated groups was determined by capillary gas chromatography (Smith, 1984). Serum samples were deproteinized, then directly injected into the gas chromatography (Hewlett Packard 5890A, with autosampler GC"B") with N-propanol (200 mmol/L) as the internal standard. The alcohols and glycols were separated on a Nukol (carbowax-coated) capillary column with the eluted peaks being monitored by a flame ionization detector. The heights of the resultant

peaks were compared to that of the internal standard to give peak height ratios. The latter were compared to the peak height ratios obtained for a standard preparation to determine the concentration.

3.1.0.0.7 Serum Zinc and Copper Determination

Zinc and copper were measured by flame atomic absorption spectrophotometry (Dalves, 1987). Serum samples, controls and standards were diluted 1:4 with 5% trichloroacetic acid (TCA) to precipitate protein. Samples, controls and standards were transferred into centrifuging tubes and centrifuged at 3000 RPM (centrifuge T6 Beckman RPM 3000) for 30 minutes. Following centrifugation, supernatant was transferred into autosampler tubes and analyzed for zinc at 213.9 nm and for copper at 324.7 nm. The concentration of both analytes was determined from standard curves generated from a protein based certified reference material containing a given amount of zinc and copper.

3.1.0.1 Day-12 Zinc Experiment

This experiment was designed to investigate whether or not zinc supplementation prevents the embryopathic effects of ethanol in whole rat embryos. The experiment was expected to reveal any growth and developmental anomalies that might not have been evident in the near-term fetuses due to possible compensatory growth and development.

Pregnant animals were randomly assigned to ad libitum control, pair-fed control, Group III (zinc supplemented), Group IV (ethanol) and Group V (ethanol + zinc) groups. There were 10 to 12 animals per group.

Group I received the control liquid diet ad libitum; Group II and Group III were restricted-fed the control liquid diet, and served as controls for the ethanol and the ethanol + zinc treated groups, respectively. In addition, Group III was injected with zinc (15 mg/kg). Group IV received the ethanol liquid diet, and Group V the ethanol liquid diet and zinc (15 mg/kg) from day 6 through day 12 of gestation. The rationale for the administration of zinc from day 6 through day 12 of gestation is because this period represents a period of active embryogenesis and organogenesis. Embryonic days 9 to 12 represent the critical period of development in the rat. Groups and treatment schedule are presented in Table 2.

On day 12 of gestation, at 1200 hours, the animals were anesthesized with ether and the uterine horns were removed and placed in Hank's balanced salt solution (Whittaker M.A. Bioproducts). The uterine horns were then incised along the antimesometrial border to reveal the embryos. With the aid of fine forceps (5- Dumont type) and a dissecting microscope, the membranes surrounding the embryo were removed to reveal the underlying visceral yolk sac. The yolk sac circulation and allantois development were evaluated. The embryo was then explanted and the development of the circulatory, nervous, visual, auditory, olfactory, and skeletal systems as well as craniofacial

Table 2. Treatment schedule: day-12 zinc experiment.

Treatment Groups	Number of Animals	Treatment
Ad libitum control	10	Maltose-dextrin liquid diet (unrestricted)
Pair-fed control	10	Maltose-dextrin liquid diet (restricted-fed)
Ethanol	12	Ethanol liquid diet (unrestricted)
Zinc supplemented	10	Maltose-dextrin liquid diet and zinc
Ethanol + zinc	11	Ethanol liquid diet and zinc

Pure ethanol was incorporated in the liquid diet (6.4%) and maltose-dextrin substituted the ethanol derived calories when the control (maltose-dextrin) liquid diet was used. Both the ethanol and control liquid diets were isocalorically balanced. Zinc, in the form of $ZnSO_4$, was administered i.p. (15 mg/kg).

development were assessed according to the criteria of Brown and Fabro (1981). The embryonic growth and development evaluation chart is presented in Appendix 2. In addition, the number of somites was counted, crown-rump length measured and protein contents of whole embryos also was analyzed.

3.1.0.1.0 Whole Embryo Protein Determination

The protein content of whole embryos in each of the five groups was determined by the Lowry method (Lowry et al., 1951; Peterson, 1977). Whole embryos placed in microcentrifuge tubes were homogenized using an ultra-sound sonicator. The optic density (OD) of each homogenized sample was read at 750 nm using a spectrophotometer. The OD values were then read against a standard curve obtained using bovine serum albumin (BSA) and protein concentrations of each sample was determined.

3.1.0.2 Day-20 Methionine Experiment

This experiment was designed to investigate whether or not methionine supplementation of ethanol-fed animals reduces the embryopathic effects of ethanol in near-term fetuses.

Pregnant animals were randomly assigned to methionine supplemented, methionine control and ethanol + methionine groups. The same ad libitum control, pair-fed control and ethanol groups, as those already described in section 3.1.0.0, were used in this experiment.

Each animal in the methionine supplemented group received the control liquid diet and methionine (200 mg/kg) dissolved in physiological saline throughout the treatment period, which was from days 6 to 12 of gestation. The animals on the ethanol + methionine group received the ethanol liquid diet and methionine (200 mg/kg). The methionine control group was given the control liquid diet and an equal volume of saline as methionine. The animals were lightly anesthetized and a volume of 0.5 ml of methionine or saline was administered by gastric intubation. Grouping and treatment schedule is presented in Table 3.

On gestational day 20, pregnant animals were killed with ether. The number of implantation sites, resorptions, and dead or live fetuses were determined. Fetal weight, crown-rump length and placental weight were recorded immediately. Later, gross external and visceral examination, skeletal staining and evaluation, and placental histology were carried out using the same procedures described under section 3.1.0.0 (3.1.0.0.1, 3.1.0.0.2, 3.1.0.0.3, 3.1.0.0.4, and 3.1.0.0.5).

3.1.0.3 Day-12 Methionine Experiment

The purpose of this experiment was to investigate the effect of methionine supplementation, of ethanol treated pregnant animals, on ethanol embryopathy in the absence of possible post-treatment compensatory growth and development.

Table 3. Treatment schedule: day-20 methionine experiment.

Treatment Groups	Number of Animals	Treatment
Ad libitum control	10	Maltose-dextrin liquid diet (unrestricted)
Pair-fed control	10	Maltose-dextrin liquid diet (restricted diet)
Ethanol	10	Ethanol liquid diet
Methionine control	10	Maltose-dextrin liquid diet and saline
Methionine supplemented	10	Maltose-dextrin liquid diet and methionine
Ethanol + Methionine	10	Ethanol liquid diet and methionine

Pure ethanol was incorporated in the liquid diet (6.4%) and maltose-dextrin substituted the calories derived from ethanol when the control (maltose-dextrin) liquid diet was used. The ethanol and the control diets were isocalorically balanced. Methionine was administered by gastric intubation (200 mg/kg).

Once pregnancy was confirmed, animals were assigned to ethanol, pair-fed control, ad libitum control, ethanol + methionine and methionine supplemented groups. The treatment period was from days 6 to 12 of gestation.

The ethanol + methionine group received the liquid ethanol diet and methionine (200 mg/kg) and the methionine supplemented group was given methionine (200 mg/kg) and the control liquid diet. In animals fed a low protein diet, supplementation with methionine, 3-4 times the estimated requirement, resulted in a marked suppression of voluntary food intake and near-cessation of growth (Benevenga and Steele, 1984). The methionine dose used in the present study (200 mg/kg) was selected from three different doses and was less than three times the recommended dietary intake and did not produce any adverse effects. The animals were lightly anesthetized with ether when administering methionine by gavage. The feeding protocol of the animals is the same as that described in section 3.1.0.0. The general treatment schedule is presented in Table 3.

On gestational day 12, at 1200 hours, the animals were anesthetized with ether and the uterine horns were excised and transferred into Hank's balanced salt solution. The materials and procedures used to retrieve embryos and the end points evaluated are described under section 3.1.0.1. Assessment of the embryos was based on the criteria developed by Brown and Fabro (1981) (see Appendix 2). The protein content of whole embryos was measured using the

Lowry method which is described under section 3.1.0.1.0.

3.1.0.4 Day-20 Zinc and Methionine Experiment

Simultaneous administration of zinc salts and thiols (sulfhydryl compounds) have been reported to synergistically protect against ethanol induced toxicity or fatality in mice (Floerscheim, 1987). The purpose of this experiment was to investigate whether simultaneous administration of zinc and methionine in ethanol treated pregnant animals has any protective effect against ethanol embryopathy in near-term fetuses.

Pregnant animals were randomly assigned to ethanol, ethanol + zinc & methionine, and zinc & methionine supplemented groups. An ad libitum control and a pair-fed (a restricted-fed) ethanol control group also were included.

The ethanol + zinc & methionine group received the ethanol liquid diet, zinc sulfate (15 mg/kg) and methionine (200 mg/kg). The zinc and methionine supplemented group received the liquid control diet, zinc sulfate (15 mg/kg) and methionine (200 mg/kg). Zinc and methionine were administered i.p. and by gavage respectively. The treatment schedule is presented in Table 4.

The animals were killed on gestational day 20, at 1200 hours. Exactly the same materials, procedures, and same growth and developmental parameters as in section 3.1.0.0 were used to evaluate fetuses.

Table 4. Treatment schedule: day-20 zinc & methionine experiment.

Treatment Groups	Number of Animals	Treatment
Ad libitum control	10	Maltose-dextrin liquid diet (unrestricted)
Pair-fed control	10	Maltose-dextrin liquid diet (restricted-fed)
Ethanol	10	Ethanol liquid diet
Zinc & methionine supplemented	10	Maltose-dextrin liquid diet and zinc & methionine
Ethanol + zinc & methionine	10	Ethanol liquid diet and zinc & methionine

Pure ethanol was incorporated in the liquid ethanol diet (6.4%) and maltose-dextrin substituted the ethanol derived calories when the control liquid diet was used. The ethanol and the control liquid diets were isocalorically balanced. Zinc (in the form of $ZnSO_4$) was administered i.p. (15 mg/kg) and methionine (200 mg/kg) was given by gavage (p.o.).

3.1.0.5 Day-12 Zinc and Methionine Experiment

This experiment was designed to investigate the combined influences of zinc and methionine on embryopathic effects of ethanol in day 12 embryos.

Pregnant animals were assigned to ethanol, ethanol + zinc & methionine, zinc & methionine supplemented, pair-fed control for ethanol and ad libitum control groups.

The treatment protocol for the respective groups and route of administration and amount of zinc methionine, and physiological saline are exactly the same as those described under section 3.1.0.3. The treatment schedule is presented in Table 4.

On gestational day 12, the animals were anesthetized with ether and embryos were recovered. The materials and methods used to retrieve embryos, the growth and developmental parameters and the assessment criteria used were exactly the same as those described under section 3.1.0.1.

3.1.1 In Vitro Experiment

An embryo culture system that can support growth and development of embryos, indistinguishable from that of the in vivo condition, have been used and reported by Brown and coworkers (1979). Under in vivo condition, it is difficult to establish direct ethanol embryo toxicity because of possible confounding variables, sources of alternative explanations, such as primary or secondary malnutrition and altered maternal metabolism and functions.

This experiment was designed to establish direct embryo toxic effects of ethanol and to investigate whether S-adenosyl methionine (AdoMet), the activated form of methionine, administration protects rat embryos from ethanol toxicity.

The whole rat embryo culture system described by Brown and coworkers (1979) was used in the present study.

3.1.1.0 Preparation of Serum for Culture

Heavy male Sprague-Dawley rats (over 350 grams) were used to obtain the serum for culture. The animals were lightly anesthetized with ether and a longitudinal mid-abdominal incision was made to expose the viscera. The viscera and intestine were exteriorized and the abdominal aorta was carefully exposed. Blood was collected from the aorta by inserting a 21-gauge needle at the bifurcation of the abdominal aorta. The blood was transferred into a 15 ml sterile polystyrene tube and was immediately centrifuged for five minutes to separate blood cells from the plasma. Serum was obtained by squeezing the plasma clot and was transferred into 4.5 ml centrifuging tubes and stored at -20°C.

3.1.1.1 Recovery of Embryos

For details regarding animal care, breeding and determination of pregnancy see section 3.0.

On embryonic day 9.5 (gestational day 10), at 1200 hours, pregnant animals were lightly etherized and the gravid uterus was excised and placed in a petri dish containing Hanks Balanced Salt Solution (Isotonic Saline; Whittaker M.A. Bioproducts).

The uterine horns were incised longitudinally along the antimesometrial border. With the help of a cataract knife and a dissecting microscope the decidual swellings were explanted from the uterus. Each decidual swelling was then split open into two halves by using two pairs of fine forceps (Type 5 Dumont Forceps) and one of the two halves would bear the embryo. The embryo, enclosed in its membrane, was then gently eased from the decidua. The Reichert's membrane, with its attached trophoblast and parietal yolk sac, was completely opened using the fine forceps and the visceral yolk sac, amnion and ectoplacental cone were left intact. The embryo was now ready for culturing. The whole process of retrieving 9.5 day old embryos is shown, step by step, in Figure 1.

3.1.1.2 Preparation of Culture Medium and Treatments

Serum was heat inactivated at 57°C in a water bath for 30 minutes to denature the compliment protein and remove the ether residual. The serum was then filtered using a sterile micro-filter, 0.45 micron, and a syringe (Nalgene Labware, USA) and collected in small, sterile 2 ml tubes (Nalgene Cryoware). The serum was transferred into 30 ml glass culture bottles (4 ml

of serum per bottle) and antibiotic was added at a dose of 10 ul/ml serum. The antibiotic used was a premixed combination of penicillin-streptomycin (5000 I.U./ml. and 500 MCG/ml; ICN Biomedicals, Inc., Costa Mesa, CA).

Finally, the treatment regimen were administered into the respective culture medium at a dose of 3 mg/ml serum of ethanol and 3 mM, 1 mM, 0.1 mM and 0.05 mM of S-adenosyl methionine (AdoMet). Embryos (4/bottle) were then placed in the culture bottles containing the medium. AdoMet, the stable salt form, (mol. wt. 766.8) was purchased from RBI: Research Biochemicals Inc., Natick, MA.

3.1.1.3 Treatment Groups and Incubation of Embryos

The 9.5 day old whole rat embryos were randomly assigned to an ethanol group, control group, and 0.05 mM AdoMet, ethanol + 3 mM AdoMet, ethanol + 1 mM AdoMet, ethanol + 0.1 mM AdoMet and ethanol + 0.05 mM AdoMet groups.

The ethanol group received ethanol (300 mg/100 ml), ethanol + 3 mM AdoMet, ethanol + 1 mM AdoMet, ethanol + 0.1 mM AdoMet and the ethanol + 0.05 mM AdoMet groups received ethanol (300 mg/100 ml) and 3 mM, 1 mM, 0.1 mM, and 0.05 mM AdoMet respectively. The 0.05 mM AdoMet group was treated with 0.05 mM AdoMet alone and the control group received an equal volume of physiological saline, the same volume as ethanol.

Following the first gas phase, embryos in the culture bottle (4 embryos/bottle) were incubated at 38±C for 48 hours in an incubator custom-made for embryo culture studies (Astell Hearson, England). The bottles were closed tightly and placed horizontally on rollers located in the incubator. The rollers rotated continuously (at 60 RPM) as to ensure maximum and even exposure of embryos to the treatments. The treatment schedule is presented in Table 5.

3.1.1.4 Gas Phases

Embryos were gassed with varying concentrations of oxygen at three intervals:

(i) First gas phase: This was carried out immediately after the treatment regimen and embryos were placed in the culture bottles and lasted for the first 18 hours of incubation. The embryos were gassed with 5% O_2, 5% CO_2, and 90% N_2 for 5 minutes.

(ii) Second gas phase: This took place 18 hours after the first gas phase and lasted for the next 8 hours. The embryos were gassed with 20% O_2, 5% CO_2, and 75% N_2 for 5 minutes.

(iii) Third gas phase: Embryos were gassed, for the rest of the 48 hours culture period, with 40% O_2, 5% CO_2, and 5% N_2.

Figure 1. Sequential processes in the explantation of 9.5 days old rat embryos for in vitro culture.

a. Gravid uterus.

b. Decidual swellings exposed by opening the uterus.

c. Pear-shaped decidual swellings explanted from the uterus.

d. Decidual swellings split into two halves to expose and explant the embryo.

e. Explanted embryo covered with Reichert's membrane (R) and attached with the ectoplacental cone (EC).

f. Embryo in embryonic membrane (EM), Reichert's membrane removed.

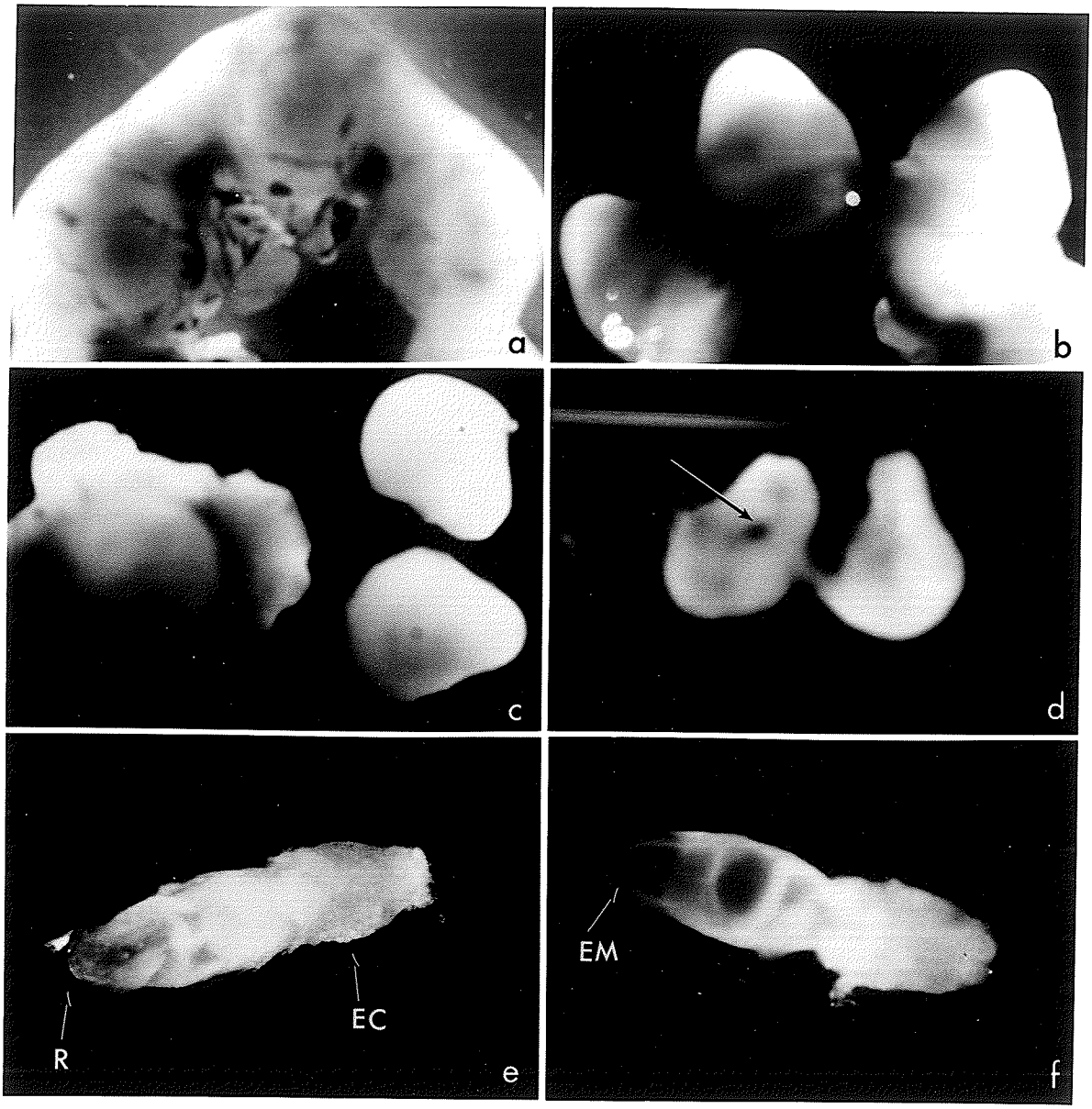

a
b
c
d
R
EC
e
EM
f

Table 5. Treatment schedule: in vitro experiment.

Groups	Treatment	Number of embryos
I	Saline	18
II	Ethanol	16
III	0.05 mM AdoMet	12
IV	Ethanol + 0.05 mM AdoMet	16
V	Ethanol + 0.1 mM AdoMet	15
VI	Ethanol + 1 mM AdoMet	15
VII	Ethanol + 3 mM AdoMet	15

* Treatment period was from embryonic day 9.5 through 11.5. Embryos were explanted from the gravid uterus on embryonic day 9.5 and were cultured in serum culture medium (4 embryos/4 ml serum) containing varying concentrations of S-adenosyl methionine (AdoMet).

3.1.1.5 Evaluation of Embryos

The incubation of the embryos was terminated at 1400 hours on the third day of incubation. This stage corresponds to embryonic day 11.5 or gestational day 12 (Brown et al., 1979). Embryos after 48 hours of culture are shown in Figure 10.

Examination and assessment of the embryos were carried out according to the criteria developed by Brown and Fabro (1981) (see Appendix 2). The materials and methods used to reveal the embryos and the parameters used as indices of growth and development and the assessment criteria were exactly the same as those described under section 3.1.0.1.

For embryos to be included in the evaluation, a detectable heart beat and visible individual semite segments must be present. In addition, the embryo must be intact, without any visible damage.

4. RESULTS

4.0 Day-20 Zinc Experiment

The results are summarized in Tables 6 to 11.

4.0.0 Maternal Food Intake and Weight Gain

The data were analyzed using one-way analysis of variance (ANOVA). The results revealed no statistically significant differences in food intake between the ethanol-fed and its pair-fed control or the ethanol + zinc groups (Table 6). Therefore, the dietary nutrient calorie and zinc intakes of the various groups of animals, except for the ad libitum control group therefore were similar. The food intake of the ad libitum group was significantly higher compared to that of the other groups (Table 6).

Ethanol consumption in the ethanol and ethanol + zinc groups also were found to be similar, 3.67±0.09 and 3.68±0.1 g/day, respectively (Table 6). This amounts to about 36% of the total calories obtained from the diet daily throughout the duration of the treatment.

Maternal weight gain in the various treatment groups, during two different periods of gestation (day 6 to 12, and day 6 to day 20), were compared. Weight-gain from days 6 to 12 of gestation were significantly ($p<0.05$) lower in the ethanol-fed animals (-1.7±5.9 grams), compared to either the pair-fed control group (6.5±3.1 grams) or the ad libitum control group

(10.7±3.4 grams). Weight gain in the ethanol group during this gestational period, was also significantly ($p<0.05$) reduced compared to that of the zinc-supplemented group (7.1±2.3 grams). Maternal weight gain during gestational days 6 to 20, however, was not significantly different when the ethanol-fed (109±12 grams) and the pair-fed control groups (112±9 grams) were compared. Thus, it appeared that the weight lost by the ethanol-fed animals during the treatment period (days 6 through 12) was regained towards the end of gestation.

However, maternal weight increase in the ad libitum control group (127±12 grams) or in the zinc supplemented group (122±9 grams), during gestational days 6 to 20, was significantly higher compared to that of the ethanol group (Table 7). The ad libitum control group received the liquid control diet unrestricted while the zinc supplemented group was given the liquid control diet restricted. The ethanol group received the ethanol containing liquid diet, while the pair-fed control group was restricted-fed the control diet, the amount consumed by the respective pair-mate in the ethanol group.

4.0.1 Pregnancy Outcome

The results are summarized in Table 7. Implantation sites, live fetuses, resorptions, and number of dead plus resorbed fetuses were used as indices of pregnancy outcome. One-way analysis of variance (ANOVA) was used to

statistically analyze the data.

The number of live fetuses as well as dead and resorbed fetuses in the ethanol-fed group was significantly different ($p<0.05$) from those in the pair-fed control, ad libitum control, ethanol + zinc and from the zinc supplemented groups (Table 7). Implantation sites, however, were not affected by ethanol treatment. The total litter weight and crown-rump length of the ethanol group were significantly lower compared to those of the pair-fed control group and also to the ethanol + zinc group. The placental weight of fetuses of ethanol-fed animals, however, was significantly increased ($p<0.01$) compared to the pair-fed control and all the other groups (Table 8).

4.0.2 Developmental Anomalies

There were more developmental anomalies per litter in the ethanol treated group. The total number of developmental anomalies per litter in the ethanol group (7.6±1.7) was significantly different ($p<0.01$) from the pair-fed control group (1.3±0.9) as well as the ethanol + zinc group (5.7±2.2). The ethanol + zinc group, however, revealed a significantly higher incidence of developmental anomalies ($p<0.01$) compared to the pair-fed control, or the zinc supplemented or the ad libitum control groups (Table 9). External anomalies were observed only in the fetuses of animals treated with ethanol (ethanol group) and in those treated with both ethanol and zinc (ethanol + zinc group).

In order to assess the degree of skeletal developmental, ossification-

centres of the sternum, metacarpals, metatarsals, thoracic centra, and the hyoid were used as indices (Table 10). Ossification of the sternebrae, metacarpals, metatarsals, thoracic centra and the hyoid was significantly delayed in the ethanol-group compared to either the pair-fed control, or ad libitum control or the zinc supplemented groups. In the ethanol group, 58% of the fetuses had less than two sternal ossification centres, and 91% or 83% showed less than three ossified metacarpal or metatarsal centres respectively. In the pair-fed control group, however, the proportions were significantly lower; that is, 32%, 56% and 52% respectively (Table 10). The degree of ossification in a pair-fed control fetus and an ethanol treated fetus is shown in Figure 2.

4.0.3 Serum Levels of Ethanol and Zinc

The data were analyzed by using one-way ANOVA. The serum ethanol level was significantly higher ($p<0.05$) in the ethanol treated group (11.7±7.9 mMol/l) than levels in the group treated with both ethanol and zinc (5.5±2.9 mMol/l) (Table 11).

The zinc concentration in serum was significantly reduced ($p<0.05$) in the ethanol group (16.9±3.8 µMol/l) compared to that in the pair-fed control group (21.9±5.5 µMol/l). However, the copper level in the ethanol group was relatively elevated. On the other hand, the serum zinc concentrations in the ethanol + zinc group (118±43 µMol/l) was significantly higher, about five and six times the concentrations found in the pair-fed control and in the ethanol

groups, respectively (Table 11).

4.0.4 Placental Histology

Photographs of placental sections, prepared from pair-fed control and ethanol treated rats, stained with haematoxylin and eosin (H&E), are shown in Figures 3 to 8. Placental sections in the ethanol treated group appeared to have more stagnated blood in the basal-decidual and in basal-labyrinthine junctions. Intervillous spaces in the labyrinthine zone were more dilated, and filled with maternal blood more frequently in the ethanol than in the pair-fed control group. In addition, the giant cells, located in the basal zone of the placenta, also appeared larger in the ethanol group compared to the pair-fed control. The frequency of occurrence of stagnated blood in either the labyrinthine zone or in the basal-labyrinthine junction appeared to be lower in the ethanol + zinc group compared to the ethanol group.

4.1 Day-12 Zinc Experiment

The results are summarized in Tables 12 to 17. The data regarding pregnancy outcome, and embryonic growth (protein content, crown-rump length, number of somites and morphological score) were analyzed by using General Linear Model (GLM). Embryonic organs or systems development data were analyzed using Chi-Square Analysis.

4.1.0 Pregnancy Outcome

Maternal weight gain during the period of treatment (gestational days 6-12) was significantly reduced ($p<0.05$) in the ethanol group (-2.3±5.8 grams) compared to the pair-fed control (6.4±4.6 grams), ad libitum control (11.3±4.0 grams) or to the zinc supplemented group (6.9±2.9 grams). There was, however, no significant difference, in maternal weight gain, between the ethanol and the ethanol + zinc groups (Table 12). Like in the ethanol group, maternal weight gain in the ethanol + zinc group (1.2±5 grams) also was significantly lower compared to the ad libitum control, pair-fed control, and the zinc supplemented groups despite similar caloric/nutrient intakes across the groups.

Although implantation of embryos was not affected by any of the treatments, the rate of resorptions in the ethanol and the ethanol + zinc groups were significantly higher ($p<0.05$) compared to the rate in the pair-fed control, ad libitum control, and the zinc supplemented groups (Table 12). This suggests that ethanol results in increased resorptions and that zinc supplementation does not influence ethanol induced embryonic resorption.

4.1.1 Embryonic Growth

The results are summarized in Table 13. The embryonic protein content, crown-rump length, number of somites and morphological score were used as indices of embryonic growth.

The results revealed that ethanol treatment of pregnant rats significantly reduced ($p<0.05$) the protein content (186±44 µg) of embryos compared to that of the pair-fed control (276±71 µg), ad libitum control (289±49 µg) and the zinc supplemented (297±58 µg) groups. The protein content of embryos in the ethanol + zinc group (237±127 µg) was found to be higher than that in the ethanol group.

In the ethanol group, the crown-rump length (3.3±0.3 mm), number of somites (22.8±2.9), and the morphological score (38.5±1.5) were also significantly lower ($p<0.001$), compared to those in the pair-fed control ad libitum control and zinc supplemented groups. These embryonic growth indices also were significantly reduced ($p<0.001$) in the ethanol + zinc group. However, zinc supplementation of ethanol treated pregnant rats significantly ($p<0.01$) improved the number of somites developed in the respective embryos (Table 13).

4.1.2 Embryonic Development

The results are summarized in Tables 14 to 17. The degree of embryonic development of the primordia of the various systems is shown in Figure 9. Circulatory system development, as assessed by yolk sac circulation, was retarded in 8% ($p<0.001$) of the 104 embryos assessed in the ethanol group compared to none in the other groups. The development of the heart, however, was not affected by any of the treatments. Auditory primordium was also not

affected (Table 14).

Embryonic nervous system development was assessed using forebrain, midbrain, hindbrain, and caudal neural tube development as indices. The results showed that the development of the nervous system (forebrain, hindbrain and caudal neural tube) was significantly ($p<0.001$) affected by ethanol treatment compared to the pair-fed control, ad libitum control and zinc supplemented groups. In addition, supplementation of the ethanol treated pregnant animals with zinc did not improve the degree of development of the embryonic nervous system (Table 15).

Embryonic craniofacial development in the ethanol and ethanol + zinc groups was significantly delayed ($p<0.01$) compared to that in the pair-fed control, ad libitum control and the zinc-supplemented groups (Table 16).

The musculoskeletal system (number of somites, somite score and forelimb) development in embryos of the ethanol and ethanol + zinc groups also was retarded compared to that in the other groups. The number of somites, however, was significantly increased ($p<0.01$) in the ethanol + zinc group compared to that in the ethanol group (Table 17).

4.2 Day-20 Methionine Experiment

The results are shown in Tables 18 to 22. The data, regarding maternal food intake and weight gain, pregnancy outcome, fetal growth, and developmental anomalies were analyzed using one-way analysis of variance (ANOVA).

4.2.0 Maternal Food Intake and Weight Gain

There were no significant differences in food intake between the ethanol group and its pair-fed control or the ethanol + methionine group, or the methionine supplemented group. However, dietary intake in the ad libitum control group was significantly higher ($p<0.01$) than any of the other treatment groups. Thus, with exception of the ad libitum control group, the dietary caloric and methionine intake of the various groups were the same. In addition, ethanol consumption of both the ethanol and the ethanol + methionine groups were similar as well (Table 18). Methionine administration in ethanol treated pregnant rats did not reduce serum ethanol levels (Table 18).

Despite similar dietary nutrient intakes, in all the groups, maternal weight gain, during the treatment period, in the ethanol group was significantly lower ($p<0.05$), compared to the methionine supplemented or the pair-fed control group. In fact, animals in the ethanol group lost weight during gestational days 6 to 12. Maternal weight gain in the ethanol group during gestational days 6-20, however, was not significantly different from either the methionine supplemented or the pair-fed group. Moreover, methionine supplementation of ethanol treated pregnant animals did not influence weight gain (Table 19).

4.2.1 Pregnancy Outcome

The results are shown in Tables 19 and 20. The data regarding pregnancy outcome, were analyzed using one-way analysis of variance.

The results revealed that the rate of live births in both the ethanol and ethanol + methionine groups was significantly lower ($p<0.05$) compared to either the pair-fed control or the methionine supplemented groups. The rate of resorptions also was significantly higher ($p<0.05$) in the ethanol group and methionine supplementation of ethanol-fed animals did not reduce the frequency of resorptions (Table 19). Implantation rates, however, were not significantly affected by any of the treatments.

The growth of fetuses was affected by ethanol treatment of pregnant animals and methionine supplementation of the ethanol-fed animals failed to prevent or lower the ethanol induced growth retardation. The litter weight and crown-rump length in the ethanol and ethanol + methionine groups were significantly lower ($p<0.01$), compared to either the pair-fed control or the methionine supplemented group (Table 20).

4.2.2 Developmental Anomalies

The incidence of gross developmental anomalies was significantly higher ($p<0.01$) in the fetuses of the ethanol + methionine groups compared to either the pair-fed control, or the methionine supplemented groups (Table 21). There was, however, no significant difference in the rate of developmental anomalies,

between the methionine supplemented and either the pair-fed or the ad libitum control groups. Both internal and external anomalies were observed only in the ethanol and in the ethanol + methionine groups.

In addition, ossification of the sternebrae, metacarpals, metatarsals, thoracic centra and that of the hyoid was delayed in both the ethanol and in the ethanol + methionine groups. The degree of ossification in the offspring of ethanol-fed animals did not differ compared to that in the fetuses of ethanol plus methionine treated animals (Table 22). Difference in ossification between an ethanol treated and a pair-fed fetuses is shown in Figure 2.

4.2.3 Placental Histology

It appeared that there was an increased volume and frequency of stagnated maternal blood in either the labyrinth or in the basal-labyrinthine junction in placentas from the ethanol, as well as the ethanol plus methionine treated animals (Figures 3 to 8).

4.3 Day-12 Methionine Experiment

Results are summarized in Tables 23 to 28. The data regarding pregnancy outcome, and growth (protein content, crown-rump length, number of somites, and morphological score) were analyzed using the General Linear Model (GLM). Embryonic development data were analyzed using Chi-Square Analysis.

4.3.0 Pregnancy Outcome

The results are summarized in Table 23 to 28. Maternal weight gain, during the treatment period was significantly decreased ($p<0.05$) in both the ethanol and in the ethanol + methionine groups, compared to either the methionine supplemented, or the pair-fed control or the ad libitum control groups. There was, however, no significant difference between the ethanol and the ethanol + methionine groups. The ethanol and the ethanol + methionine groups also showed significantly ($p<0.05$) higher incidence of resorptions (Table 23).

4.3.1 Embryonic Growth

The results are shown in Table 24. Embryonic growth indices used were embryonic protein content, crown-rump length, number of somites, and morphological score.

The protein content of embryos of the ethanol-fed animals was significantly lower ($p<0.05$), compared to the pair-fed control, or ad libitum control, or the methionine supplemented group. Although the protein content in the ethanol + methionine group was significantly reduced ($p<0.05$), compared to the methionine supplemented group, it did not appear to be different from all the other groups.

The crown-rump length, number of somites, and the morphological score were significantly lower ($p<0.001$) in the ethanol and ethanol + methionine

groups, compared to either the pair-fed control, or the methionine supplemented group (Table 24).

4.3.2 Embryonic Development

The results are shown in Tables 25 to 28. Developmental status of the primordia of the various systems is shown in Figure 9. The degree of development of the yolk sac circulation was affected ($p<0.01$) in embryos of both the ethanol and the ethanol + methionine groups compared to the pair-fed control and to the methionine supplemented groups (Table 25).

The forebrain, hindbrain and caudal neural tube development was retarded ($p<0.001$) in both the ethanol and the ethanol + methionine groups (Table 26).

Embryonic craniofacial development, the optic system ($p<0.01$), olfactory system ($p<0.001$), and branchial bars ($p<0.01$), were delayed in both the ethanol, and in the ethanol + methionine groups (Table 27).

In addition, the development of the forelimb ($p<0.001$), and the somites ($p<0.001$) the indices of musculoskeletal system development, in the ethanol group, was considerably affected. The ethanol induced delay in musculoskeletal system development was not prevented or reduced by methionine supplementation of the ethanol treated pregnant animals (Table 28).

4.4 Day-20 Zinc and Methionine Experiment

The results are given in Tables 29 to 33. Maternal food intake and weight gain, pregnancy outcome, fetal growth and developmental-anomalies data were all analyzed using one-way analysis of variance.

4.4.0 Maternal Food Intake and Weight Gain

The food intake, i.e. dietary calorie, zinc, and methionine intake, was similar in the ethanol, pair-fed control, ethanol + zinc & methionine, and in the zinc & methionine supplemented groups. Dietary intake, however, was significantly higher ($p<0.01$) in the ad libitum control group compared to all the other groups (Table 29).

Maternal weight gain in the ethanol group during gestational-days 6-12, was significantly reduced ($p<0.05$) compared to all the other groups (Table 30).

4.4.1 Pregnancy Outcome

The results are summarized in Tables 30 and 31. In the ethanol group, the rate of live births was significantly lower ($p<0.05$) and resorptions were higher ($p<0.05$) compared to either the pair-fed control or the zinc & methionine supplemented group (Table 30).

In addition, litter weight and crown-rump length were significantly less ($p<0.05$) in the ethanol group (Table 31) and zinc & methionine supplementation of the ethanol treated pregnant animals did not improve

pregnancy outcome (Table 30 and Table 31).

4.4.2 Developmental Anomalies

The incidence of gross developmental anomalies ($p<0.01$) or internal anomalies ($p<0.01$) and external anomalies ($p<0.01$) were significantly higher in both the ethanol and ethanol + zinc & methionine groups compared to either the pair-fed control, or the zinc & methionine supplemented groups (Table 32). In addition, ossification of the sternebrae, metacarpals, and metatarsals also was significantly retarded ($p<0.05$) in the ethanol as well as in the ethanol + zinc & methionine groups (Table 33). Ossification levels for an ethanol treated and a pair-fed fetuses are shown in Figure 2.

In addition, the placental histology in both the ethanol as well as in the ethanol plus zinc & methionine treated animals did not appear different (Figures 3 to 8).

4.5 Day-12 Zinc and Methionine Experiment

The results are shown in Tables 34 to 39. The data concerning protein content, crown-rump length, number of somites and morphological score of embryos as well as that of pregnancy outcome were analyzed using the General Linear Model procedure and that on embryonic development using the Chi-Square Analysis.

4.5.0 Pregnancy Outcome

The results are shown in Table 34. In the ethanol as well as in the ethanol + zinc & methionine groups there were significantly higher ($p<0.05$) rates of resorptions, and in addition, maternal weight gain was lower ($p<0.05$) compared to the pair-fed control or to the zinc & methionine supplemented group (Table 34).

4.5.1 Embryonic Growth

The results are summarized in Table 35. The embryonic protein content was lower ($p<0.05$) in the ethanol group. Moreover, the crown-rump length, number of somites and the morphological score were significantly reduced ($p<0.001$) in both the ethanol and the ethanol + zinc & methionine groups compared to either the pair-fed control or to the zinc & methionine supplemented groups (Table 35).

4.5.2 Embryonic Development

The results are summarized in Tables 36 to 39. The level of embryonic development of the primordia of the various systems is shown in Figure 9. In embryos of the ethanol and of the ethanol + zinc & methionine groups, the circulatory, nervous, craniofacial and the musculoskeletal systems were significantly retarded ($p<0.001$) in development, compared to either the pair-fed control, or the ad libitum control, or to the zinc & methionine

supplemented group (Tables 36 to 39).

4.6 In Vitro S-Adenosyl Methionine Experiment

The results are summarized in Tables 40-44. The protein content of embryos (in µg), number of somites, crown-rump length, yolk sac diameter and morphological score were end points used to determine level of growth of embryos. One-way analysis of variance and Chi-Square analysis were used to analyze the data. Embryos cultured with ethanol alone (Group II) revealed significant retardation ($p<0.01$) in growth compared to control embryos (Group I). The number of somites ($p<0.05$), protein content, crown-rump length, and yolk sac diameter ($p<0.01$) of Group II embryos were also retarded compared to those in embryos treated with ethanol and 0.05 mM S-adenosyl-L-methionine (Group IV). The protein content, crown-rump length and morphological score of Group IV embryos were not significantly different from those of the control group. The mean number of somites, crown-rump length, morphological score and protein content were 28.9±3.2, 2.7±0.2mm, 42.7±1.6 and 330.0±68µg, respectively, in Group I embryos; 22.4±3.5, 2.3±0.2mm, 37.8±3.5 and 193±54µg, respectively, in Group II and 25.3±3.1, 2.6±0.2mm, 39.8±1.6 and 297±45µg, respectively, in Group IV embryos (Table 40). Embryos after 48 hours of culture are shown in Figure 10.

Delays in the development of the cardiovascular system were observed in Group II embryos. The frequency of developmental retardation of the heart

and yolk sac circulation were 10% and 12.5% respectively in Group II, compared to 0% in either Group I or Group IV embryos. However, this delay was not significantly different (Table 41).

The frequency of developmental retardation of the nervous system was higher in Group II embryos compared to Group I or Group IV (Table 42).

The musculoskeletal system (number of somites and somite score) and craniofacial primordia (branchial bars and maxilla) were significantly delayed ($p<0.01$) in Group II embryos, compared to those of Group I (Tables 43 & 44). Musculoskeletal (somite score) and craniofacial (branchial bars) development was also significantly retarded in Group IV embryos.

There was no significant difference between Groups I and III or Group IV and Group V embryos in growth and development. The highest degree of growth and developmental retardation were observed in Group VI (ethanol + 1mM AdoMet) and Group VII (ethanol + 3mM AdoMet). Group VII embryos showed severe retardation in all of the growth and developmental endpoints.

Table 6. Dietary intakes of animals: day-20 zinc experiment.

Treatment groups	Energy intake (Kcal/day)	Zinc intake* (mg/day)	Ethanol intake (g/day)
Ad libitum control	86.5±3.4	0.65±0.02	-
Pair-fed control	71.7±1.5[a]	0.54±0.01[a]	-
Ethanol	71.4±1.7[a]	0.54±0.01[a]	3.70±0.01
Zinc control	72.2±0.8[a]	0.55±0.01[a]	-
Zinc-supplemented	72.8±0.4[a]	0.55±0.01[a]	-
Ethanol + zinc	71.5±2.1[a]	0.54±0.01[a]	3.70±0.01

Results are expressed as mean ± SDM.

a = Results significantly different ($p<0.01$) from ad libitum control group. (ANOVA)

* Zinc intake is only from dietary source and does not include supplemental zinc.

Table 7. Pregnancy outcome following treatment with ethanol and zinc.

	Ad libitum control (n=10)	Pair-fed control (n=10)	Ethanol fed (n=10)	Zinc control (n=10)	Zinc supplemented (n=10)	Ethanol + zinc (n = 10)
Number of fetuses	139	140	129	137	142	140
Maternal weight gain (g):						
a) Days 6-12	10.7±3.4	6.5±3.1	-1.7±5.9 [a,b,d]	6.7±3.1	7.1±2.3	2.6±4.65 [a,d]
b) Days 6-20	127.3±11.9	111.7±8.6[a]	108.7±12.4 [a,d]	112±10.5	121.5±9.2	113.2±10.0 [a]
Implantation sites/dams	14.3±0.6	14.6±1.3	14.7±1.5	13.9±1.5	14.6±0.8	15.1±1.0
Live fetuses/ litter	13.7±0.9 (98.5%)	13.5±1.1 (96.5%)	11.9±1.2 [a,b,c,d] (90.8%)	13.1±1.2 (95%)	13.8±0.9 (98.0%)	13.4±1.0 (95%)
Resorptions/ litter	0.4±0.7 (2.8%)	0.6±0.8 (4.1%)	1.8±0.9 [a,b,d] (12.2%)	0.5±0.7 (3.8%)	0.4±0.9 (2.8%)	1.1±0.7 (7.3%)
Dead + resorbed fetuses/litter	0.6±0.7 (5%)	1.1±1.0 (7.9%)	2.8±0.8 [a,b,c,d] (21.4%)	0.9±1.0 (7%)	0.9±1.0 (5.6%)	1.7±0.9 [a,d] (12%)

Results are summarized as mean ± SDM

a = significantly different ($p<0.05$) from ad libitum control group.
b = significantly different ($p<0.05$) from pair-fed control group.
c = significantly different ($p<0.05$) from ethanol + zinc group.
d = significantly different ($p<0.05$) from zinc supplemented group.
(ANOVA)

Table 8. Growth of rat fetuses following treatment with ethanol and zinc.

	Ad libitum control (n=10)	Pair-fed control (n=10)	Ethanol fed (n=10)	Zinc control (n=10)	Zinc supplemented (n=10)	Ethanol + zinc (n = 10)
Litter wt. (g)	24.1±1.4	23.5±0.9	21.7±1.4[a]	23.5±1.5	23.9±1.4	23.2±0.5[b]
Crown-rump length/litter (cm)	28.5±0.8	27.8±0.4	26.5±0.9[a]	27.71±0.3	27.8±0.6	27.3±0.4[b]
Placental wt. (g)	4.1±0.1	4.2±0.1	5.1±0.1[a]	4.2±0.1	4.1±0.1	4.4±0.1[c]

Results are summarized as mean ± SDM

a = significantly different ($p<0.01$) from ad libitum and pair-fed control groups, ethanol + zinc and zinc supplemented groups.
b = significantly different ($p<0.01$) from the ad libitum control group.
c = significantly different ($p<0.01$) from the zinc supplemented, ad libitum and pair-fed control groups.
(ANOVA)

Table 9. Developmental anomalies in rat fetuses after ethanol and zinc treatment.

	Ad libitum control (n=10)	Pair-fed control (n=10)	Ethanol fed (n=10)	Zinc control (n=10)	Zinc supplemented (n=10)	Ethanol + zinc (n = 10)
No. of fetuses examined	100	100	100	100	100	100
Internal anomalies/litter	0.4±0.5	1.3±1.0	6.7±1.6[a]	1.0±0.7	0.8±0.8	5.1±1.7[b]
External anomalies/litter	0	0	0.9±0.9[b]	0	0	0.6±1.3[b]
Total anomalies/litter	0.4±0.5	1.3±0.9	7.6±1.7[a]	1.0±0.7	0.8±0.8	5.7±2.2[b]

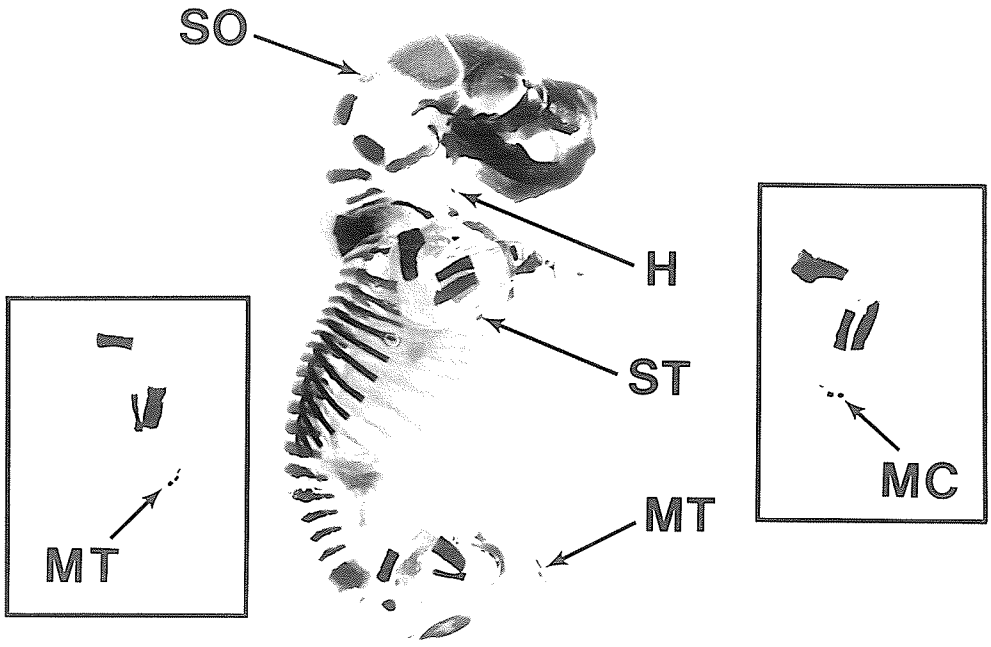
All results are expressed as mean ± SDM

a = significantly different ($p<0.01$) from ad libitum, pair-fed, zinc supplemented and ethanol+ zinc groups.
b = significantly different ($p<0.01$) from ad libitum, pair-fed and zinc supplemented.
(ANOVA)

Figure 2. Ossification centres in 20 days old rat fetuses.

A. A fetus of a pair-fed control animal showing the supraoxipital (SO), hyoid (H), and sternebrae (S) ossification centres. Inserts show metacarpal (MC) and metatarsal (MT) ossification centres.

B. A fetus of an ethanol treated animal.



A.

B.

Table 10. Fetal skeletal development after treatment of pregnant rats with ethanol and zinc.

Treatment Groups	Sternum* (%)	Metacarpus (%)**	Metatarsus (%)**	Thoracic centra*+ (%)	Hyoid + (%)
Ad libitum control (n=22)	18	41	22	5	91
Pair-fed control (n=25)	32	56	52	28	76
Ethanol (n=24)	58[a]	91[a]	83[a]	67[a]	58[a]
Zinc control (n=21)	28	57	47	33	72
Zinc supple-mented (n=22)	14	50	36	5	86
Ethanol + zinc (n=25)	40[b]	76[a]	76[a]	60[a]	64[a]

Results are summarized as:

* - fetuses having <2 sternal ossification centres
** - fetuses having <3 ossified metacarpal or metatarsal centres
*+ - fetuses having <12 thoracic centra
\+ - fetuses showing ossified hyoid
n - number of fetuses
a - significantly different ($p<0.05$) from ad libitum control, pair-fed control and zinc supplemented groups
b - significantly different ($p<0.05$) from ad libitum control and zinc supplemented groups
(CHI-SQUARE)

Table 11. Serum ethanol and zinc levels.

	Pair-fed control (n=10)	Ethanol fed (n=10)	Ethanol + zinc (n = 10)
Ethanol (mMol/l)	-	11.7±7.9	5.5±2.9[a]
Zinc (µMol/l)	21.9±5.5	16.9±3.8[b]	118±42.6[c]
Copper (µMol/l)	26.8±3	38.3±8[b]	35.6±14

Results are expressed as mean ± SDM

a = significantly different ($p<0.05$) from ethanol fed group.
b = significantly different ($p<0.05$) from pair-fed group.
c = significantly different ($p<0.05$) from ethanol-fed and pair-fed control groups. (ANOVA)

Figure 3. Placental sections (x 168) showing the basal zone (BZ), the labyrinthine zone (LZ) and the basal-labyrinthine junction (arrow).

A. Placenta of a pair-fed control animal.

B. Placenta of an ethanol treated animal showing intervillous spaces in the labyrinthine zone (LZ) and in the basal-labyrinthine junction (arrows) more filled with maternal blood.

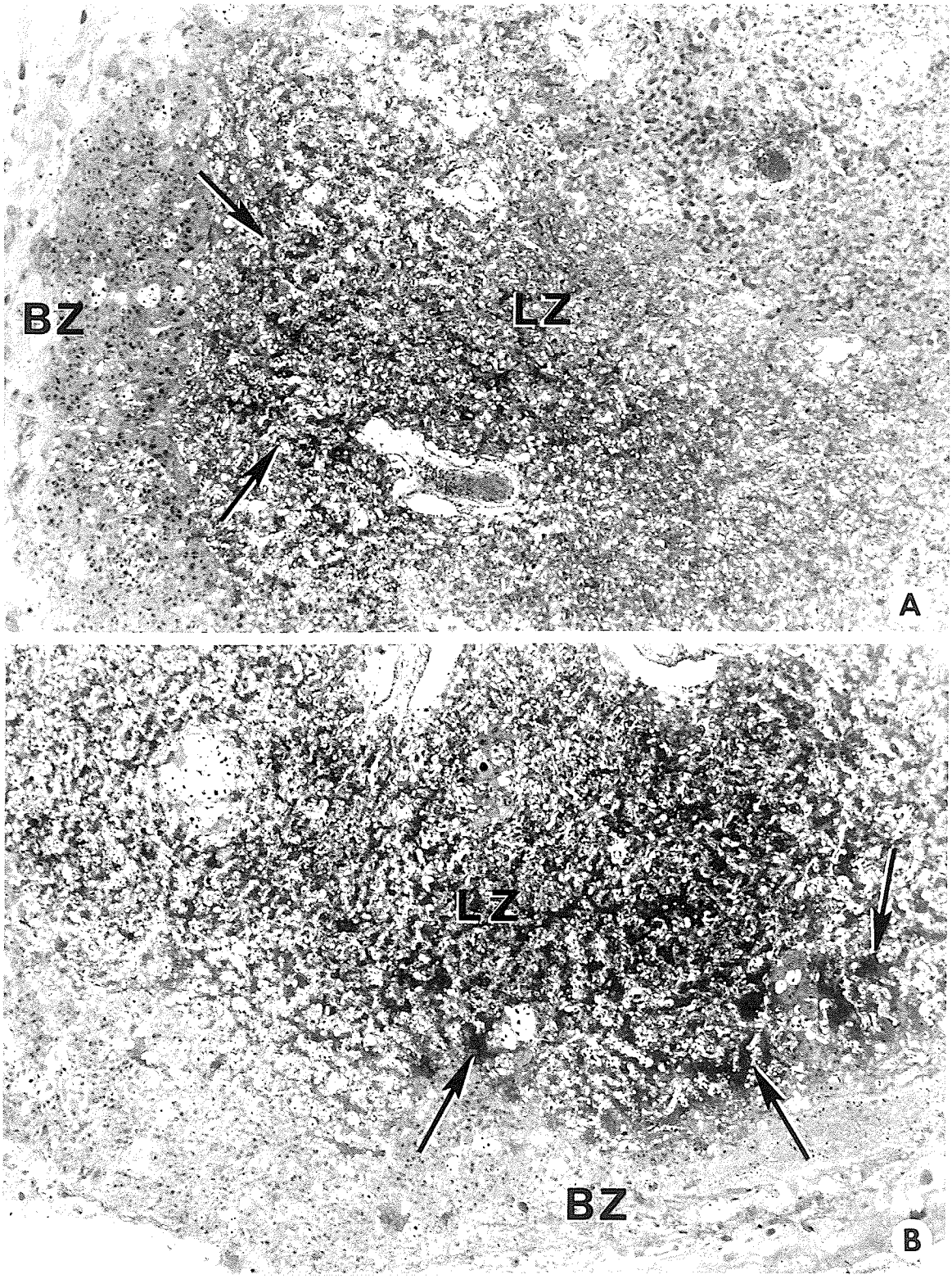

BZ
LZ
A
LZ
BZ
B

Figure 4. Placental sections showing the labyrithine zone (x 420).

A. Placenta of a pair-fed control animal showing chorionic villi (CV) and intervillous spaces (IV).

B. Placenta of an ethanol treated animal showing increased stagnating blood and blood corpuscles in the intervillous spaces (arrow). Intervillous spaces (IV) also appear dilated.

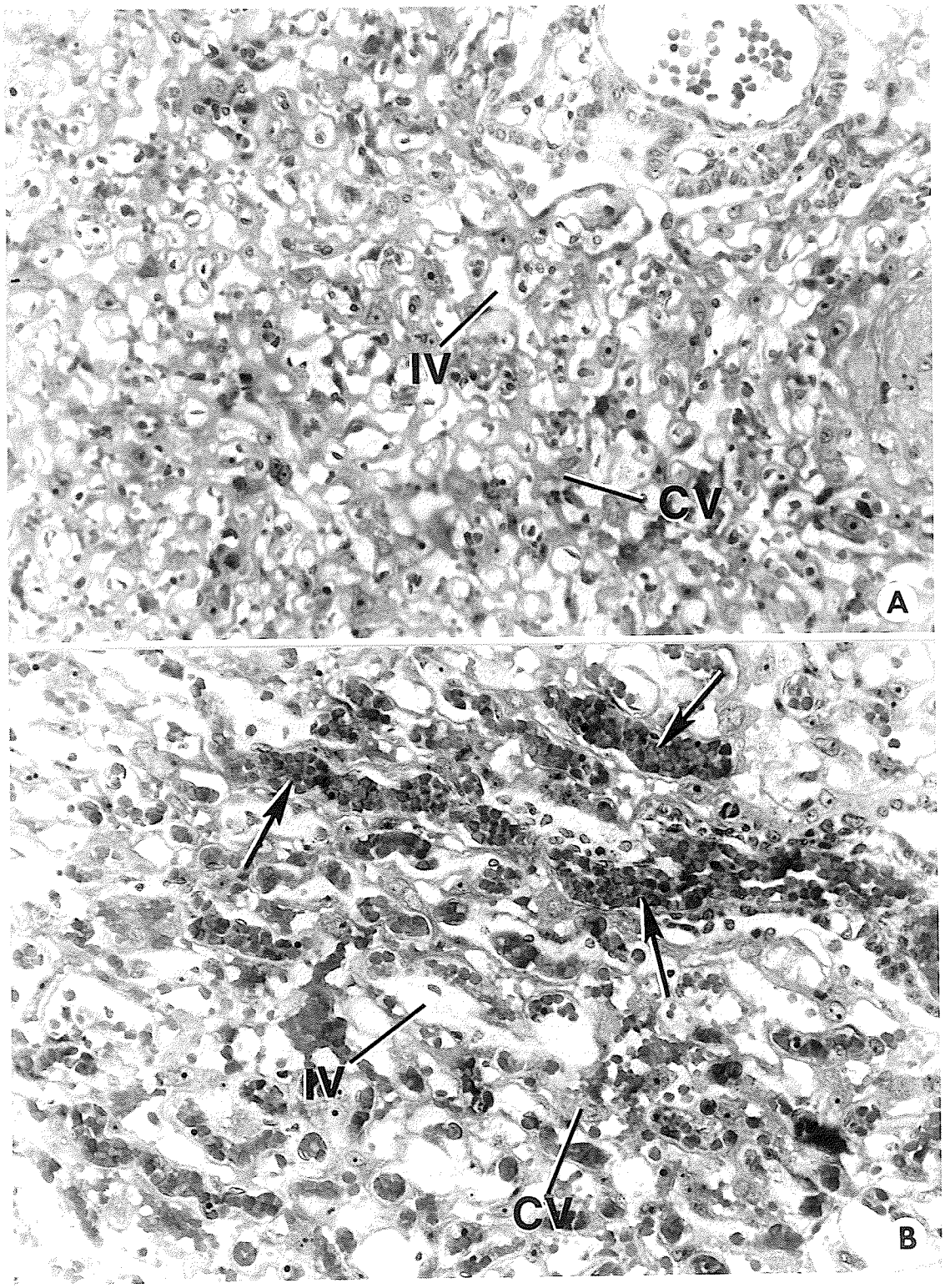

IV
CV
A
IV
CV
B

Figure 5. Placenta of an ethanol plus zinc treated animal showing the labyrinthine zone with abundant chorionic villi (CV). The size of the intervillous space (IV) and the maternal blood (arrow) in it appeared to be reduced, compared to only ethanol treatment (x 420).

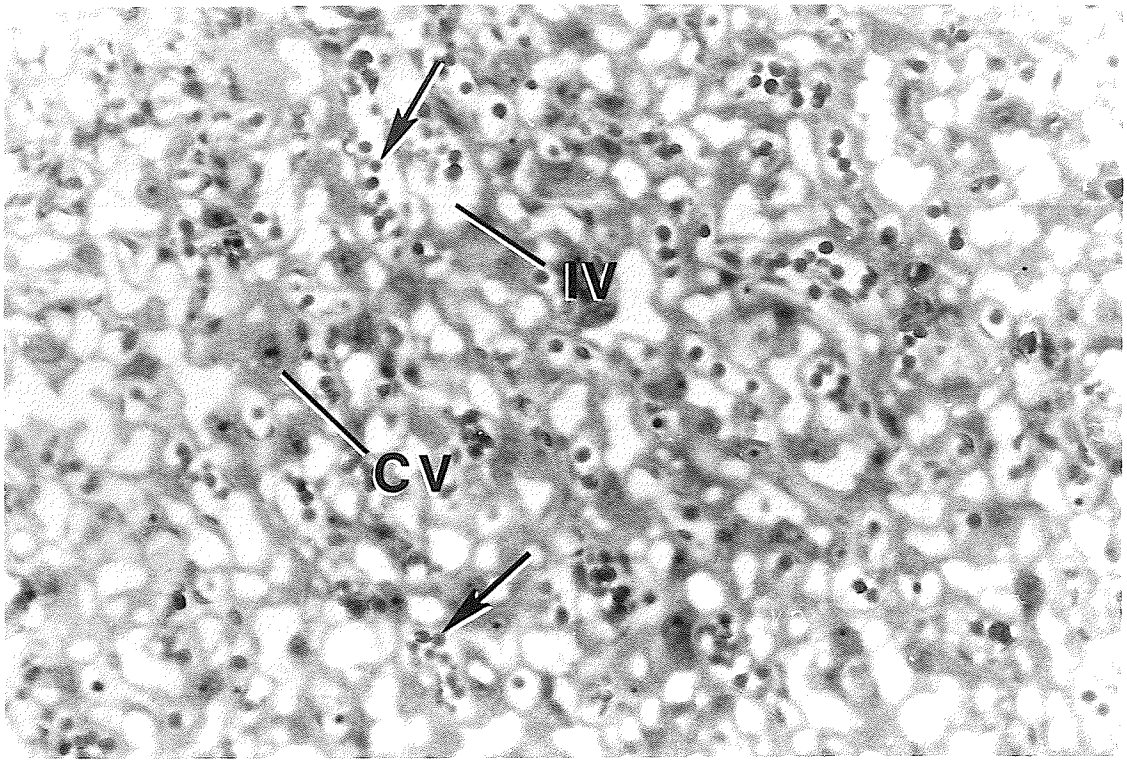

LV
CV

Figure 6. Placental sections showing the basal zone with decidua basalis (DB), giant cells (JC), trophoblast (TC) and glycogen cells (GC) (x 420).

A. Placenta of a pair-fed control animal.

B. Placenta of an ethanol treated animal: the basal-labyrinthine junction is more filled with maternal blood (arrows).

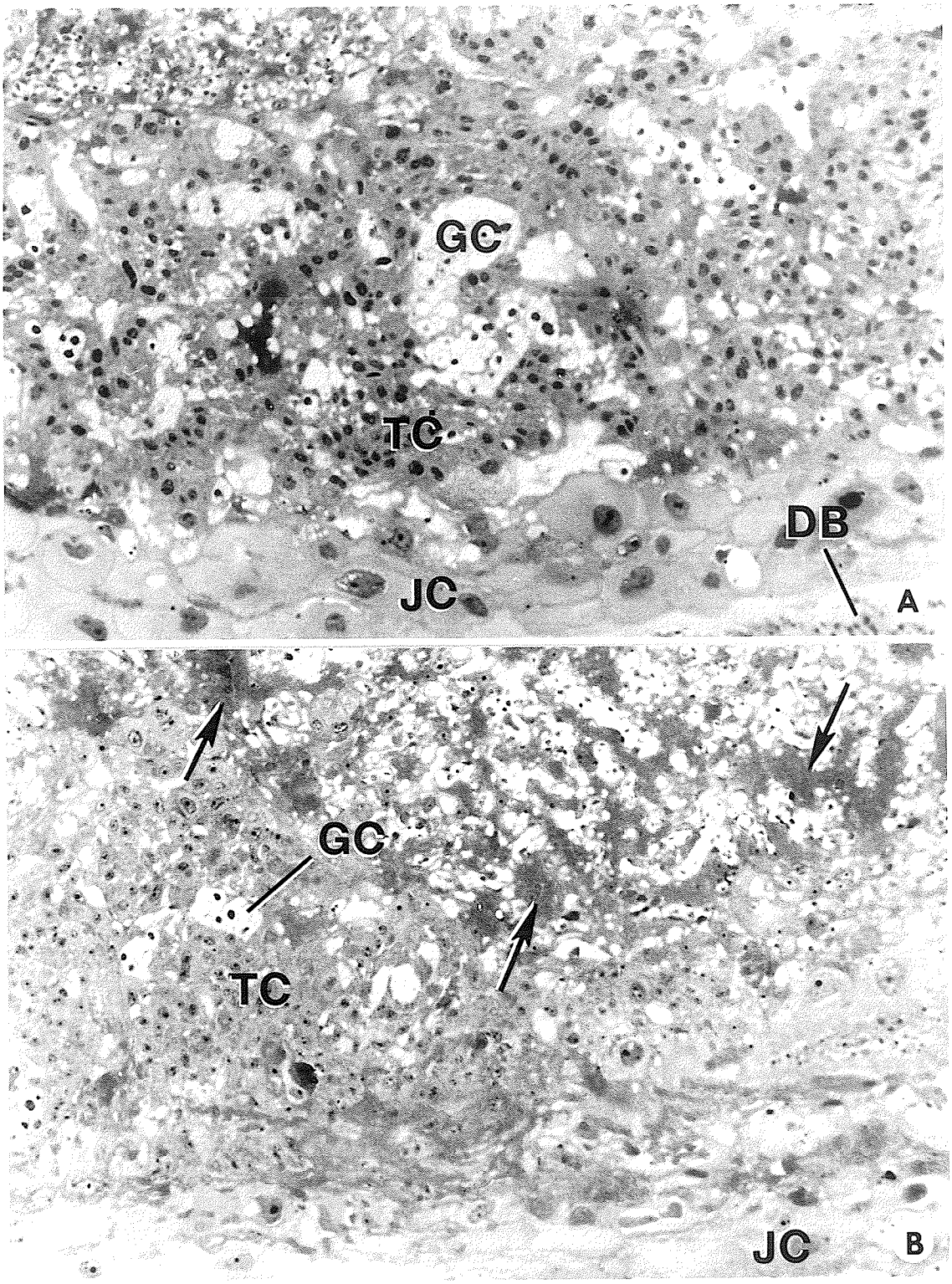

GC
TC
DB
JC
A
GC
TC
JC
B

Figure 7. Placental sections showing the basal zone (x 840).

A. Placenta of a pair-fed control animal.

B. Placenta of an ethanol treated animal showing decidua basalis (DB), trophoblast, and larger giant cells (JC).

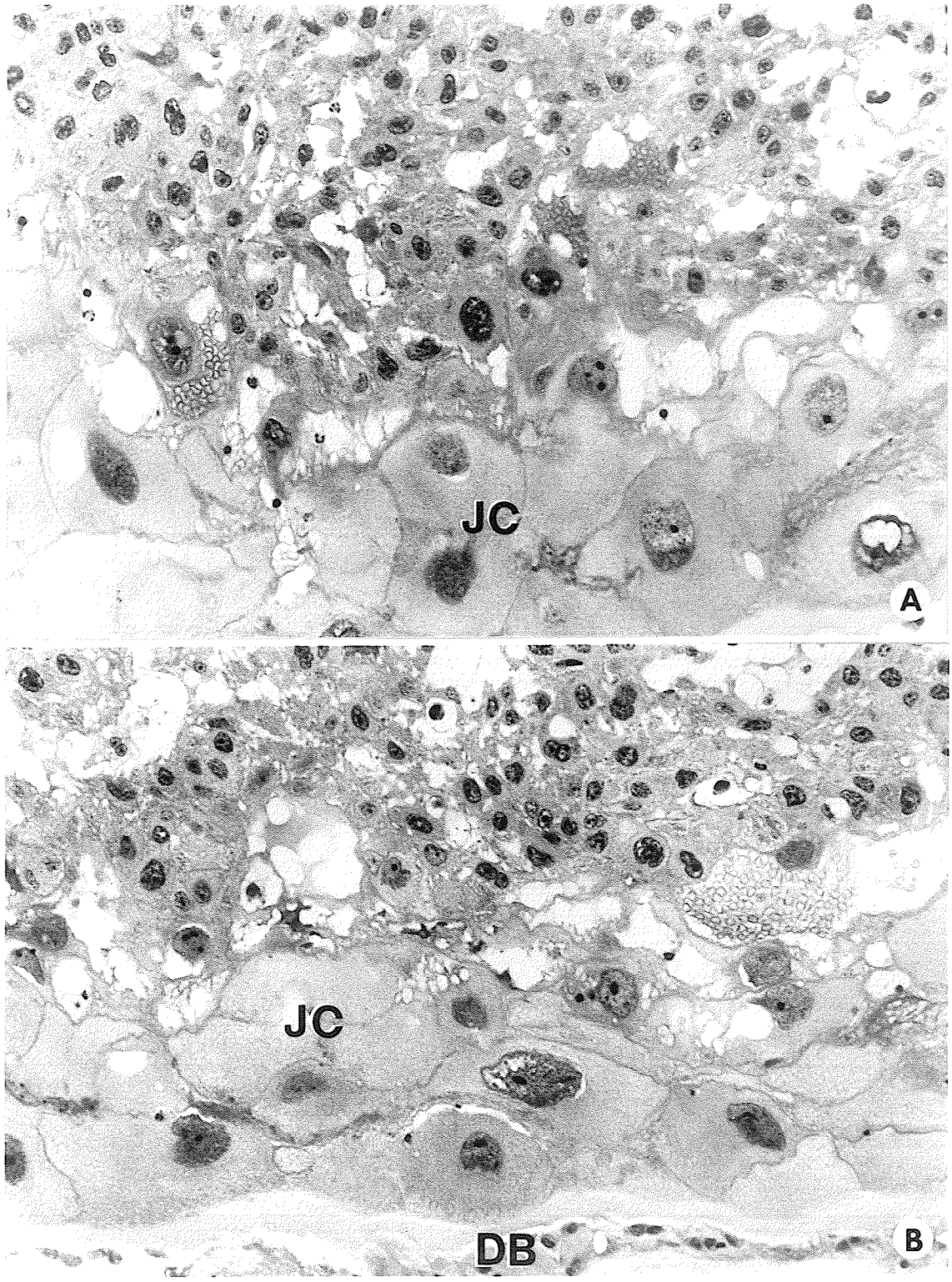

JC
A
JC
DB
B

Figure 8. Placental sections showing the basal-zone with decidua basalis (DB), giant cells (JC), trophoblast (TC) and glycogen cells (GC) (x 420).

A. Placenta of a pair-fed control animal.

B. Placenta of an ethanol treated animal showing maternal blood and blood corpuscles at the junction of the giant cell (JC) and trophoblast cell layers (arrows). Note also lots of blood corpuscles, probably degenerating erythrocytes, in the cytoplasm of the giant cells.

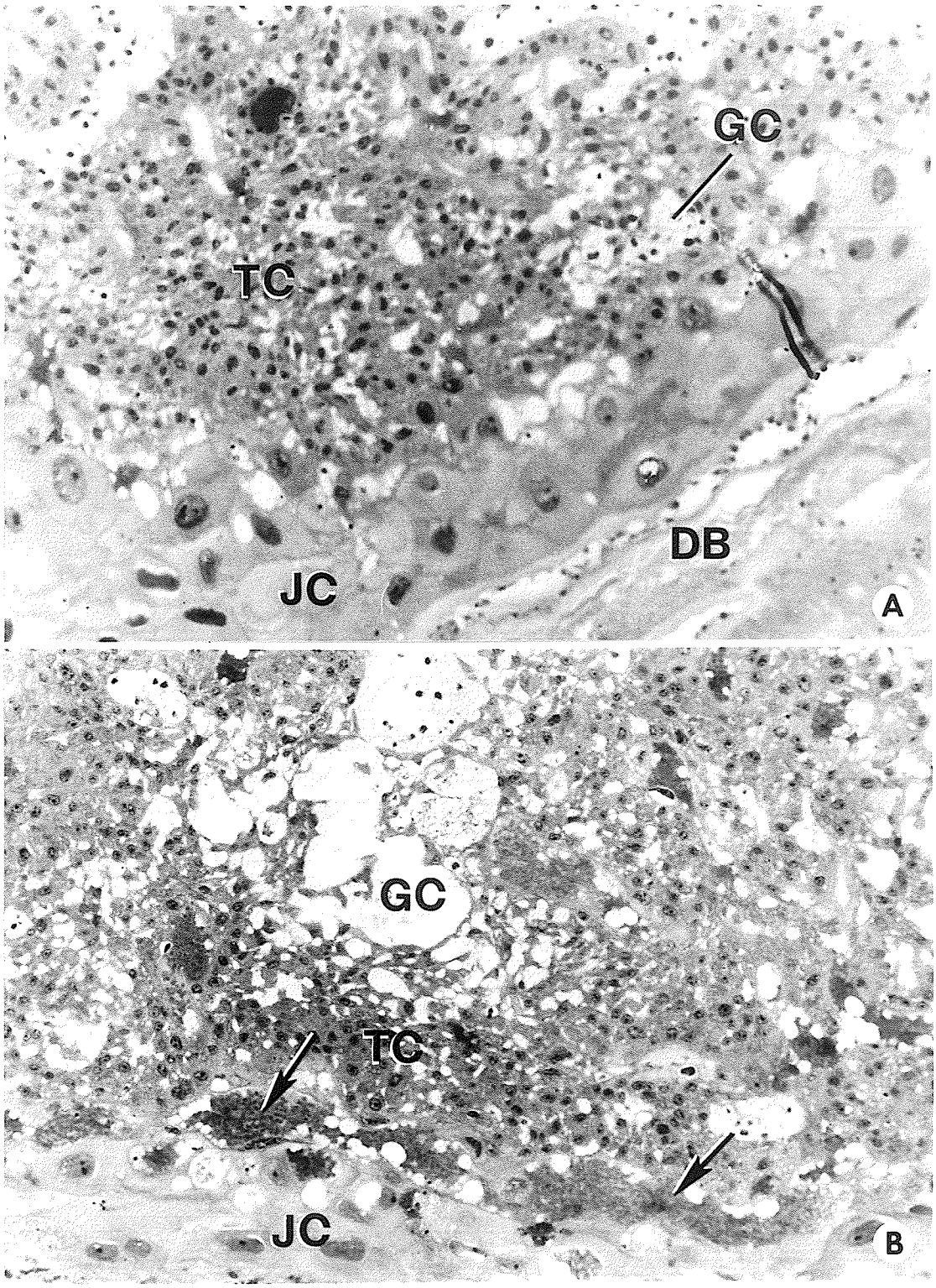

GC
TC
DB
JC
A
GC
TC
JC
B

Table 12. Pregnancy outcome following treatment of pregnant rats with ethanol and zinc: day-12 experiment.

Treatment Groups	Maternal weight gain (g)	Implantation sites/litter	Resorptions/ litter
Ad libitum control	11.3±4.0	15.4±1.0	0.6±0.8
Pair-fed control	6.4±4.6	15.7±1.5	0.9±0.8
Ethanol	-2.3±5.8[a]	16.2±1.5	2.6±0.6[a]
Zinc-supplemented	6.9±2.9	15.5±1.3	0.9±0.9
Ethanol + zinc	1.2±5.0[a]	15.0±1.6	2.1±0.7[a]

Results are summarized as mean ±SDM.

a = results significantly different ($p<0.05$) from ad libitum control, pair-fed control, and zinc-supplemented groups.
(ANOVA)

Table 13. Embryonic growth following treatment with ethanol and zinc.

Treatment Groups	Protein* content (µg)	Number of somites	Crown-rump length (mm)	Morphological score
Ad libitum control	289±49	29.7±2.0	4.5±0.4	42.4±1.4
Pair-fed control	276±71	29.1±2.2	4.3±0.4	41.4±2.0
Ethanol	186±44[c]	22.8±2.9[a,b]	3.3±0.3[a,c]	38.5±1.5[a]
Zinc-supplemented	297±58	29.4±2.4	4.2±0.3	41.8±1.2
Ethanol + zinc	237±127	24.1±2.6[a]	3.4±0.4[a,c]	39.0±1.7[a]

Results are summarized as mean ± SDM.

a = significantly different ($p<.001$) from ad libitum control, pair-fed control and zinc supplemented group.
b = significantly different ($p<0.01$) from ethanol + zinc group.
c = significantly different ($p<0.05$) from ad libitum control, pair-fed control and zinc supplemented groups.
(*-ANOVA; GLM)

Figure 9. Twelve days old rat embryos showing various organ primordia (Brown and Fabro, 1981).

A. An embryo enclosed in the yolk sac, and yolk sac circulation showing vitalline artery (VA) and vitalline vein (VV).

B. With the yolk sac removed, the embryo shows telencephalon (TL), Olfactory plate (OL), forelimb (FL).

C. An embryo showing optic primordia (OP), mesencephalon (ME), rhombencephalon (RE) and dorsal recess of otocyst (DR).

D. An embryo showing otocyst (OC), atrium commune (AC), ventriculus communis (VC), and somite (SO).

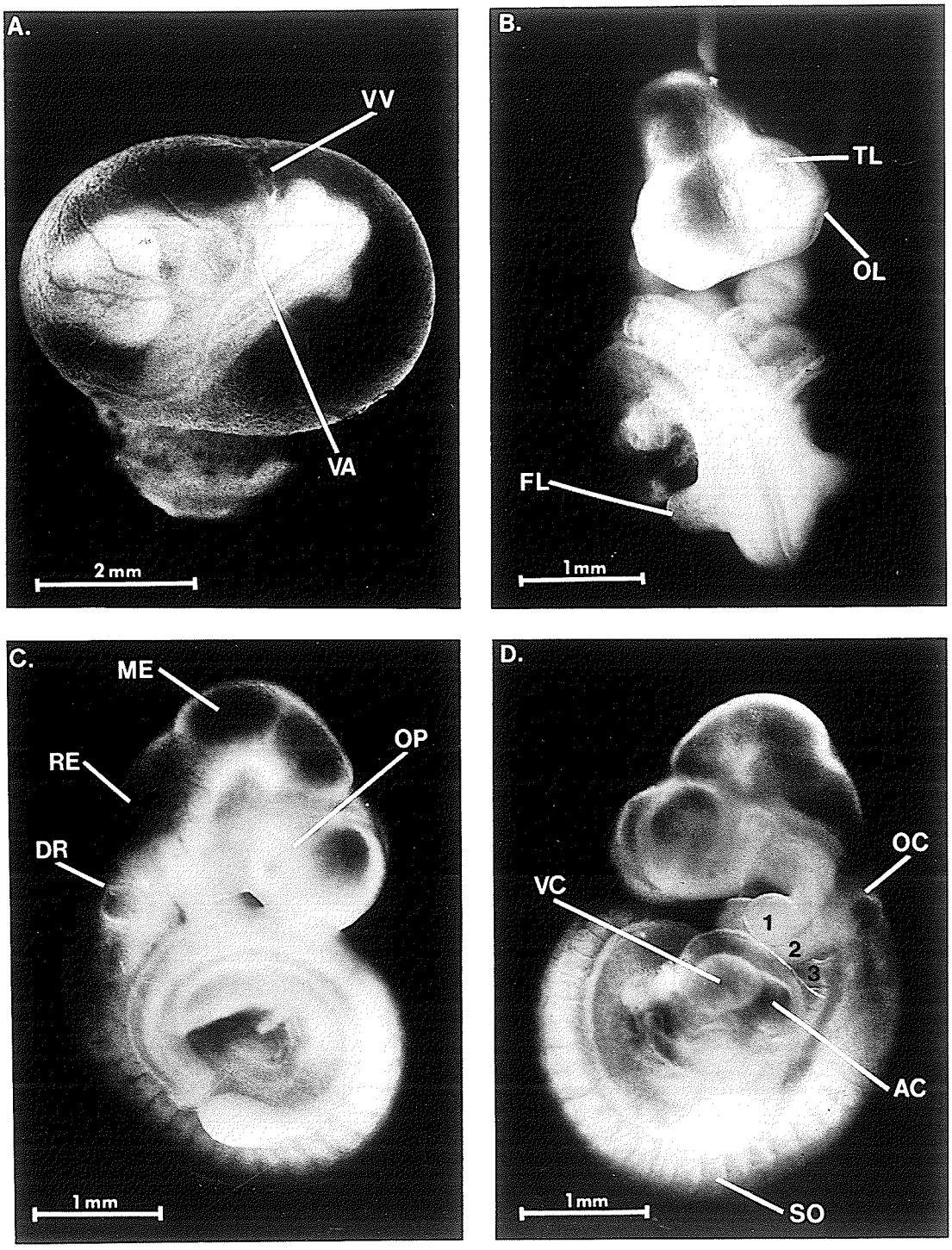

A.
VV
VA
2 mm
B.
TL
OL
FL
1 mm
C.
ME
OP
RE
DR
1 mm
D.
OC
VC
1
2
3
AC
1 mm
SO

Table 14. Embryonic circulatory and auditory systems development after treatment with ethanol and zinc.

	% Retarded Development		
Treatment Group	Yolksac circulation	Heart	Otic system
Ad libitum control	0	0	12
Pair-fed control	0	0	15
Ethanol	8[a]	1	12
Zinc-supplemented	0	0	14
Ethanol + zinc	0	0	19

Results are summarized as percentages of retarded development

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control, zinc supplemented and ethanol + zinc groups.
(CHI-SQUARE)

Table 15. Embryonic nervous system development following treatment with ethanol and zinc.

Treatment Groups	% Retarded Development			
	Caudal-neural tube	Forebrain	Midbrain	Hindbrain
Ad libitum control	2	0	0	13
Pair-fed control	4	0	0	17
Ethanol	43[a]	16[a]	0	71[a]
Zinc-supplemented	2	0	0	15
Ethanol + zinc	45[a]	12[a]	2	68[a]

Results are summarized as percentages of retarded development.

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control and zinc supplemented groups.
(CHI-SQUARE)

Table 16. Embryonic craniofacial development after treatment with ethanol and zinc.

	% Retarded Development			
Treatment Groups	Optic system	Olfactory system	Branchial-bars	Maxillary process
Ad libitum control	2	26	16	0
Pair-fed control	6	33	18	0
Ethanol	17[a]	83[b]	34[a]	0
Zinc-supplemented	8	30	14	0
Ethanol + zinc	20[a]	88[b]	33[a]	0

Results are summarized as percentages of retarded development.

a = significantly different ($p<0.01$) from ad libitum control, pair-fed control and zinc-supplemented groups.
b = significantly different ($p<0.001$) from ad libitum control, pair-fed control, and zinc-supplemented groups.
(CHI-SQUARE)

Table 17. Embryonic musculoskeletal system development following treatment with ethanol and zinc.

	% Retarded Development		
Treatment Group	Number of somites*	Somite score	Forelimb
Ad libitum control	29.7±2.0	13	0
Pair-fed control	29.1±2.2	17	0
Ethanol	22.8±2.9[a,b]	100[a]	13[c]
Zinc-supplemented	29.4±2.4	16	0
Ethanol + zinc	24.1±2.6[a]	100[a]	8[c]

Results are expressed as mean ±SDM (*) (ANOVA) or as percentages of retarded development (CHI-SQUARE).

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control and zinc-supplemented groups.
b = significantly different ($p<0.01$) from ethanol + zinc group.
c = significantly different ($p<0.001$) from ad libitum control, pair-fed control and zinc-supplemented groups.

Table 18. Dietary intakes of animals: day-20 methionine experiment.

Treatment Groups	Calorie intake (Kcal/day)	Methionine intake* (mg/day)	Ethanol intake (g/day)	Serum ethanol (mMol/l)
Ad libitum control	86.5±3.4	26±1.6	-	-
Pair-fed control	71.7±1.5[a]	22±1.2[a]	-	-
Ethanol	71.4±1.7[a]	21±1.3[a]	3.7±0.01	11.7±7.9
Methionine control	72.0±1.1[a]	22±1.0[a]	-	-
Methionine-supplemented	72.3±1.0	22±1.0[a]	-	-
Ethanol + methionine	70.9±1.3[a]	21±1.1[a]	3.7±0.01	13.4±5.3

Results are summarized as mean ±SDM.

a = results significantly different ($p<0.01$) from the ad libitum control group. (ANOVA)

* Methionine intake refers to the amount obtained from the diet and does not include supplemental methionine.

Table 19. Pregnancy outcome following treatment with ethanol and methionine.

Parameters	Ad libitum control (n=10)	Pair-fed control (n=10)	Ethanol fed (n=10)	Methionine control (n=10)	Methionine supple-mented (n=10)	Ethanol + Methionine (n = 10)
Number of fetuses	139	140	129	136	144	127
Maternal weight gain (g):						
a) Days 6-12	10.7±3.4	6.5±3.1	-1.7±5.9 [a,b,c]	6.9±2.2	7.3±2.7	-1.0±6 [a,b]
b) Days 6-20	127.3±11.9	111.7±8.6[a]	108.7±12.4 [a]	119±5.6	114.4±7.7	111.3±16.2[a]
Implantation sites/dams	14.3±0.6	14.6±1.3	14.7±1.5	14.1±1.5	14.9±1.4	14.4±1.1
Live fetuses per litter	13.7±0.9	13.5±1.1	11.9±1.2 [a,b,c]	13.3±1.0	14.0±1.0	11.8±1.0[a,b,c]
Resorptions	0.4±0.7	0.6±0.8	1.8±0.9 [a,b,c]	0.5±0.5	0.5±0.7	1.7±1.0[a,b,c]
Dead + resorbed	0.6±0.7	1.1±1.0	2.8±0.8 [a,b,c]	0.8±0.8	0.9±1.0	2.6±1.0[a,b,c]

Results are summarized as mean ± SDM

a = significantly different ($p<0.05$) from ad libitum control group.
b = significantly different ($p<0.05$) from pair-fed control group.
c = significantly different ($p<0.05$) from methionine supplemented group.
(ANOVA)

Table 20. Growth of fetuses following treatment with ethanol and methionine.

Treatment Groups	Litter wt. (g)	Crown-rump length/litter (cm)	Placental wt./ litter (g)
Ad libitum control	24.1±1.4	28.5±0.8	4.1±0.1
Pair-fed control	23.5±0.9	27.8±0.4	4.2±0.1
Ethanol	21.7±1.4[a]	26.5±0.9[a]	5.1±0.1[a]
Methionine control	23.4±1.6	27.7±0.4	4.1±0.1
Methionine-supplemented	23.5±1.2	27.8±0.4	4.8±0.1
Ethanol + Methionine	21.8±1.0[a]	26.57±0.8[a]	5.0±0.1[a]

Results are summarized as mean ± SDM

a = Significantly different ($p<0.01$) from ad libitum and pair-fed controls and methionine control and non-methionine supplemented groups.
(ANOVA)

Table 21. Developmental anomalies in rat fetuses after ethanol and methionine treatment.

Treatment Groups	Number of fetuses	Internal anomalies (per litter)	External anomalies (per litter)	Total anomalies (per litter)
Ad libitum control	100	0.4±0.5	0	0.4±0.5
Pair-fed control	100	1.3±1.0	0	1.3±0.9
Ethanol	100	6.7±1.6[a]	0.9±0.9[a]	7.6±1.7[a]
Methionine control	96	0.9±0.9	0	0.9±0.9[a]
Methionine-supplemented	98	0.9±0.6	0	0.9±0.6
Ethanol + methionine	100	6.4±1.8[a]	0.8±0.9[a]	7.2±1.5[a]

Results are expressed as mean ± SDM

a = Significantly different ($p<0.01$) from ad libitum and pair-fed controls and methionine supplemented group.
(ANOVA)

Table 22. Fetal skeletal development after treatment of pregnant rats with ethanol and methionine.

Treatment Groups	Sternum* (%)	Metacarpus (%)**	Metatarsus (%)**	Thoracic centra*+ (%)	Hyoid + (%)
Ad libitum control (n=22)	18	41	22	5	91
Pair-fed control (n=25)	32	56	52	28	76
Ethanol (n=24)	58[a]	91[a]	83[a]	67[a]	58[a]
Methionine control (n=22)	31	59	54	32	73
Methionine supple-mented (n=23)	22	57	48	22	74
Ethanol + methionine (n=25)	44[b]	88[a]	80[a]	56[a]	56[a]

Results are summarized as:

* - fetuses having <2 sternal ossification centres
** - fetuses having <3 ossified metacarpal or metatarsal centres
*+ - fetuses having <12 thoracic centra
\+ - fetuses showing ossified hyoid
n - number of fetuses
a - significantly different ($p<0.05$) from ad libitum control, pair-fed control and methionine supplemented groups
b - significantly different ($p<0.05$) from ad libitum control and methionine supplemented groups
(CHI-SQUARE)

Table 23. Pregnancy outcome following treatment of pregnant rats with ethanol and methionine: day-12 experiment.

Treatment Groups	Maternal weight gain (g)	Implantation sites/litter	Resorptions/ litter
Ad libitum control	11.3±4.0	15.4±1.0	0.6±0.8
Pair-fed control	6.4±4.6	15.7±1.5	0.9±0.8
Ethanol	-2.3±5.8[a]	16.2±1.5	2.6±0.6[a]
Methionine-supplemented	7.1±4.5	16.0±1.3	1.2±0.6
Ethanol + methionine	0.4±5.3[a]	15.8±1.1	2.8±0.9[a]

Results are summarized as mean ±SDM.

a = results significantly different ($p<0.05$) from the ad libitum control, pair-fed control and the methionine-supplemented groups.
(ANOVA)

Table 24. Embryonic growth after treatment with ethanol and methionine.

Treatment Groups	Protein* content (μg)	Number of somites	Crown-rump length (mm)	Morphological score
Ad libitum control	289±49	29.7±2.0	4.5±0.4	42.4±1.4
Pair-fed control	276±71	29.1±2.2	4.3±0.4	41.4±2.0
Ethanol	186±44[b]	22.8±2.9[a]	3.1±0.3	38.5±1.5[a]
Methionine-supplemented	307±53	28.8±2.0	4.0±0.3	41.6±1.8
Ethanol + methionine	205±51[c]	22.4±2.3[a]	3.3±0.3	38.7±1.2[a]

Results are summarized as mean ±SDM.

a. significantly different ($p<0.001$) from ad libitum control, pair-fed control and methionine supplemented group.
b. significantly different ($p<0.05$) from ad libitum control, pair-fed control, and methionine supplemented groups.
c. significantly different ($p<0.05$) from the methionine supplemented group.
(* ANOVA; GLM)

Table 25. Embryonic circulatory and auditory systems development after treatment with ethanol and methionine.

	% Retarded Development		
Treatment Group	Yolk sac circulation	Heart	Otic system
Ad libitum control	0	0	12
Pair-fed control	0	0	15
Ethanol	8[a]	1	12
Methionine-supplemented	0	0	13
Ethanol + methionine	5	6[a]	22

Results are summarized as percentages of retarded development.

a = significantly different ($p<0.01$) from all the other groups. (CHI-SQUARE)

Table 26. Embryonic nervous system development following treatment with ethanol and methionine.

	% Retarded Development			
Treatment Groups	Caudal-neural tube	Forebrain	Midbrain	Hindbrain
Ad libitum control	2	0	0	13
Pair-fed control	4	0	0	17
Ethanol	43[a]	16[a]	0	71[a]
Methionine-supplemented	6	0	0	22
Ethanol + methionine	51[a]	14[a]	0	77[a]

Results are summarized as percentages of retarded development.

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control, and methionine supplemented groups.
(CHI-SQUARE)

Table 27. Embryonic craniofacial development after treatment of pregnant rats with ethanol and methionine.

	% Retarded Development			
Treatment Groups	Optic system	Olfactory system	Branchial-bars	Maxillary process
Ad libitum control	2	26	16	0
Pair-fed control	6	33	18	0
Ethanol	17[a]	83[b]	34[a]	0
Methionine-supplemented	6	35	15	0
Ethanol + methionine	23[a]	86[b]	35[a]	0

Results are summarized as percentages of retarded development.

a = significantly different ($p<0.01$) from ad libitum control, pair-fed control, and methionine-supplemented groups.
b = significantly different ($p<0.001$) from ad libitum control, pair-fed control, and methionine-supplemented groups.
(CHI-SQUARE)

Table 28. Embryonic musculoskeletal system development following treatment of pregnant rats with ethanol and methionine.

	% Retarded Development		
Treatment Group	Somite* number	Somite score	Forelimb
Ad libitum control	29.7±2.0	13	0
Pair-fed control	29.1±2.2	17	0
Ethanol	22.8±2.9[a]	100[a]	13[b]
Methionine-supplemented	28.8±2.5	19	0
Ethanol + methionine	22.4±2.3[a]	100[a]	12[b]

Results are expressed as mean ±SDM (*) (ANOVA) or as percentages of retarded development (CHI-SQUARE).

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control, and methionine-supplemented groups.
b = significantly different ($p<0.001$) from ad libitum control, pair-fed control, and methionine-supplemented groups.

Table 29. Dietary intakes of animals: day-20 zinc and methionine experiment.

Treatment Groups	Calorie intake (Kcal/day)	Zinc intake (mg/day)	Methionine intake (mg/day)	Ethanol intake (g/day)
Ad libitum control	86.5±3.4[a]	0.65±0.02[a]	26±1.6[a]	-
Pair-fed control	71.7±1.5	0.54±0.01	22±1.2	-
Ethanol	71.4±1.7	0.54±0.01	21±1.3	3.7±0.01
Zinc & methionine-supplemented	72.2±1.0	0.55±0.01	22±1.0	-
Ethanol + zinc & methionine	71.0±1.5	0.54±0.01	21±1.0	3.7±0.01

Results are summarized as mean ±SDM.

a = results significantly different ($p<0.01$) from all the other groups. (ANOVA)

Table 30. Pregnancy outcome following treatment with ethanol plus zinc and methionine.

Parameters	Ad libitum control (n=10)	Pair-fed control (n=10)	Ethanol fed (n=10)	Zinc & methionine supplemented (n=10)	Ethanol + zinc & methionine (n = 10)
Number of fetuses	139	140	129	141	134
Maternal weight gain (g):					
a) Days 6-12	10.7±3.4	6.5±3.1	-1.7±5.9 [a,b,c]	6.8±3.3	3.6±9.0[a]
b) Days 6-20	127.3±11.9	111.7±8.6[a]	108.7±12.4 [a,c]	118±8.0	114.3±15[a]
Implantation sites/dams	14.3±0.6	14.6±1.3	14.7±1.5	14.5±0.8	14.9±1.7
Live fetuses per litter	13.7±0.9	13.5±1.1	11.9±1.2 [a,b,c]	13.8±0.7	12.6±1.3[c]
Resorptions	0.4±0.7	0.6±0.8	1.8±0.9 [a,b,c]	0.4±0.7	1.5±1.0[a,b,c]
Dead + resorbed	0.6±0.7	1.1±1.0	2.8±0.8 [a,b,c]	0.7±0.7	2.3±0.8[a,b,c]

Results are expressed as mean ± SDM

a = significantly different ($p<0.05$) from ad libitum control group.
b = significantly different ($p<0.05$) from pair-fed control group.
c = significantly different ($p<0.05$) from zinc and methionine supplemented group.
(ANOVA)

Table 31. Growth of fetuses following treatment with ethanol plus zinc and methionine.

Treatment Groups	Litter Wt. (g)	Crown-rump length/litter (Cm)	Placental wt. (g)
Ad libitum control	24.1±1.4	28.5±0.8	4.1±0.1
Pair-fed control	23.5±0.9	27.8±0.4	4.2±0.1
Ethanol	21.7±1.4[a]	26.5±0.9[a]	5.1±0.1[a]
Zinc & methionine-supplemented	23.7±1.1	27.7±1.0	4.2±0.2
Ethanol + zinc & methionine	22.0±1.3[a]	26.7±0.8[a]	5.3±0.2[a]

Results are summarized as mean ± SDM

a = Significantly different ($p<0.05$) from ad libitum control, pair-fed control and zinc & methionine supplemented groups.
(ANOVA)

Table 32. Developmental anomalies in rat fetuses following treatment with ethanol plus zinc and methionine.

Treatment Groups	Number of fetuses	Internal anomalies (per litter)	External anomalies (per litter)	Total anomalies (per litter)
Ad libitum control	100	0.4±0.5	0	0.4±0.5
Pair-fed control	100	1.3±1.0	0	1.3±1.0
Ethanol	100	6.7±1.6[a]	0.9±0.9[a]	7.6±1.7[a]
Zinc and methionine-supplemented	98	0.8±0.9	0	0.8±0.9
Ethanol plus zinc and methionine	100	5.7±1.3[a]	0.7±0.8[b]	6.4±1.5[a]

Results are summarized as mean ± SDM

a = Significantly different ($p<0.01$) from ad libitum control, pair-fed control and zinc & methionine supplemented groups.
b = Significantly different ($p<0.05$).
(ANOVA)

Table 33. Fetal skeletal development after treatment of pregnant rats with ethanol, zinc, and methionine.

Treatment Groups	Sternum* (%)	Metacarpus (%)**	Metatarsus (%)**	Thoracic centra*+ (%)	Hyoid + (%)
Ad libitum control (n=22)	18	41	22	5	91
Pair-fed control (n=25)	32	56	52	28	76
Ethanol (n=24)	58[a]	91[a]	83[a]	67[a]	58[a]
Zinc & methionine supple-mented (n=23)	9	48	35	4	82
Ethanol + zinc + methionine (n=23)	39[b]	83[a]	78[a]	74[a]	61[a]

Results are summarized as:

* - fetuses having <2 sternal ossification centres
** - fetuses having <3 ossified metacarpal or metatarsal centres
*+ - fetuses having <12 thoracic centra
\+ - fetuses showing ossified hyoid
n - number of fetuses
a - significantly different ($p<0.05$) from ad libitum control, pair-fed control and zinc + methionine supplemented groups
b - significantly different ($p<0.05$) from ad libitum control and zinc + methionine supplemented groups (CHI-SQUARE)

Table 34. Pregnancy outcome following treatment of pregnant rats with ethanol, zinc and methionine.

Treatment Groups	Maternal weight gain (g)	Implantation sites/litter	Resorptions/ litter
Ad libitum control	11.3±4.0	15.4±1.0	0.6±0.8
Pair-fed control	6.4±4.6	15.7±1.5	0.9±0.8
Ethanol	-2.3±5.8[a]	16.2±1.5	2.6±0.6[a]
Zinc & methionine-supplemented	7.5±5.2	15.3±1.3	0.9±1.0
Ethanol + zinc & methionine	0.8±4.4[a]	15.5±1.1	2.4±0.5[a]

Results are summarized as mean ± SDM

a = results significantly different ($p<0.05$) from the ad libitum control, pair-fed control and the zinc & methionine supplemented groups.
(ANOVA)

Table 35. Embryonic growth following treatment of pregnant rats with ethanol, zinc and methionine.

Treatment Groups	Protein* content (μg)	Number of somites	Crown-rump length (mm)	Morphological score
Ad libitum control	289±49	29.7±2.0	4.5±0.4	42.4±1.4
Pair-fed control	276±71	29.1±2.2	4.3±0.4	41.4±2.0
Ethanol	186±44[b]	22.8±2.9[a]	3.3±0.3[a]	38.5±1.5[a]
Zinc & methionine-supplemented	284±88	28.3±2.3	4.3±0.3	41.2±1.8
Ethanol + zinc & methionine	235±110	23.3±2.4[a]	3.4±0.3[a]	39.2±1.6[a]

Results are summarized as mean ± SDM

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control, and zinc plus methionine supplemented groups.
b = significantly different ($p<0.05$) from ad libitum control, pair-fed control, and zinc plus methionine supplemented groups.
(GLM, and * ANOVA)

Table 36. Embryonic circulatory and auditory systems development after treatment of pregnant rats with ethanol, zinc and methionine.

	% Retarded Development		
Treatment Group	Yolk sac circulation	Heart	Otic system
Ad libitum control	0	0	12
Pair-fed control	0	0	15
Ethanol	8[a]	1	12
Zinc & methionine-supplemented	0	0	16
Ethanol + zinc & methionine	0	0	22

Results are summarized as percentages of retarded development.

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control, and zinc plus methionine supplemented groups.
(CHI-SQUARE)

Table 37. Embryonic nervous system development following treatment of pregnant rats with ethanol, zinc and methionine.

	% Retarded Development			
Treatment Groups	Caudal-neural tube	Forebrain	Midbrain	Hindbrain
Ad libitum control	2	0	0	13
Pair-fed control	4	0	0	17
Ethanol	43[a]	16[a]	0	71[a]
Zinc & methionine-supplemented	6	0	0	15
Ethanol + zinc & methionine	40[a]	14[a]	0	70[a]

Results are summarized as percentages of abnormal development.

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control and zinc plus methionine supplemented groups.
(CHI-SQUARE)

Table 38. Embryonic craniofacial development after treatment of pregnant rats with ethanol, zinc and methionine.

	% Retarded Development			
Treatment Groups	Optic system	Olfactory system	Branchial-bars	Maxillary process
Ad libitum control	2	26	16	0
Pair-fed control	6	33	18	0
Ethanol	17[a]	83[a]	34[a]	0
Zinc & methionine-supplemented	4	29	13	0
Ethanol + zinc & methionine	16[a]	78[a]	30[a]	0

Results are summarized as percentages of retarded development.

a = significantly different ($p<0.01$) from ad libitum control, pair-fed control and zinc plus methionine supplemented groups.
(CHI-SQUARE)

Table 39. Embryonic musculoskeletal system development after treatment of pregnant rats with ethanol, zinc and methionine.

	% Retarded Development		
Treatment Group	Somite number*	Somite score**	Forelimb**
Ad libitum control	29.7±2.0	13	0
Pair-fed control	29.1±2.2	17	0
Ethanol	22.8±2.9[a]	76[a]	13[b]
Zinc & methionine-supplemented	29.8±2.8	14	0
Ethanol + zinc & methionine	23.0±2.6[a]	81[a]	10[b]

Results are summarized as mean ± SDM (*) or as percentages of retarded development (**).

a = significantly different ($p<0.001$) from ad libitum control, pair-fed control and zinc plus methionine supplemented groups.
b = significantly different ($p<0.001$) from ad libitum control, pair-fed control, and zinc plus methionine supplemented groups.

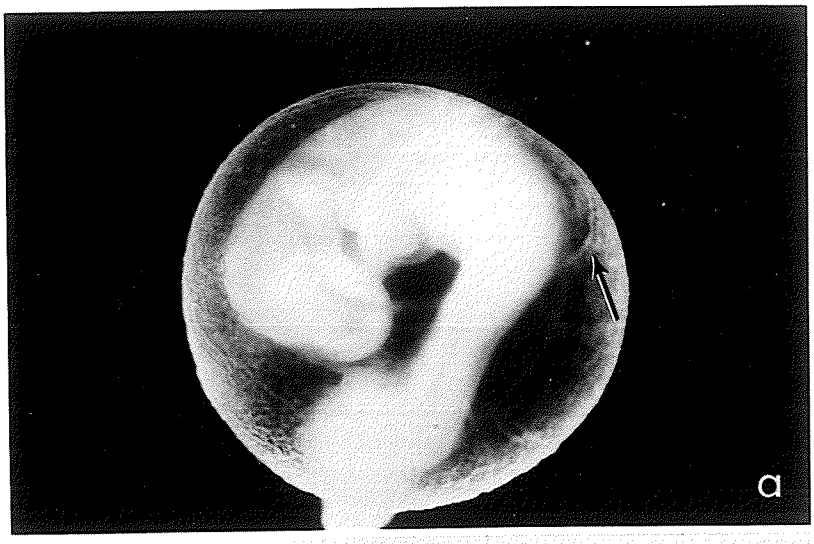
* GLM
** CHI-SQUARE

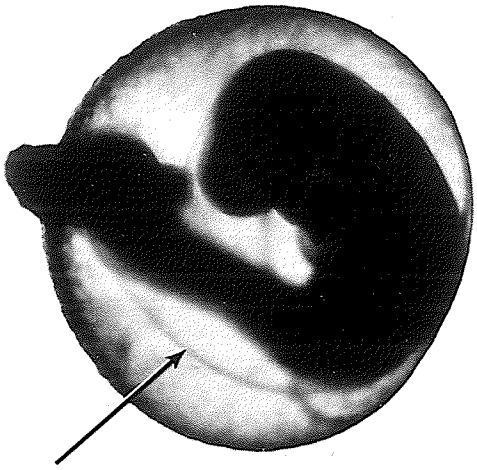
Figure 10. In vitro grown 12 days old rat embryos (x 16).

a. Embryo enclosed in the yolk sac. Vitalline blood vessels (arrow) shows yolk sac circulation.

b. Embryo enclosed in amniotic-membrane (arrow).

c. Embryo showing abnormal size and curvature.


a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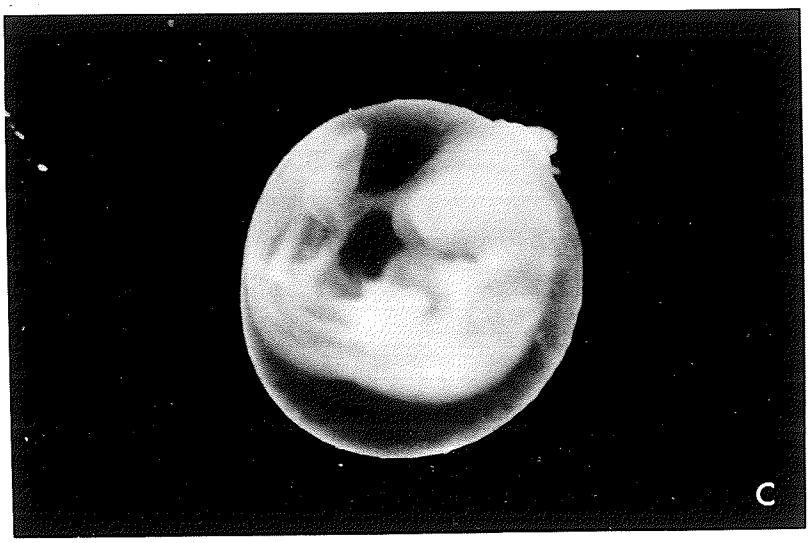
c

Table 40. Embryonic growth following treatment with ethanol and S-adenosyl methionine

		Embryological end-points			
Treatment Groups	Protein content (µg)	Number of somites	Crown-rump length (mm)	Yolk sac diameter (mm)	Morphological score
I (Saline)	330±68	28.93.2	3.8±0.3	4.6±0.5	42.7±1.6
II (Ethanol)	193±54[e]	22.4±3.5[a]	3.2±0.3[d]	3.8±0.4[a]	37.8±3.5[b]
III (0.05 mMol AdoMet)	-	27.2±3.1	3.6±0.3	4.3±0.3	40.8±2.6
IV (Ethanol + 0.05mM AdoMet)	297±45	25.3±3.1[b]	3.6±0.3	4.2±0.3	39.8±1.6
V (Ethanol + 0.1mM AdoMet)	-	24.5±3.3[b]	3.6±0.3	4.2±0.3	39.7±6.1
VI (Ethanol + 1mM AdoMet)	-	21.5±3.8[b]	3.1±0.4[b]	4.0±0.5[b]	35.1±6.1
VII (Ethanol + 3mM AdoMet)	-	15.4±1.4[c]	2.2±0.1[c]	3.3±0.3[c]	27.2±6.1[a]

a = significantly different from Group I, III ($p<0.01$) and Group IV ($p<0.05$).
b = significantly different ($p<0.01$) from Group I.
c = significantly different ($p<0.01$) compared to Groups I, II, III, IV, V and VI.
d = significantly different ($p<0.01$) from Groups I, III and IV.
e = significantly different ($p<0.01$) from Groups I and IV.
(ANOVA)

Table 41. Cardiovascular system development following treatment with ethanol and S-adenosyl methionine.

	% Retarded Development		
Treatment groups	Yolk sac circulation	Heart	Allantois
I (Saline)	0	0	0
II (Ethanol)	12.5	10	0
III (0.05 mMol AdoMet)	0	0	0
IV (Ethanol + 0.05mM AdoMet)	0	0	0
V (Ethanol + 0.1mM AdoMet)	0	0	0
VI (Ethanol + 1mM AdoMet)	13.3	33.3[a]	0
VII (Ethanol + 3mM AdoMet)	23.6[a]	35.3[a]	11.8[a]

a = significantly different ($p<0.01$) compared to Groups I, II, III, IV and V. (CHI-SQUARE)

Table 42. Nervous system development following treatment with ethanol and S-adenosyl methionine.

Treatment Groups	% Retarded development			
	Caudal neural tube	Hindbrain	Midbrain	Forebrain
I (Saline)	11.1	5.6	0	0
II (Ethanol)	26.7	13.3	13.5	6.7
III (0.05 mMol AdoMet)	16.6	8.3	0	0
IV (Ethanol + 0.05mM AdoMet)	18.8	12.5	0	0
V (Ethanol + 0.1mM AdoMet)	16.7	20.0	6.7	21.0[a]
VI (Ethanol + 1mM AdoMet)	40.0	40.0[a]	26.7[a]	64.7[a]
VII (Ethanol + 3mM AdoMet)	100[a]	100[a]	35.3[a]	

a = significantly different from Group I ($p<0.01$).
(CHI-SQUARE)

Table 43. Craniofacial development following treatment with ethanol and S-adenosyl methionine.

	% Retarded Development		
Treatment groups	Branchial bar	Maxilla	Mandible
I (Saline)	22.2	22.2	0
II (Ethanol)	53.3[a]	66.7	0
III (0.05 mMol AdoMet)	33.3	25.0	0
IV (Ethanol + 0.05mM AdoMet)	56.3[b]	68.8	0
V (Ethanol + 0.1mM AdoMet)	60.0[b]	60.0[b]	0
VI (Ethanol + 1mM AdoMet)	100[a]	46.7[a]	0
VII (Ethanol + 3mM AdoMet)	100[a]	70.6[a]	0

a = significantly different ($p<0.01$) from Group I and Group III.
b = significantly different ($p<0.05$) from Group I.
(CHI-SQUARE)

Table 44. Musculoskeletal system development following treatment with ethanol and S-adenosyl methionine.

Treatment Groups	No. of* somites	% Retarded development: Somite score	Forelimb	Hind-limb	Flexion
I (Saline)	28.9±3.2	22.2	5.6	0	0
II (Ethanol)	22.4±3.6[a]	93.3[a]	6.7	0	33.3[c]
III (0.05 mMol AdoMet)	27.7±3.1	33.3	8.2	0	16.6
				0	37.5
IV (Ethanol + 0.05mM AdoMet)	25.3±3.1[a]	81.2[a]	12.5	0	33.3
V (Ethanol + 0.1mM AdoMet)	24.5±3.3[a]	80.0[a]	20.0	0	46.7[a]
				0	100[b]
VI (Ethanol + 1mM AdoMet)	21.5±3.8[a]	100[a]	46.7[a]		
VII (Ethanol + 3mM AdoMet)	15.4±1.4[b]	100[a]	76.4[a]		

a = significantly different ($p<0.01$) from Group I and Group III.
b = significantly different ($p<0.01$) from Groups I, II, III, IV, V and VI.
c = significantly different ($p<0.05$) from Group I.
(* ANOVA; CHI-SQUARE)

5. DISCUSSION

5.0 Method of Ethanol Administration

The method of chronic administration of ethanol is a critical condition in the design of studies investigating the effects of ethanol. This is especially crucial in reproductive studies regarding the teratogenic potential of ethanol. Thus, the method of ethanol exposure in pregnant rats is considered as an essential element of any rodent model of FAS. The criteria for an ideal method of exposure include that the method should: allow modification of the amount of ethanol administered, be able to produce intoxicating blood ethanol levels, provide adequate nutrition and should allow for control of environmental factors such as variability in food intakes (Wiener et al., 1981). In reproductive studies, ethanol is usually administered by gastric intubation, incorporation of ethanol in liquid diets, or by placing it in the drinking water or by i.p. injection.

Gastric intubation is good for controlling dosage and time of exposure and for inducing higher blood ethanol levels. However, this method is unlike human usage and stressful for the animal. Moreover, it could cause gastric-irritation, reduction in flood and fluid intake and increased resorptions and abortions. Self-administration of ethanol by incorporating it in the drinking water results in a wide variability in ethanol consumption, low blood ethanol level, and a decreased food and fluid intake, which introduces maternal

nutrition as a confounding variable.

Administration of ethanol by way of incorporation in a liquid diet has proven to be a more efficient procedure (Chernoff, 1977; Randall et al., 1977; Lieber and DeCarli, 1982). Higher blood ethanol levels are achieved and strict pair-feeding of the experimental and control animals is possible. When the liquid diet is the sole source of food and fluid, a high daily ethanol intake of 12 to 18 g/kg and blood ethanol levels of 100 to 150 mg/100 ml are common (Lieber and DeCarli, 1982). In addition, the use of the liquid diet procedure allows for the control and measurement of the intake of various nutrients. Therefore, the Lieber-DeCarli (1982) liquid diet formulation was used in the present investigation.

5.1 Zinc Dosage

In the present study, pregnant rats were supplemented with zinc in order to investigate the influence of zinc supplementation on the embryopathic effects of ethanol. The zinc salt used was zinc sulfate ($ZnSO_4$). Zinc sulphate administration has been reported to protect against ethanol-induced gastric ulcers in rats (Cho et al., 1985; Wong et al., 1986). Its protective effect is attributed to the strengthening of the gastric mucosal membrane and to the maintenance of the integrity of biomembranes (Chvapil, 1973). Zinc sulfate administration has also been reported to prevent the depressive action of ethanol on the activity of glutathione transferase which, in turn, increases the

excretion of active metabolites of ethanol through conjugation with glutathione in the liver (Cho and Fong, 1990).

There have been reports regarding the toxic effects of zinc supplementation. Chronic administration of zinc, in the form of zinc chloride (100 μg/ml in the drinking water), in mice has been observed to produce a decrease in hepatic ADH activity and in blood ethanol clearance (Dar et al., 1986). Single doses of 12.5, 20.5 or 25 mg/kg/day of $ZnCl_2$ given i.p. on days 8 to 11 of gestation produced skeletal anomalies in mice fetuses (Chang et al., 1977). Thus, a preliminary experiment was carried out to establish a non-toxic zinc dose. The zinc dose used (15 mg/kg) in the present study was chosen from doses of 50 mg/kg, 40 mg/kg, 25 mg/kg, 15 mg/kg and 5 mg/kg. The amount of zinc administered (15 mg/kg/day) was about six times and three times greater than the daily intakes recommended by NAS (National Academy of Sciences) and AIN (American Institute of Nutrition) or by Lieber and DeCarli (1982) respectively.

Zinc sulfate was administered intraperitoneally in order to avoid the influence of certain factors that affect intestinal zinc absorptions. Chronic ethanol consumption has been associated with decreased zinc absorption (McClain and Su, 1983). In addition, dietary factors such as fiber, phytate, calcium, and iron reduce intestinal zinc absorption (Cousins, 1985).

5.2 Methionine Dosage

Methionine is the most toxic among the amino acids that are required for protein synthesis (Benevenga and Steele, 1984). The consumption of excess methionine has been reported to result in a marked suppression in voluntary food intake, growth retardation, erythrocyte membrane damage (Harper et al., 1970), a reduction in copper-zinc superoxide dismutase activity and in increased hepatic lipid peroxidation (Lynch and Strain, 1989). In addition, excess dietary methionine has been associated with abnormal fetal growth (Viau and Leathem 1973).

In the present study the supplemental dose of methionine used was chosen among doses of 320 mg/kg, 200 mg/kg and 100 mg/kg.

L-methionine was used in the present study because of its much more rapid intestinal absorption than the D-isomer and because L-methionine is the in vivo precursor of L-cysteine (Nagasawa et al., 1980).

5.3 Ethanol Embryopathy

Alcohol is now recognized as the most common chemical teratogen resulting in congenital anomalies and mental retardation in humans (Smith, 1979). Chronic ethanol consumption during pregnancy is the most common cause of abnormal human development (Abel and Sokol, 1987) and is a major cause of mental retardation in children (Wright, 1983; Chudly, 1991). In humans, a characteristic pattern of congenital anomalies, known as the fetal

alcohol syndrome, has been identified in the offspring of chronic alcoholic mothers. The growth and developmental abnormalities observed in FAS include prenatal and postnatal growth retardation, microcephaly, mental retardation, characteristic facial dysmorphism as well as various non-specific malformations (Jones and Smith, 1973). Similar developmental anomalies have also been reported in experimental animals (Abel and Dintcheff, 1978; Chernoff, 1980; Randall, 1981; Persaud, 1988).

The fetal alcohol syndrome is associated with heavy drinking. However, even moderate drinking (about two drinks a day) can have adverse consequences on the fetus ranging from spontaneous abortions or resorptions to prenatal growth retardation (Kaminski et al., 1976; Streissguth et al., 1981; Olson et al., 1983).

One of the most common abnormalities associated with intrauterine exposure to ethanol is prenatal growth retardation, such as low birth weight, reduced crown-rump length, and smaller head circumference. Other fetal alcohol effects include central nervous system defects, anomalies of the heart, the kidney and urogenital tract, liver, limbs and the skeleton.

Results of the present study also have shown that ethanol consumption during pregnancy had teratogenic effects in the rat. Prenatal growth retardation such as reduced litter weight and crown-rump length, developmental anomalies, and gross internal and external organ malformations were observed in near term fetuses of ethanol treated animals in excess of

those in the pair-fed control group. These adverse effects of ethanol, thus, were independent of undernutrition. Growth retardation and developmental anomalies were also observed in twelve days old embryos of ethanol treated rats, as well as in cultured whole rat embryos.

5.4 Zinc Experiments

The mechanism underlying ethanol-induced teratogenesis is not known. Chronic ethanol consumption affects the absorption, transport, metabolic activation, storage and excretion of nutrients (Lieber, 1988). As a result of this, nutrient availability to the fetus will be reduced.

Because ethanol ingestion interferes with the normal metabolism of essential trace elements, such as zinc, it is plausible that the growth and developmental anomalies observed in the offspring of chronic mothers could be the result of ethanol-induced zinc deficiency or impaired zinc metabolism.

Chronic ethanol consumption has been associated with decreased absorption, increased urinary loss of zinc and lower serum zinc levels in humans (McClain and Su, 1983). Lower levels of hepatic-zinc and reduced hepatic activity of alcohol dehydrogenase, the principal ethanol metabolizing enzyme, have been documented (Prasad et al., 1967; Sullivan, 1962). In addition, inverse relationship between maternal alcohol dehydrogenase activity and maternal blood ethanol level or the frequency of fetal anomalies (Chernoff, 1980), and also between maternal plasma zinc concentration and the incidence

of fetal anomalies similar to FAS (Flynn et al., 1981) have been reported. The congenital anomalies observed in zinc deficiency and in FAS are similar (Hurley, 1979).

Acute ethanol administration has been observed to induce and increase many fold hepatic metallothionein (Waalkes et al., 1989; Bracken and Klaassen, 1987) which leads to increased binding of zinc to metallothionein in the liver and, thus, plasma zinc concentration is decreased, which in turn results in developmentally adverse embryonic zinc deficiency (Daston et al., 1991).

Therefore, ethanol-induced zinc deficiency is one of the more plausible mechanisms of the teratogenic effects of ethanol.

In the present study, pregnant rats were treated with ethanol alone or with ethanol plus zinc during the period of organogenesis. Despite the same amount of food intake, ethanol-fed dams did not gain weight in contrast to the pair-fed controls. A similar failure to gain weight or loss of weight had been observed in pregnant rats exposed to ethanol chronically (Das et al., 1984; Shetlar et al., 1986). The significant difference in maternal body weight gain between the ethanol and the pair-fed groups could have been due to impaired gluconeogenesis and increased mobilization of body fat. When ethanol is metabolized by ADH, the excess reducing equivalent (NADH) produced inhibits gluconeogenesis and this results in hypoglycemia. In order to maintain blood glucose level, glucagon mobilizes stored body fat. There were increased

resorptions and higher mortality rates in the ethanol treated group and this is consistent with other reports (Weinberg, 1985; Sanchis et al., 1987; Zidenberg-Cher et al., 1988). Maternal ethanol consumption retarded the growth of fetuses which was reflected in a significantly lower litter weight and reduced crown-rump length in twenty day old fetuses. Similar observations have been reported by others (Leichter and Lee, 1979; Keppen et al., 1985; Sanchis et al., 1987; Leichter, 1991). Chronic ethanol feeding also resulted in increased external and internal anomalies in the fetuses. The organ developmental anomalies observed in the fetuses included the brain (microcephaly, hydrocephaly), the heart (ventricular septal defect), and the kidneys (hydronephrosis, ectopic kidney). Similar developmental anomalies have been reported in fetuses of mice exposed to ethanol (Chernoff, 1977; Randall and Taylor, 1979).

In the present study, ethanol feeding of pregnant rats resulted also in changes in the histological structure of the placenta. Increased stagnation of maternal blood in the labyrinth and in the basal-labyrinthine junction, and enlarged giant cells in the basal zone of the placenta in excess of those in the corresponding pair-fed control group, were observed and this could have accounted for the enlarged placenta in the ethanol treated rats. The fact that these alterations in the placental structure were apparent on day-20 of gestation, long after treatment of the animals was discontinued, appear to suggest that the effect of ethanol on the placenta is long lasting. The

stagnating maternal blood in the maternal blood channels could impair the placentral transfer of nutrients and oxygen to the fetus. As a result, fetal malnutrition and hypoxia will occur and enhance the embryopathic effects of ethanol. Similar vascular and cellular changes in the placenta also have been reported in pregnant rats treated with ethanol (Eguchi et al., 1989). Maternal blood channels in the labyrinth were more dilated and filled with blood and the giant cells were larger in size and more in number in the ethanol treated group. These structural and cellular changes were suggested to be responsible for the significantly increased placental weight observed in the ethanol treated animals compared to that in the pair-fed controls (Eguchi et al., 1989). The placental weight in the ethanol treated animals was significantly higher also in the present study.

Zinc supplementation of the ethanol treated pregnant rats improved the intrauterine growth of the offspring. There was a significant increase in the weight and crown-rump length of litters of ethanol plus zinc treated dams compared to litters of dams fed only the ethanol liquid diet. This finding was consistent with the observations of Shetlar and coworkers (1986) who reported increased litter weight in zinc-supplemented ethanol treated rats compared to that of animals treated with ethanol alone. A lower fetal weight, a higher incidence of external anomalies, and a higher frequency of resorptions in mice fed a low zinc diet and ethanol, compared to mice fed an ethanol diet with adequate levels of zinc, have also been reported (Keppen et al., 1985). In the

present study, although the frequency of resorptions in the ethanol plus zinc treated dams was not significantly different from the ethanol-fed dams, zinc supplementation of the ethanol diet improved the viability of fetuses which was evidenced by a significantly higher rate of live fetuses. In addition, there was a lower incidence of developmental anomalies in the offspring of zinc supplemented-ethanol treated dams. Zinc supplementation of dams on chronic ethanol diet ameliorated the growth, viability and frequency of organ anomalies. These improvements, however, failed to reach control levels. Similarly, Amemiya and coworkers (1986) have reported that maternal dietary zinc supplementation partially prevented the toxicity of 6-mercaptopurine. 6-mercaptopurine toxicity produced similar forms of developmental toxicity as zinc-deficiency (Hirsch and Hurley, 1978; Hurd et al., 1983).

The serum zinc concentrations were significantly depressed and the copper levels were elevated in the ethanol treated pregnant animals compared to those in pair-fed controls. In addition, circulating levels of ethanol were lower in the ethanol + zinc treated animals than in those treated with ethanol alone. These results are in agreement with the observations of other investigators who have reported reduced serum/plasma zinc (Ghishan et al., 1982; Das et al., 1984) and liver zinc levels (Zidenberg-Cher et al., 1988), and higher levels of plasma (Zidenberg-Cher et al., 1988) and liver copper (Shetlar et al., 1986; Zidenberg-Cher et al., 1988) in ethanol-fed pregnant rats. A decrease in ADH activity and a slower rate of ethanol elimination also have

been reported in zinc deficient rats (Das et al., 1984).

The histological study of the placenta showed that the increased appearance of stagnated maternal blood in the labyrinth or in the basal-labyrinthine junction, in ethanol treated animals, appeared to have been reduced in the animals treated with ethanol plus zinc. Chronic ethanol feeding during gestation has been reported to decrease placental blood flow in the rat (Jones et al., 1981) and as a result nutrient availability and transfer to the fetus could be affected. These ethanol-induced impairment may be ameliorated by anything that lowers circulating ethanol levels. Reduced serum ethanol concentrations were observed, in the present study, following supplementation of ethanol treated animals with zinc.

In the investigation involving 12 days old embryos (day-12 zinc experiment), growth in embryos of ethanol treated animals was retarded as evidenced by significantly lower embryonic protein content, shorter crown-rump length, and a reduced number of somites compared to the pair-fed controls. The embryonic circulatory system (yolk sac circulation) and the nervous system (forebrain, hindbrain and causal neural tube) development also were retarded in the embryos of ethanol treated rats. In addition, embryonic craniofacial and musculoskeletal system (number of somites, somite score, and forelimb) development were significantly retarded.

Although supplementation of ethanol-fed pregnant animals with zinc did not reverse all of the ethanol-induced growth and developmental retardation,

it significantly improved the development of somites and the circulatory-system. In addition, the embryonic protein content in the zinc supplemented-ethanol treated group was higher than that of the ethanol-fed group, but the difference was not significant. It appears that the improvement in the growth indices, observed in day-20 fetuses of ethanol plus zinc treated animals, resulted from the relatively increased number of somites during embryonic development.

5.5 Methionine Experiments

Methionine is an essential amino acid for protein synthesis, methylation of DNA and membrane phospholipids and for the formation of glutathione. It protects against the formation of fatty liver and plays a role in maintaining hepatic integrity. Methionine is also a precursor of AdoMet, which is a universal methyl donor for a number of biological reactions and an activator of the transsulfration pathway which results in the biosynthesis of glutathione (GSH), the major cellular antioxidant, responsible for the detoxification of xenobiotics (Friedel et al., 1989; Ross, 1988).

Chronic ethanol consumption has been shown to interfere with methionine metabolism in animals. A decrease in methionine synthetase activity due to chronic ethanol treatment of rats (Finkelstein et al., 1974; Barak et al., 1987), which results in increased loss of methionine via the transsulfuration pathway, has been reported. In addition, ethanol

consumption has been shown to alter hepatic levels of AdoMet and GSH (Speisky et al., 1985; Pierson and Mitchell, 1986; Lieber et al., 1990).

Methionine administration in ethanol treated rats produced a reduction in both hepatic and circulating levels of acetaldehyde (Tabakoff et al., 1989). AdoMet administration significantly improved hepatic levels of AdoMet and GSH in the baboon (Lieber et al., 1990), prevented depletion of GSH, decreased the liver and blood acetaldehyde levels and liver triglyceride concentrations in rats (Feo et al., 1986). Moreover, simultaneous administration of AdoMet and ethanol in healthy humans has been shown to lower the plasma ethanol and acetaldehyde concentrations (DePadova et al., 1984).

In the present study, involving day-20 fetuses, pregnant rats were treated with ethanol, ethanol plus methionine and with methionine alone. A pair-fed and an ad libitum control group were included. The pregnant animals treated with ethanol and with ethanol plus methionine lost weight during the treatment period and this was not related to undernutrition. Weight loss due to ethanol consumption is consistent with other reports (Das et al., 1984; Shetlar et al., 1986). Ethanol treatment resulted also in retarded fetal growth, and increased mortality and resorptions. Gross external and internal development anomalies were increased and skeletal ossification also was retarded. Methionine supplementation of the ethanol-fed pregnant rats did not protect the offspring against the growth retardation and developmental anomalies induced by ethanol.

In addition, the investigation involving day-12 embryos showed marked growth retardation and developmental delays in the circulatory, nervous, optic, olfactory, and in the musculoskeletal systems of animals tested with both ethanol alone and ethanol plus methionine. Methionine supplementation of ethanol treated pregnant animals, thus, did not prevent the embryopathic effects of ethanol. Methionine supplementation of animals that were not treated with ethanol did not produce any adverse effects either on the mother, the embryos or the fetuses. Thus, the adverse effects observed in the offspring of animals treated with both ethanol and methionine did not appear to be due to methionine toxicity.

The lack of any protective effect of methionine supplementation against the ethanol-induced growth and developmental anomalies is not surprising. This is because there is an adaptive increase in methionine recycling during methionine deficiency or altered methionine metabolism. Ethanol administration impairs methionine synthesis through the folate dependent reaction, i.e. trapping of folate in the liver and decrease in the activity of methionine synthetase. Rats possess choline oxidase, the enzyme that catalyzes the conversion of choline to betaine. In the presence of betaine, normal production of methionine occurs, in chronic ethanol consumption, as a result of a compensatory increase in betaine utilization and adaptive increase in betaine-homocysteine methyl transferase activity (Barak et al., 1987; Barak and Beckenhauer, 1988). This explains why hepatic levels of methionine start

to decline only after a prolonged ethanol feeding in the rat. In addition, because the trans-sulfuration pathway enzymes are not fully active during embryonic or fetal life, methionine is conserved by the remethylation of homocysteine instead of being lost via the transsulfuration pathway (Sturman et al., 1970; Gaull et al., 1973). Therefore, the ethanol related growth and developmental abnormalities observed in the present study may not have been due to ethanol-induced methionine deficiency.

5.6 Zinc and Methionine Experiments

The nutrients adversely affected by chronic ethanol consumption include zinc and methionine. Chronic or transient zinc deficiency results in abnormal embryonic or fetal development (Hurley et al., 1971; Keen and Hurley, 1989). Throughout development, there is a significant flux of zinc and amino acids, including methionine, and a decrease in maternal plasma zinc (Daston et al., 1991) and a decrease in placental transfer of zinc (Ghishan et al., 1982) or of amino acids (Gordon et al., 1985) results in reduced zinc and amino acid accumulation by the embryo. Because both zinc and methionine are essential for the normal growth and development of the embryo/fetus, ethanol-induced alteration in their metabolism and bio availability could result in growth retardation and developmental anomalies.

In addition, organic zinc salts and sulfhydryl compounds or thiols administered simultaneously, have been reported to protect mice against the

toxicity of ethanol (Floersheim, 1987).

In order to investigate whether zinc plus methionine supplementation of ethanol treated pregnant rats reduces the embryopathic effects of ethanol, pregnant animals were treated either with ethanol, or ethanol plus zinc & methionine or with zinc & methionine alone. A pair-fed and an ad libitum control group also were included.

Maternal weight gain during the treatment period was impaired by ethanol treatment despite the same food intakes across the various groups. The excess rate of resorptions and mortality, the growth retardation, gross external and internal anomalies and the delayed skeletal ossification observed in day-20 fetuses of ethanol treated rats were not reduced by zinc and methionine supplementation. Moreover, zinc and methionine supplementation of ethanol-fed rats failed to prevent or improve the increased resorptions, growth retardation, as well as the developmental delays in the nervous, optic, olfactory, and musculoskeletal systems observed in 12 days old embryos. However, zinc plus methionine administration in pregnant animals, maintained on the control liquid diet, did not affect adversely the growth and development of either the day-12 embryos or the day-20 fetuses.

5.7 In Vitro Experiment

The growth and developmental anomalies associated with FAS may be the result of a direct effect of ethanol, or its metabolic byproducts, such as

acetaldehyde, or alcohol induced nutrient deficiencies, or altered maternal function or a combination of these and other similar factors.

The current study was carried out to establish the embryopathic effects of ethanol in vitro and to investigate whether S-adenosyl-L-methionine (AdoMet) supplementation of cultured whole rat embryos reduces the embryopathic effects of ethanol.

AdoMet is a universal methyl donor for a number of biological reactions involving transmethylation. Transmethylation reactions are essential for the synthesis and metabolism of nucleic acids, proteins and phospholipids (Friedel et al., 1989). AdoMet is also important in the synthesis of glutathione, the major cellular antioxidant (Ross, 1988).

A series of preliminary trials were carried out to standardize the conditions for the embryo culture system. The embryo culture system used, which is similar to the one used by Brown et al., 1979, supported the growth and development of embryos during the period of organogenesis. Our observation is consistent with that reported by Brown et al. (1979) who demonstrated a direct effect of ethanol on the developing rat embryos in the absence of confounding variables such as maternal/fetal nutrition, altered maternal function or metabolism.

The growth of embryos cultured with ethanol alone was significantly retarded. Similar growth retardation was also reported by other investigators (Brown et al., 1979; Wynter et al., 1983; Fadel and Persaud, 1992). Retarded

development of the craniofacial and musculoskeletal system was detected. However, delay in the development of the cardiovascular and nervous system was not significant.

In rats on chronic ethanol diet, administration of S-adenosyl-L-methionine prevented the depletion of glutathione, the major cellular antioxidant, and production of acetaldehyde (Pascale et al., 1989; Feo et al., 1986). AdoMet supplementation of ethanol treated human hepatocytes also prevented the decrease in intracellular glutathione. Simultaneous administration of AdoMet and ethanol has been shown to lower plasma ethanol and acetaldehyde levels in healthy humans (DiPadova et al., 1984).

In the current investigation, administration of AdoMet (0.05mM) significantly improved the growth of embryos cultured in ethanol containing medium as evidenced by higher embryonic protein content, greater crown-rump length, and increased number of somites. The frequency of developmental anomalies of the craniofacial and musculoskeletal systems was lower in embryos cultured with AdoMet and ethanol than in those cultured with ethanol alone. S-adenosyl-L-methionine administration in culture appears to have a protective influence against the adverse effects of ethanol on the growth and development of rat embryos. At higher doses (3mM), AdoMet was observed to be toxic and resulted in growth retardation and developmental anomalies.

6. SUMMARY AND CONCLUSION

In the present investigation, ethanol resulted in embryopathic effects in vivo and in vitro. The observed embryotoxic and teratogenic effects of ethanol were independent of ethanol-induced nutrient deficiencies.

Zinc supplementation of ethanol treated pregnant rats partially ameliorated the embryopathic effects of ethanol. Similarly, 6-mercaptopurine toxicity, which produces similar forms of developmental toxicity as zinc deficiency, was partially remedied by maternal supplementation of dietary zinc. Comparable growth and developmental anomalies are manifested in the case of zinc deficiency and in FAS. Thus, the developmental anomalies observed in children with FAS may be partly due to ethanol-induced alterations in zinc metabolism. The fact that the embryopathic effects of ethanol were ameliorated only partially by zinc supplementation suggests that the cause of ethanol-induced embryopathy may be multifactorial.

Methionine supplementation of ethanol treated pregnant rats failed to protect the embryo against the effects of ethanol. This may be due to the presence of alternate pathways which compensate for any ethanol-induced disturbances in methionine metabolism. Thus, circulating and liver levels of methionine could be maintained for a long period of time without any significant decline in concentration.

Zinc and methionine supplementation, although it appeared to improve

pregnancy outcome and the incidence of developmental anomalies, did not significantly influence the embryopathic effects of ethanol. This result was unexpected, in view of the reported synergistic protection of mice by zinc and thiols against ethanol toxicity, and considering the fact that both zinc and methionine are important in vital biochemical processes, such as DNA synthesis and membrane integrity.

Simultaneous administration of both S-adenosyl methionine and ethanol prevented some of the embryotoxic effects of ethanol in cultured whole rat embryos. S-adenosyl methionine is a universal methyl donor, essential in DNA and membrane phospholipids methylation, and it is a source of glutatione, the major cellular antioxidant.

The observation that ethanol administration produced teratogenic and embryotoxic effects in vivo and in vitro suggested that the embryopathic effects of ethanol could be the results of both the direct and indirect actions of ethanol.

The presence of structural changes in the placenta in the ethanol treated pregnant rats suggested that ethanol induced alterations in placental functions could be partly responsible for the observed adverse effects of ethanol in vivo.

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APPENDIX 1. Skeletal scoring chart of ossification centers in the rat fetus.*

SKULL:	Right	Left
Frontal		
Parietal		
Interparietal		
Supraoccipital		
Exoccipital		
Basioccipital		
Basisphenoid		
Presphenoid		
Alisphenoid		
Nasal		
Lacrimal		
Premaxilla		
Maxilla		
Zygoma		
Squamosal		
Tympanic bulla		

HYOID (BODY): _____

STERNUM: _____

	Right	Left
RIBS (13 pair)		

General Remarks: _____

_____

FORELIMBS:	Right	Left
Clavicle		
Scapula		
Humerus		
Radius		
Ulna		
Metacarpus		
Phalanges		

HINDLIMBS:	Right	Left
Femur		
Tibia		
Fibula		
Metatarsus		
Phalanges		

VERTEBRAL COLUMN:

	Centra	Arches
Cervical (7)		
Thoracic (13)		
Lumbar (6)		
Sacral (4)		
Caudal (28-30)		

HIP:	Right	Left
Ilium		
Ischium		
Pubis		

* From Nash and Persaud (1989).

APPENDIX 2. A morphological scoring system for quantifying rat embryonic development (Brown and Fabro, 1981).

		0	1	2	3	4	5	SCORE
A	YOLK SAC CIRCULATORY SYSTEM	no visible, or scattered, blood islands	Corona of blood islands w or w/o anastamoses	Vitelline vessels with few yolk sac vessels	Full yolk sac plexus of vessels	Yolk stalk obliterated, vitelline artery & vein well separated		
B	ALLANTOIS	Allantois free in exocoelome	Allantois fused with chorion	Umbilical vessels	Separate aortic origins of umbilical and vitelline vessels			
C	FLEXION	Ventrally convex	Turning	Dorsally convex	Dorsally convex with spiral torsion			
D	HEART	Endocardial rudiment not visible, or visible but not beating	Beating 's' shaped cardiac tube	Convoluted cardiac tube	Bulbus cordis, atrium commune and ventriculus communis	Dividing atrium commune		
E	CAUDAL NEURAL TUBE	Neural plate or neural folds	Closing, but unfused neural folds (groove)	Neural folds fused at level of somites 4/5	Posterior neuropore formed, but open	Posterior neuropore closed		
F	HIND BRAIN	Neural plate	Rhombomeres A and B	Anterior neuropore formed but open	Anterior neuropore closed, rhombencephalon formed	Pronounced pontine flexure with transparent roof of 4th ventricle		
G	MID BRAIN	Neural plate	Mesencephalic brain folds	Closing or fusing mesencephalic folds	Completely fused mesencephalon	Visible division between mesencephalon & diencephalon		
H	FORE BRAIN	Neural plate or no visible prosencephalon	Prosencephalic brain folds	Completely fused prosencephalon	Visible telencephalic evaginations	Well elevated telencephalic hemispheres		
J	OTIC SYSTEM	No sign of otic development	Flattened or indented otic primordium	Otic pit	Otocyst	Otocyst with dorsal recess	Otocyst with endolymphatic duct	
K	OPTIC SYSTEM	No sign of optic development	Sulcus opticus	Elongated optic primordium	Primary optic vesicle with open optic stalk	Indented lens plate	Lens pocket or lens vesicle	
L	OLFACTORY SYSTEM	No sign of olfactory development	Olfactory plate	Olfactory plate with rim	Distinct olfactory ridges	Lateral nasal process and medial rim		
M	BRANCHIAL BARS	None visible	I visible	I and II visible	I, II and III visible	II overgrowing and obscuring III		
N	MAXILLARY PROCESS	No sign of maxillary development	Maxillary process demarkated, visible cleft anterior to bar I	Maxillary process fused with nasal process				
P	MANDIBULAR PROCESS	No sign of mandibular development from bar I	First brancial bars fused and forming mandibular process					
Q	FORE LIMB	No sign of fore limb development	Distinct evagination of wolfian crest at level of somites 9-13	Fore limb bud	Paddle shaped fore limb bud	Distinct Apical ridge on fore limb bud		
R	HIND LIMB	No sign of hind limb development	Distinct evagination of wolfian crest at level of somites 26-30	Hind limb bud	Paddle shaped hind limb bud			
S	SOMITES	0 - 6	7 - 13	14 - 20	21 - 27	28 - 34	35 - 41	